Cloning and Targeted Deletion of the Mouse Fetuin Gene*

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We proposed that the α2-Heremans Schmid glycoprotein/fetuin family of serum proteins inhibits unwanted mineralization. To test this hypothesis in animals, we cloned the mouse fetuin gene and generated mice lacking fetuin. The gene consists of seven exons and six introns. The cystatin-like domains D1 and D2 of mouse fetuin are encoded by three exons each, whereas a single terminal exon encodes the carboxyl-terminal domain D3. The promoter structure is well conserved between rat and mouse fetuin genes within the regions shown to bind transcription factors in the rat system. Expression studies demonstrated that mice homozygous for the gene deletion lacked fetuin protein and that mice heterozygous for the null mutation produced roughly half the amount of fetuin protein produced by wild-type mice. Fetuin-deficient mice were fertile and showed no gross anatomical abnormalities. However, the serum inhibition of apatite formation was compromised in these mice as well as in heterozygotes. In addition, some homozygous fetuin-deficient female ex-breeder mice developed ectopic microcalcifications in soft tissues. These results corroborate a role for fetuin in serum calcium homeostasis. The fact that generalized ectopic calcification did not occur in fetuin-deficient mice proves that additional inhibitors of phase separation exist in serum.

Fetuins are plasma proteins that occur in high concentration (up to several grams/liter) during fetal life and gradually decrease in amount toward adulthood. The human species homologue is α2-HS1 glycoprotein (1); hence, the genetic symbol for fetuins is “Ahsg.” Fetuins are members of the cystatin superfamily of proteins possessing two tandemly arranged cystatin domains and a third domain rich in proline and glycine. Secondary modifications, N-glycosylation, O-glycosylation (2, 3), Ser phosphorylation (4), and proteolytic processing (5) have all been described for a variety of fetuins from several species. Despite this wealth of information on the structure of fetuins, their biological function is still far from clear. Functions proposed for fetuins are based on their ability to influence processes as diverse as opsonization, lipid transport, cell proliferation, tyrosine kinase inhibition of the insulin receptor, protease inhibition, and hematopoietic cell homing, all reviewed in a recently published monograph (6), as well as transforming growth factor-β and bone morphogenetic protein cytkine binding (7) and hepatocyte growth factor binding (8). Confusing but not necessarily contradictory, these results indicate molecular binding of fetuins to diverse target molecules. Binding is mediated by structural features of the protein part, as shown for transforming growth factor-β and apatite binding (7, 9), or by binding of the carbohydrate moiety to lectin-like molecules, as shown for binding of the fetuin homologue he-mo-nectin (10) to hematopoietic cells or binding of bovine fetuin to bacterial toxins (11). In two cases, inhibition of the insulin receptor tyrosine kinase and inhibition of hepatocyte growth factor signaling (8), the activity of fetuin is specific in that only serine-phosphorylated fetuin (phosphofetuin) is active, whereas the dephosphorylated form is inactive. In summary, fetuins could, like serum albumins, act as generic molecular carriers facilitating the transport of otherwise insoluble cargo molecules.

Based on the finding that fetuins accumulate in bones and teeth as a major fraction of noncollagenous bone (12), a role in bone metabolism and turnover has been suggested. Our knowledge of molecular mechanisms initiating skeleton formation and mineralization has substantially increased over the past few years (13). Recently, in a series of independent studies, it was shown that one critical component required for the very start of osteoblast differentiation and hence of skeleton mineralization is the transcription factor Osf2/Cbfa1 (14–17). Regarding the opposite end, termination of mineralization and prevention of unwanted mineralization, we are still faced with the problem that most extracellular fluids are supersaturated salt solutions that must in fact be hindered from mineralizing spontaneously (18). One effective inhibitor of calcification was discovered by targeted disruption of the gene for matrix GLA (γ-glutamic acid) protein (19). This protein is a tissue-bound inhibitor of spontaneous calcification, as lethal calcification of arteries and cartilage occurred in mice lacking matrix GLA protein.

Recently, we determined that fetuins might be soluble, serum-derived inhibitors of unwanted mineralization, as fetuins inhibited apatite formation both in cell culture and in vitro (9). This inhibitory effect is mediated by acidic amino acids clustering in the cystatin-like domain D1. Fetuins account for roughly half of the capacity of serum to inhibit salt precipitation. Interestingly, despite extensive use of α2-HS/human fetuin as a forensic marker (20), no complete deficiency has been reported to date. Therefore, lack of α2-HS is either phenotypically silent or prohibitive of full-term development. Evidence...
for either possibility can be obtained in a transgenic mouse model. To distinguish both possibilities, we cloned the mouse fetuin gene and produced mice lacking the entire fetuin gene. The Ahsg null mutant mice developed normally and reproduced four generations (through up to now). Biochemical analyses show that the ability of serum to inhibit apatite formation was reduced in the mutant mice. In addition, some homozygous fetuin-deficient female ex-breeders developed ectopic microcalcifications in soft tissues. This observation is consistent with a role for fetuins as inhibitors of phase separation in serum.

**EXPERIMENTAL PROCEDURES**

**Cloning of the Mouse Fetuin Gene and Construction of a Replacement Vector**—To construct a vector for the targeting of the α2-HS glycoprotein/fetuin gene (Ahsg), we screened a mouse genomic library (derived from D3 ES cells of strain 129, kindly provided by Drs. B. Bettenhausen and A. Gessler) with rat fetuin cDNA (21). We isolated two overlapping AEMBL3 clones containing a combined 28.4-kb DNA fragment that included the entire Ahsg gene (6.4 kb), 7 kb of 5’-upstream sequence, and 15 kb of 3’-downstream sequence. The identity of the gene was confirmed by restriction mapping, probing with oligonucleotides corresponding to parts of the mouse fetuin cDNA sequence (22), and automated sequencing using a combined shotgun cloning and primer walking/sequencing approach. Contiguous traces were assembled and edited using SEQUENCER software (Gene Codes, Ann Arbor, MI). Sequence data were analyzed by BLAST searches of the GenBank™/EMBL Data Bank. Repeat sequences were detected at the RepeatMasker Web Server. 3 Rat and mouse fetuin gene promoters were aligned using CLUSTAL V software (23). Transcription factor-binding sites were scored using MATINSPECTOR software and the TRANSFAC database. 4

A replacement vector was constructed in several steps. The final targeting construct (see Fig. 3) was assembled in a vector consisting of an NdeI-AatII fragment of the pBR322 plasmid containing ORI and AmpR (24), polylinker 1, a pMC1TKA9 cassette (25) in opposite transcriptional orientation to the adjacent pPGKNeo9A cassette (26), polylinker 2, and a pGKTKA9 cassette (26) in the same transcriptional orientation as the pPGKNeo9A cassette. Into polylinker 1, we inserted a 2.2-kb HindIII fragment of Ahsg genomic sequence (ending 2.5 kb upstream of the start of the cDNA sequence as determined in Ref. 22), and into polylinker 2, we cloned a 13-kb BamHI fragment of genomic sequence (beginning 3 kb downstream of the fetuin gene polyadenylation signal). Thus, a 10.8-kb HindIII-BamHI genomic fragment containing the entire coding region of the fetuin gene was replaced by the pPGKNeo9A9 cassette positioned in the same transcriptional orientation as the Ahsg gene.

**Gene Targeting in ES Cells**—R1 embryonic stem cells (passage 16) (27) were grown and electroporated with 150 μg of PouI-linearized Ahsg-neo replacement vector DNA as described (28). After positive-negative selection using G418 (300 μg/ml final concentration) and ganciclovir (2 μM final concentration), resistant clones were frozen, and replicas were used for DNA preparation (25).

**DNA Analysis**—DNA from individual ES cell clones was digested with the ScaI restriction enzyme, separated by agarose gel electrophoresis, and transferred to nylon membrane. Blots were probed with a 0.9-kb EcoRI-HindIII fragment located immediately upstream of the 2.2-kb HindIII fragment contained in the replacement vector. This probe detects a 13-kb ScaI fragment in wild-type DNA and a 6.3-kb ScaI fragment in the correctly targeted mouse Ahsg locus (see Fig. 3).

**Blastocyst Injection**—Blastocysts were harvested from 3.5-day postcoitus naturally mated C57BL/6 mice (29). About 15 ES cells were injected into each blastocoele, and blastocysts were implanted into the uterine horns of pseudopregnant female mice. Two chimeras were obtained in two independent blastocyst injection/transfer experiments. Both chimeric males transmitted the targeted Ahsg locus through the germline.

**Genotyping**—DNA was prepared by proteinase K digestion and extraction (30) of tail and ear biopsies taken at the time of weaning. Southern analysis was performed as described above for ES cells (see Figs. 1 and 2). In addition, the blots were stripped and reprobed with a 1.2-kb EcoRI fragment from mouse fetuin cDNA (22), generously provided by Dr. F. Yang (University of Texas Health Science Center, San Antonio, TX). This probe detects the identical 13-kb ScaI fragment in the wild-type Ahsg locus, but is not contained in the correctly targeted mouse Ahsg locus.

**Histological and Anatomical Analyses**—Embryos and tissues were dissected and fixed in Bouin’s fluid overnight and processed for paraffin embedding. Sections were stained with hematoxylin and eosin. For skeletal analysis, mice were eviscerated, fixed in ethanol, cleared in KOH, and consecutively stained with Alcan blue SGX and alizarin S (as described (29, 31)).

**Estimation of Serum Fetuin Levels**—Mouse sera were obtained from spontaneously clotted blood drawn by cardiac puncture. Serum fetuin level was measured by indirect ELISA using anti-mouse fetuin anti-serum (diluted 1:5000), secondary peroxidase-labeled antibody, and the chromogenic substrate 2-azo-ninios-(3-ethylbenzathionine-6-sulfonic acid) (ABTS, Boehringer Mannheim). Serum was prediluted 1000-fold and coated (50 μl) onto microtiter plates in a 2-fold dilution series.

**Inhibition of Apatite Formation**—To estimate the inhibition of salt precipitation by serum, assays were done in 20 mM Tris, pH 7.4, 100 mM NaCl, 1.6 mM NaHPO4, and 4.8 mM CaCl2 containing a trace amount of 43CaCl2. Sera were dialyzed by repeated spin dialysis against incubation buffer using Microcon 10 centrifuge concentrators (Amicon, Inc.). Inhibition of calcium salt precipitation was determined by incubation at 37°C for 30 min. Salt precipitates formed were collected by centrifugation (15,000 × g for 5 min at room temperature), dissolved in 0.5% acetic acid, and quantified by liquid scintillation counting. All incubations were done in triplicates and independently repeated at least two times.

**RESULTS**

**Structure of the Ahsg Gene Locus**—Two overlapping genomic clones containing 28.5-kb contiguous genomic sequence were obtained from a mouse 129Sv liver. Fig. 1A shows the structure of Ahsg, the fetuin gene locus. The restriction map of the combined clones comprises the fetuin gene of 7.5 kb, 8 kb 5’ to the first exon of the fetuin gene, and 13 kb 3’ to the fetuin gene. Upstream of the fetuin gene, we determined sequences rich in simple repeat structures. A similarity search of downstream sequence revealed extensive sequence similarity to mouse ORR1 transposon-like elements (GenBank™/EMBL Data Bank accession numbers U17092–U17095, also detected in noncoding regions of the mouse c-abl oncogene (GenBank™/EMBL Data Bank accession number U17271).

Sequence analysis of the fetuin gene proper revealed simple repeat sequences, (GAAA)₄ and (GAAA)₉, and SINE-type repeats (MBBF1) at positions 2099–2486. Additional simple repeats, (GAAA)₇, (CAGA)₉, (GAAA)₉, and (GA)₉, occurred dispersed throughout the introns at positions 3105–3241, 5594–5726, 6163–6227, 7105–7214, and 7165–7214, respectively. The exon-intron structure was similar to that of the rat fetuin gene (32) in that seven exons were also determined in the mouse fetuin gene. The exon-intron borders (Fig. 1B, short underlines) were conserved between the rat and mouse genes, but introns were larger in the mouse gene than had been estimated for the rat gene (32). A comparison of the aligned mouse fetuin promoter (Fig. 1B, long underline) and the rat fetuin promoter (GenBank™/EMBL accession number M56574) is presented in Fig. 2. Extensive sequence identity exists within 500 bases upstream of the transcription start point. Farther upstream, the promoter sequences diverge. We determined transcription factor-binding sequences by computer searching of the mouse fetuin promoter. Matching binding motifs were returned for c-Myc-like factors involved in growth regulation and differentiation (n = 4) (33), C/EBP-like enhancer-binding proteins known to regulate acute-phase proteins (n = 1) (34), AP1-like transcriptional activators (n = 4) (35), growth factor independence-1-like transcriptional repressors (n = 3) (36), hepatic nuclear factor-3B hepatocyte-specific transcriptional activator (n = 3) (37), and NF-1-like transcription factors (n = 1) (38).

**Generation of Ahsg Null Mutant Mice**—The genomic clones

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2 Available at http://ftp.genome.washington.edu/cgi-bin/mrs/mrs_req.
3 Available at http://www-hgc.lnl.ibi/cgi-bin/promoter.pl.
4 Available at http://transfac.gbf-braunschweig.de/cgi-bin/matSearch/matsearch.pl.
Knockout of the Mouse α2-HS Glycoprotein/Fetuin Gene

FIG. 1

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were used to construct a targeting vector for homologous recombination and positive-negative selection of recombinants (25) as shown in Fig. 3. Single clones resistant to G418 and ganciclovir were screened by Southern blot hybridization using probe A. Restriction enzyme digestion with ScaI generated a 13-kb fragment in the wild-type Ahsg locus, whereas the correctly recombined locus generated a 6.3-kb fragment (Fig. 4A). Three correctly targeted clones were identified among 800 resistant colonies, yielding a targeting frequency of ~1 in 270. One of these clones consistently had a signal for the 13-kb fragment twice as strong as for the 6.3-kb fragment and therefore likely contained a chromosomal aberration. In addition, genomic DNA from ES cells and from litters of heterozygous crosses was digested with HindIII and probed with a mouse cDNA. This probe detected a 7-kb fragment in wild-type DNA and no fragment in the correctly targeted mouse Ahsg locus (Fig. 4B).

Chimeras were generated by blastocyst injection from one targeted ES cell clone containing the two alleles in equimolar amounts. Two chimeras transmitted the correctly targeted locus through the germ line as demonstrated by Southern blot analysis of tail DNA. Fig. 4C illustrates germ line transmission for a litter born to two heterozygous parents. The Ahsg null mutation in its homozygous state was maintained on a mixed 129SvC57BL/6 background. For further reference, the mutant strain is named B6,129-Ahsgim1Mbl according to the rules of the Institute of Laboratory Animal Resources.

**Phenotype of Ahsg Null Mutant Mice—**Ahsg null mutant mice were born at the expected mendelian frequency from crosses of heterozygotes (example in Fig. 4C). In an assembly of 120 total offspring, 32 mice were genotyped Ahsg wild-type (Ahsg+/+), 57 mice heterozygous (Ahsg+/−), and 31 mice homozygous (Ahsg−/−) for the null mutation. All mice were fertile and appeared healthy, with no gross abnormalities observed upon macroscopic inspection. A routine histological survey of major organs likewise indicated that mice can develop normally in the absence of zygotic fetuin (data not shown). In addition, the homozygous Ahsg−/− genotype could be propagated into the F4 generation by now, indicating that Ahsg−/− need not be rescued by maternally supplied fetuin during embryonic development.

**Fetuin Serum Levels—**We estimated the amount of fetuin present in 4-week-old wild-type Ahsg+/+, heterozygous Ahsg+/−, and homozygous Ahsg−/− mice by indirect ELISA. Mice were age-matched as fetuin levels in wild-type animals vary with age. In normal animals, the peak concentration of up to 1 mg/ml occurs in newborn mice, and the concentration decreases to levels around 0.200 mg/ml in adult wild-type mice. The ELISA was standardized with recombinant mouse fetuin expressed in Sf9 insect cells (9). Fig. 5 shows that the ELISA measured 23.6 ± 7.3 ng/ml recombinant mouse fetuin at half-maximum absorbance (A405 = 1.25). Compared with this standard, sera of 4-week-old Ahsg+/+, Ahsg−/−, and Ahsg−/− mice contained 0.84 ± 0.10 mg/ml, 0.28 ± 0.02 mg/ml, and no fetuin, respectively (Fig. 5). The data reflect the Ahsg gene deletion on the protein level. Furthermore, the data indicate that fetuin gene copies are expressed independently of each other, as sera of heterozygous Ahsg+/− mice contained roughly half the amount of fetuin as sera of wild-type Ahsg+/+ mice.

**Bone Mineralization—**Fetuins accumulate in mineralized bone to high concentrations. Therefore, a biological role for fetuins has been proposed in the regulation of bone mineralization and turnover. Studies with primary osteoblasts from rat calvaria demonstrated that fetuins maintain the formation of mineralized bone nodules in this model system of bone mineralization (9). Fetuins effectively prevented the precipitation of large amounts of apatite onto these cells that occurs in the absence of fetuins probably as a consequence of the high concentration of β-glycerophosphate required to induce mineralization in this system. To study in vivo the effect of lack of fetuin on mineralization, we performed bone and cartilage staining in normal and Ahsg−/− mice. In newborn mice, no obvious differences were observed in skeletal preparations of Ahsg+/+ and Ahsg−/− mice double-stained with Alcian blue for cartilage and alizarin red for mineralized bone. Fig. 6A shows stained skeletons of mice killed 2 days after birth. As in control Ahsg+/+ mice (Fig. 6A, left), no gross skeletal deformations were present in Ahsg−/− mice (Fig. 6A, right). Upon casual inspection of the major bones, calvarial morphology and sutures, rib number and morphology, and bone length and morphology all appeared normal in Ahsg−/− mice compared with Ahsg+/+ mice and with published data on younger mice (embryonic day 18) (39). At 4 months of age, both Ahsg−/− and wild-type Ahsg+/+ mice displayed variable morphology of the costochondral junction in that the boundary between cartilage (blue) and mineralized bone (red) ranged from sharp and well defined (Fig. 6B, left) to fuzzy and extended (Fig. 6B, right). Double-blind examination of 18 Ahsg−/−, Ahsg+/−, and Ahsg+/+ mice revealed no co-segregation of this phenotypic variation with Ahsg genotype. Therefore, we conclude that deletion of the fetuin gene does not result in skeletal anomalies beyond normal variation. We observed ectopic microcalcification of soft tissues (neck, intercostal muscle, and leg muscles) in a limited number of fetuin-deficient female ex-breeders (Fig. 6C). Ectopic calcification was not detectable in age-matched wild-type ex-breeders.

**Serum Inhibition of Apatite Formation—**It is known that serum contains inhibitors of salt precipitation (40). Previously, we have shown by affinity depletion of sera that fetuins contribute roughly 30% to the inhibitory activity of whole serum (9). To test this biochemical finding, we studied the inhibition of apatite formation in sera from wild-type and mutant mice. For simplicity and improved reproducibility, we modified the original experimental setup for precipitation assays in the presence of serum (9) and included only CaCl2, Na2HPO4, and a trace amount of [45Ca]Cl2 in the precipitation buffer. Fig. 7 illustrates the results of a typical experiment in which spontaneous apatite formation from supersaturated solutions of CaCl2 and Na2HPO4 was measured as incorporation of 45Ca into the insoluble salt pellet. The precipitation reactions proceeded for 30 min at 37 °C either in the absence of serum (control) or in the presence of 10% dialyzed serum from wild-type Ahsg+/+, heterozygous Ahsg+/−, or homozygous Ahsg−/− mice. Fig. 7 shows that serum from wild-type animals effected an 80% inhibition of apatite formation relative to the control precipitation reaction lacking serum. In contrast, serum from homozygous Ahsg−/− mice did not inhibit the formation of apatite, whereas serum from heterozygous Ahsg+/− mice ef-
fected an intermediate inhibition of apatite formation by 40 ± 15%. This biochemical phenotype of the fetuin gene deletion corroborates in vivo the conclusion drawn from our previous in vitro studies, namely that fetuins contribute significantly to the apatite precipitation inhibition observed in whole serum.

**DISCUSSION**

We cloned the mouse fetuin gene from a mouse strain 129 genomic phage library. We determined the complete genomic sequence for this member of the \( \alpha_2 \)-HS glycoprotein/fetuin gene family. A partial sequence was previously published for the rat fetuin gene (32). As in two additional members of the cystatin superfamily, kininogen (41) and histidine-rich glycoprotein (42), three exons each code for the cystatin-like domains D1 (exons 1–3) and D2 (exons 4–6). The fetuin-specific domain D3 is encoded by a single long exon (exon 7). Given the known chromosomal locations for the human \( \alpha_2 \)-HS gene (3q27) and the rat fetuin gene (chromosome 11), synteny tables available at the mouse genome data base suggest that the mouse fetuin gene is located on chromosome 16. However, this point needs experimental support through direct genetic mapping.

The sequence comparison of the mouse and rat fetuin gene promoters revealed extensive sequence identity upstream of the transcription start point. Pending a detailed analysis of the mouse fetuin promoter, it is likely that the mouse fetuin gene transcriptional control is similar to that of the rat fetuin gene, as the area shown to harbor crucial transcription factor-binding sites is well conserved (43). More specifically, the promoter regions best protected in S1 nuclease protection assays of the rat promoter (shaded boxes, Fig. 3) were all conserved in the mouse promoter. An analysis of putative binding sequences for transcription factors revealed well conserved binding motifs of factors NF-1, hepatocyte nuclear factor-3B, c-Myb, and C/EBP and of the transcriptional enhancer AP-1 and the transcriptional silencer sites are overlined and labeled following the TRANSFAC data base transcription factor nomenclature. S1 nuclease-protected regions of the rat fetuin promoter (43) are shaded; the TATA box is boxed; and the promoter predicted by sequence analysis software is marked by a thick overline. The transcription start point is marked tsp.

**FIG. 3. Strategy for the targeted deletion of the fetuin gene.** From the top are shown a 13-kb genomic ScaI fragment (dashed line) hybridizing with probe A (lower left) in the wild-type locus and a 7-kb HindIII fragment (dashed line) hybridizing with the cDNA probe in the wild-type locus, but not in the mutated locus. The upper center sketch depicts part of the wild-type Ahsg locus including the seven exons of the fetuin gene (solid boxes) and six introns (open boxes). Shown below is the targeted locus derived by replacement of the wild-type gene with a gene for neomycin resistance through homologous recombination. Recombination was driven by a genomic HindIII fragment 2.2 kb in length located 5’ to the gene and a genomic BamHI fragment 13 kb in length located 3’ to the gene (shaded boxes). The deletion effected by the gene knockout covers a total of 12 kb of genomic sequence including the entire fetuin gene and flanking regions. When hybridized with probe A, a restriction fragment length polymorphism of ScaI genomic fragments (top and bottom dashed lines) distinguishes the wild-type locus (13-kb fragment) from the knockout locus (6.3-kb fragment).

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**FIG. 2. Comparison of the mouse and rat fetuin gene promoters.** Bases 536–1395 of the mouse fetuin gene described in this paper and the corresponding 5’-flanking region of the rat fetuin gene were aligned with CLUSTAL V software using default settings. Identical bases are marked with asterisks; putative transcription factor-binding sites are overlined and labeled following the TRANSFAC data base transcription factor nomenclature. S1 nuclease-protected regions of the rat fetuin promoter (43) are shaded; the TATA box is boxed; and the promoter predicted by sequence analysis software is marked by a thick overline. The transcription start point is marked tsp.
growth factor independence-1, respectively. In the rat fetuin gene promoter, two typical C/EBP sites (X1 and X3) plus a heterogeneous C/EBP/NF-1-like site (X5) separated by two classical NF-1-binding sites (X2 and X4) were identified by footprinting and gel retardation assays (43). In a concerted fashion, these transcription factors drive the strong basal fetuin expression in hepatocytes. C/EBP-like transcription factors likely also mediate the inflammatory cytokine-induced down-regulation of the fetuin gene during acute phase (44), as C/EBP-like enhancer proteins have been shown to regulate the expression of several other acute-phase serum proteins (34).

One NF-1-like factor-binding motif protected from S1 nuclease digestion (X4) (43) provides a rationale for the transient fetuin expression observed in the developing brain of embryonic sheep (45). Notably, multiple classes of NF-1 factors have been implicated in the development of the human brain (38). Considering the postulated involvement of fetuins in the homeostasis of extracellular calcium, a solid understanding of fetuin transcriptional control in response to calcium-regulating hormones like vitamin D is lacking. The cloning of the mouse fetuin gene will facilitate studies of this kind.

We produced mice lacking a functional fetuin gene. To prevent possible activity of partially expressed proteins, we deleted the entire fetuin gene including the region corresponding to the promoter previously determined in rats (32). Southern blot analysis of genomic DNA from the mutant mice confirmed the gene deletion. Consequently, the fetuin null mutant mice with genotype Ahsg^{2/2} contained no immunologically detectable fetuin protein in their sera. Fetuin alleles are independently regulated, as no compensatory up-regulation of the single remaining fetuin gene copy was observed in heterozygotes. In addition, the fact that all heterozygotes displayed half the fetuin serum levels of wild-type mice indicates that the Ahsg locus is not subject to imprinting control mechanisms.

The bone gross morphology appeared normal in both newborn and adult heterozygous and homozygous fetuin deletion mutants. This finding suggests no major role for fetuins in bone formation under physiological conditions. However, the lack of...
Fetuin might impinge on bone fine structure and on bone repair after fracture and during osteoporosis and therefore will be studied in more detail.

The results obtained with serum inhibition of apatite formation correspond well with data previously obtained by immunoaffinity depletion of sera (9). They provide independent genetic evidence to support the hypothesis derived from biochemical experimentation that fetuins are inhibitors of apatite formation in serum. The microcalcification of soft tissues observed in some fetuin-deficient female ex-breeders indeed suggests that fetuins are inhibitors of spontaneous calcification. The fact that microcalcification was only observed in female ex-breeders may be due to increased calcium mobilization during pregnancy to meet the needs of rapidly mineralizing fetal skeletons. Microcalcification was not, however, generally detected in all homozygous null mutation ex-breeders and may be subject to genetic background variation. A full description of this phenomenon therefore awaits the breeding of the fetuin knockout mutation into defined genetic backgrounds.

Fetuins are not the sole inhibitors of apatite formation since spontaneous phase separation in serum and concomitant damage of microvasculature were not generally observed in untreated fetuin null mutant mice. Serum albumin is known to also contribute to a pool of plasma protein inhibitors of phase separation (46). Furthermore, we have identified a third plasma protein, the fetuin-related protein histidine-rich glycoprotein (47), as a potential inhibitor of unwanted mineralization. Finally, a tissue-bound inhibitor of spontaneous calcification has been unequivocally identified by recent genetic experimentation (19). The targeted disruption of the gene for fetuin might impinge on bone fine structure and on bone repair after fracture and during osteoporosis and therefore will be studied in more detail.

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matrix GLA protein in mice resulted in spontaneous lethal calcification of arteries and cartilage. As a whole, these inhibi-
tors proteins readily buffer the physiological fluctuations of Ca\(^{2+}\) concentra-
tion in plasma to prevent phase separation. Whether or not ectopic calcification occurs in Ahsg\(^{-/-}\) mice after artificial elevation of serum Ca\(^{2+}\) levels or exogenous stimulation of calcification is being investigated.

Fetuin functions have been postulated also for biological processes other than bone metabolism. For example, insulin signaling (48, 49), transforming growth factor-β signaling (7), hepatocyte growth factor signaling (8), and lipogenesis (50) have all been proposed to be markedly influenced by fetuin. It is therefore surprising that Ahsg\(^{-/-}\) mice showed no signs of disturbance of any vital functions. Similar unexpected results have been reported in many null mutant mice, and this is considered evidence in favor of backup systems of critically important traits. A number of null mutant mice exist for genes with proposed functions similar to those of fetuin. For example, the singular gene deletion of Ahsg with proposed functions similar to those of fetuin. For example, the singular gene deletion of Ahsg has been described as proteinase inhibitors (52, 53) and as is therefore surprising that have all been proposed to be markedly influenced by fetuin. It hepatocyte growth factor signaling (8), and lipogenesis (50) stimulation of calcification is being investigated.

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