Differential Developmental Expression of the Rat Kininogen Genes[1]

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ABSTRACT. The rat liver expresses two low molecular weight kininogens (T-KG and K-KG). Although they share 90% of the nucleotide sequence in their 5' flanking regions, T- and K-KG genes are differentially regulated. The T-KG gene is inducible, and its protein is a potent thiol-protease inhibitor. In contrast, K-KG gene is expressed constitutively and encodes the precursor of the vasoactive nonapeptide bradykinin. To further elucidate the differential regulation of T- and K-KG genes, we examined their developmental expression in the Sprague-Dawley rat. Northern blots of total liver RNA were probed with oligonucleotides complementary to T and K-KG mRNA under high-stringency conditions. A single T-KG mRNA (1.8 kb) and two K-KG mRNA species (1.6 and 2.3 kb) were consistently detected at all ages studied. Steady state T-KG mRNA levels increased 3.5-fold at birth and remained high during the 1st week of postnatal life only to decline thereafter. T-KG immunoreactivity in the liver and plasma determined by Western blot analysis paralleled T-KG mRNA expression. In marked contrast, K-KG mRNA expression was not altered during the transition from fetal to neonatal life, nor was it affected by postnatal maturation. The results demonstrate that the fetal rat liver synthesizes kininogens and that T- and K-KG genes are differentially regulated during development. Up-regulation of T-KG synthesis after birth may serve a protective function in the newborn via its antiprotease activity. Our previous finding that the tissue kallikrein gene is up-regulated with age is in marked contrast with the constitutive expression of its substrate, suggesting that kallikrein synthesis is the rate-limiting factor for bradykinin formation during postnatal maturation. (*Pediatr Res* 32: 649–653, 1992)

Abbreviations

LMW, Low molecular weight
HMW, High molecular weight
KG, kininogen
TdT, terminal deoxynucleotide transferase
GRE, glucocorticoid response element

The liver is the main organ that synthesizes and releases KG. There are two forms of KG, the HMW and the LMW KG.

HMW and LMW KG mRNA are identical in nucleotide sequence in the 5' terminal portion but diverge completely in the 3'-terminal portion. The two mRNA species are generated from a single gene by differential splicing of the primary transcript (1). In humans, LMW KG is cleaved by tissue kallikrein releasing the vasoactive peptide Lys-bradykinin (2, 3). In the rat, two LMW KG have been characterized (T and K) (4–6). T-KG gene expression is induced dramatically by inflammation and therefore is considered an acute-phase protein (7). T-KG is kallikrein-resistant but is cleaved by T-kininogenase (8) generating T-kinin (isoleucyl-seryl-bradykinin), which shares many pharmacologic properties with bradykinin (9). In contrast, the K-KG gene is not induced by inflammation (10) and is considered the equivalent of human LMW KG (5). Rat HMW and LMW K-KG are cleaved by rat tissue kallikrein to release bradykinin (11).

In addition to biochemical and regulatory differences, T- and K-KG possess different biologic functions. T-KG is a powerful cysteine proteinase inhibitor (12), and its synthesis and release in response to tissue damage and inflammation may serve an important protective function. K-KG, on the other hand, primarily serves as the precursor of vasoactive peptide bradykinin, which plays an important role in mediating pain, capillary permeability, organ blood flow, renal hemodynamics, and solute and water excretion (2, 3).

In the present study, we provide further evidence for the differential regulation of T- and K-KG genes in the rat and speculate on the responsible molecular mechanisms. Specifically, we have investigated the effects of development on these genes for the following reasons: *1*) many tissue-specific genes are developmentally regulated—we wished to determine whether the closely related T- and K-KG genes are differentially regulated during development; and *2*) we have recently reported that the fetal and newborn rat kidneys express the tissue kallikrein gene and its protein (13), and in this study, we wished to determine whether the developing animal synthesizes the kallikrein substrate, K-KG. Given the homology in nucleotide sequence between the rat T- and K-KG (90% in exon-1 and the 5' upstream region), we used gene-specific oligonucleotides to probe for T- and K-KG transcripts. In addition, specific polyclonal T-KG antibodies were used to detect the translational products of the T-KG gene.

MATERIALS AND METHODS

Time-dated pregnant and adult Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, (Wilmington, MA) and fed regular rat Chow (Purina 5012; Ralston-Purina, St. Louis, MO) and tap water until the day of experiments. Fetuses (at 15, 17, 18, 19, and 20 d of gestation) and newborn (at 1, 5, 12, and 20 d of postnatal life) and male adult (150–250 g) rats were studied.

On the day of study, adult rats were killed by decapitation into liquid nitrogen. For the ontogeny studies, pregnant rats were

anesthetized with intraperitoneal pentobarbital (5 mg/100 g body weight); the fetuses were delivered through a midabdominal incision and immediately decapitated into liquid nitrogen. The mothers were subsequently killed by intracardiac injection of a saturated solution of potassium chloride.

Western blot analysis. Livers were pooled from five to six rats in each age group, whereas the plasma was collected from newborn and adult rats only. The livers were removed, rinsed thoroughly, and homogenized in PBS (pH 7.2) on ice. Homogenates were centrifuged at 600 × g for 20 min (4°C), and the supernatant was treated with deoxycholate (0.5% wt/vol) for 30 min at room temperature and centrifuged at 20 000 × g for 30 min at 4°C. The samples were subsequently assayed for protein concentrations according to the method of Lowry *et al.* (14) using BSA as a standard.

Aliquots of liver extract (50–100 µg, total protein) were separated by 3–12% stacking SDS-PAGE [12% acrylamide, 5 mM Bis (Bio-Rad Laboratories, Richmond, CA), 0.38 M Tris-HCl, pH 8.8, 0.1% SDS, 0.1% ammonium persulfate, and 0.025% TEMED (Bio-Rad)]. Rainbow molecular weight markers (14 300–200 000; Amersham, Arlington Heights, IL) were used to determine approximate molecular weight. Electrophoresis was carried out at 60 V for 3 h, in duplicate. One gel was stained with 0.1% Coomassie blue stain R250 to visualize the protein bands. The proteins from the second gel were electrophoretically transferred to nitrocellulose in a Genie Electrophoretic Blotter (Idea Scientific, Corvallis, OR) at 20 V for 90 min. Transfer buffer is Tris-glycine (25 mM Tris, 0.2 M glycine), pH 8.3, in 20% methanol. After transblotting, the nitrocellulose was washed in PBS, pH 7.2, and incubated overnight at 4°C in PBS containing 3% BSA as a blocking agent. After rinsing in 0.05% Tween 20 in PBS for 20 min at room temperature, the nitrocellulose was incubated with rabbit antirat T-KG antiserum (15) (diluted 1:1000 in PBS-Tween 20) for 2 h at room temperature. The nitrocellulose was then washed in PBS-Tween 20 followed by PBS alone and incubated at room temperature for 1 h with antirabbit IgG peroxidase conjugate raised in goat (Sigma Chemical Co., St. Louis, MO; diluted 1:2000 in PBS-Tween 20 with 3% BSA). After washing the blots in PBS, visualization of antigen-antibody reaction products was accomplished by adding 60 mg of 4-chloro-1-naphthol dissolved in 20 mL methanol to 0.05% H_2O_2 in PBS.

Northern blot analysis using T- and K-KG oligonucleotide probes. Total liver RNA was extracted using the guanidinium isothiocyanate-phenol-chloroform method of Chomczynski and Sacchi (16) from fetuses at 2 and 1 d before birth ($n = 10$/group); newborns at postnatal d 1 ($n = 8$), d 5 ($n = 8$), d 12 ($n = 7$), and d 20 ($n = 7$), and adults ($n = 6$). RNA was quantified spectrophotometrically by absorbance at 260 and 280 nm. Only samples with $A_{260/280}$ ratio over 1.8 were used. The extracted RNA was pooled for each age group of animals for the Northern blot analysis, whereas RNA extracted from individual animals was used for further quantitative measurements by slot blot analysis ($n = 4$–6/group). RNA was dissolved in sterile water and stored at $-70°C$ until use. Precipitated denatured RNA (15–20 µg) was subjected to 1.2% agarose/2.2 M formaldehyde gel electrophoresis. RNA was then transferred into a positively charged nylon membrane (Zetabind; AMF Cuno, Inc., Meriden, CT) and cross-linked by exposure to UV light for 1 min. Equal transfer of RNA to the membrane was confirmed by UV shadowing at 254 nm. Slot blots were prepared by dissolving 2–0.5 µg of total RNA in 0.5 mL of sterile 25-mM sodium phosphate buffer (pH 7.2) and applying this RNA to a Zetabind membrane preequilibrated with the same buffer by use of a Minifold II Slot-Blotter (Schleicher & Schuell, Keene, NH). The blot was allowed to dry at room temperature, and equal RNA loading was confirmed by UV shadowing at 254 nm.

The DNA probes consisted of synthetic HPLC-purified oligomers complementary to T-KG and K-KG mRNA (6). The TKG oligonucleotide sequence is 5'-GACACAGAGGTCCT-

CCTCTGTCTTCTTA-3'; the K-KG oligomer sequence is 5'-CAGACAGTGGTACTCGTTTGTCACTATG-3'. The oligonucleotides were 3'-end-labeled with α-^{32}P-deoxy ATP using the enzyme TdT as described by Henderson *et al.* (17) with few modifications. The reaction mixture contained 30 ng (1.5 µL) of oligonucleotide, 2.5 µL RNase-free water, 4 µL of 5× TdT buffer (BRL Gibco, Grand Island, NY), 10 µL (33 pmol) of α-^{32}P-deoxy ATP (6000 Ci/mmol, DuPont-New England Nuclear, Boston, MA), and 30 units (2 µL) of TdT (BRL, Gibco). After incubation at 37°C for 1 h, the reaction was stopped by adding 10 µL of ice-cold RNase-free water. Unincorporated nucleotides were removed from the reaction mixture using NENSORB-20 cartridges by following the manufacturer's recommendations (DuPont-New England Nuclear). Sp act was 1–3.3 × 10⁹ cpm/µg of DNA.

Northern blots were prehybridized at 54°C for 4 h in 1 × SSPE (0.15 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA, pH 7.4), 2 × Denhardt's solution, 10% dextran sulfate, 2% SDS, and 200 µg/mL herring sperm DNA. The labeled probe was added at a final concentration of 2 × 10⁶ cpm/mL, and hybridization was carried out at 54°C for 40 h. Posthybridization washes consisted of 1 × SSPE/0.5% SDS and 0.2 × SSPE/1% SDS at room temperature (30 min each) and 0.1 × SSPE/0.5% SDS (30 min at 54°C). Signals were detected by autoradiography (Kodak XOMAT-AR and -RP films Eastman Kodak Co., Rochester, NY) in the presence of one or two image-intensifying screens and quantified by scanning densitometry. The Northern blots were stripped off the oligonucleotide probe (0.1 × SSPE/0.5% SDS for 60 min at 95°C) and reprobed for the constitutively expressed nonmuscle β-actin gene using a β-actin cDNA (18) labeled with α-^{32}P-deoxycytidine triphosphate by random primer technique (19).

Comparisons of the densitometric values in the slot blots among the age groups were performed by one-way analysis of variance and Newman-Kuels test. Statistical significance is defined as $p < 0.05$, and the results are presented as means ± SEM.

RESULTS

Fetal rat liver expresses T- and K-KG genes. It is well established that the adult rat liver expresses the T- and K-KG genes (10). Recently, Mann and Lingrel (20) demonstrated that the fetal liver expresses T-KG mRNA. In this report, we confirm their results (Fig. 1) and show that the T-KG oligonucleotide hybridized to a single 1.8-kb mRNA species and that the relative amounts of T-KG mRNA in the near-term fetal liver are at least as large as those in the adult liver.

We also show that the fetal liver expresses K-KG mRNA transcripts (Fig. 1). The K-KG oligonucleotide hybridized consistently to two mRNA species (1.6 and 2.3 kb). We have also observed on occasion a band of about 4.0 kb. The 1.6-kb mRNA is considered to be the equivalent to the human LMW KG, whereas the higher mRNA species represent the HMW KG (6, 10).

Effect of birth on T- and K-KG expression. Birth had a stimulatory effect on the T-KG gene. T-KG steady state mRNA accumulation increased by more than 3.5-fold from late prenatal to postnatal life (Figs. 1 and 2). In contrast, hepatic K-KG gene expression did not appear to change during the transition from fetal to neonatal life (Fig. 1). Densitometric analysis of RNA slot blots of individual livers indicated that the 1- and 5-d-old newborn rats expressed significantly more T-KG mRNA than the late gestation fetuses ($p < 0.001$), whereas K-KG mRNA levels remained relatively stable during that period (Fig. 2).

Postnatal expression of KG genes. After delivery, T-KG mRNA levels peaked on d 1 of postnatal life and remained expressed at high levels during the first 5 d after birth (Figs. 1 and 2). The adult liver had the lowest steady state levels of T-KG mRNA across all age groups. In contrast, expression of the 1.6- or 2.3-kb K-KG mRNA did not change appreciably from the early postnatal period to adulthood (Figs. 1 and 2).
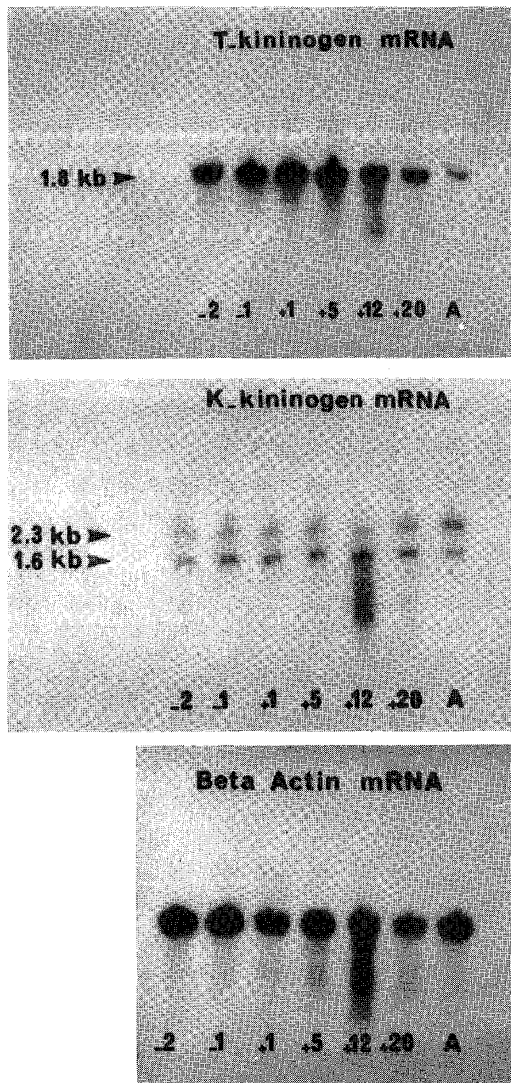
Fig. 2. Densitometric results of RNA slot blot analysis of T- and K-KG mRNA during liver maturation (means ± SEM; $n = 4–6$/age group). Negative numbers = days before birth; positive numbers = days after birth. A value of 1 was arbitrarily assigned to the level of mRNA present at 2 d before birth. $*p < 0.001$ vs d −2, +12, +20, and adult; $\#p < 0.05$ vs d −1.

Fig. 1. Ontogeny of T- and K-KG gene expression. Northern blots of total liver RNA (15–20 µg/lane) were probed with ^{32}P-labeled T- and K-KG oligonucleotides or β-actin cDNA. Negative numbers = days before birth; positive numbers = days after birth; A, adult. The near-term fetus expresses both T- and K-KG mRNA. The transition from fetal to newborn life up-regulates T-KG mRNA expression (top). In contrast, K-KG mRNA species (1.6 and 2.3 kb) did not change with maturation (middle). Bottom panel shows liver β-actin mRNA expression.

Hepatic and circulating T-KG protein expression during maturation. Western blot analysis of liver proteins using the polyclonal T-KG antibody revealed a 68-kd immunoreactive band that was first detectable at d 17 of fetal life (4 d before birth) (Fig. 3, *upper panel*). HMW KG was not detected, confirming the specificity of the antibody for LMW KG. T-KG immunoreactivity increased toward the end of gestation. Similar to the expression of its mRNA, T-KG protein levels peaked on the day after birth, remained relatively high during the first 5 d of postnatal life, and were followed by an age-related decline in T-KG immunoreactivity.

The temporal expression of T-KG was qualitatively similar in the liver and plasma. Plasma proteins obtained from newborn and adult rats expressed a 68-kd immunoreactive band when probed with the T-KG antibody (Fig. 3, *lower panel*). The intensity of the 68-kd band was higher in newborn than adult rats, indicating that synthesis and release of hepatic T-KG are coupled throughout postnatal maturation.
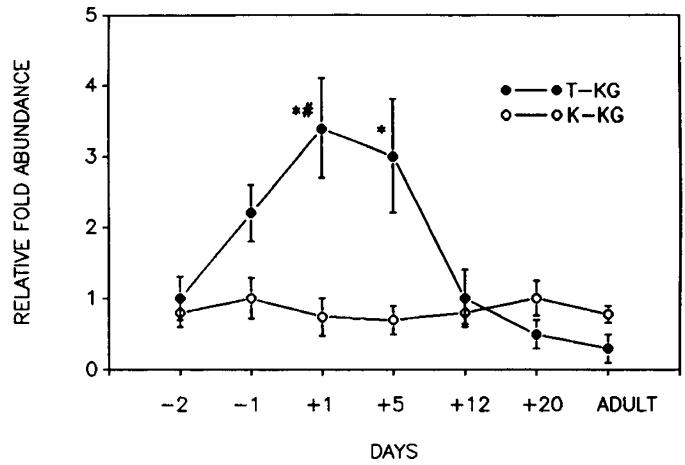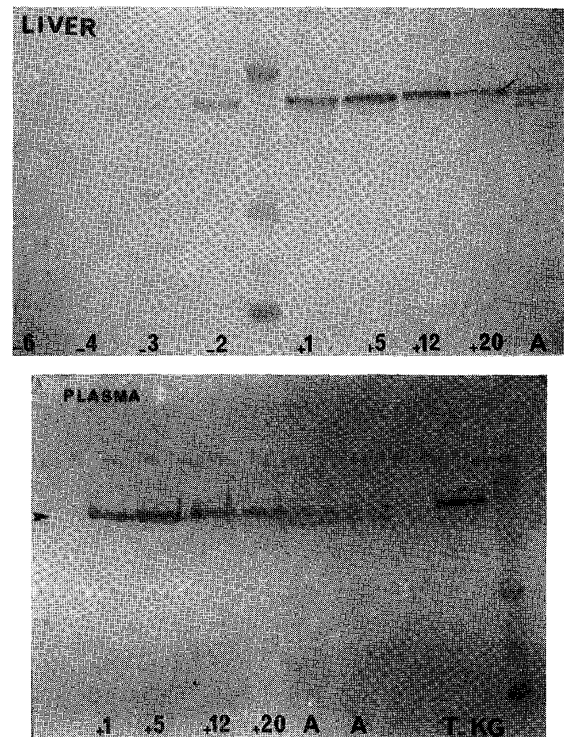

Fig. 3. Maturational changes in T-KG protein expression. Western blot of liver protein extracts (100 µg total protein/lane) was probed with polyclonal T-KG antibody (*top*). Western blot of plasma proteins (50 µg total protein/lane) was probed with the polyclonal T-KG antibody (*bottom*). Negative numbers = days before birth; positive numbers = days after birth.

DISCUSSION

Compared with other species, including humans, the rat is unique in that this species expresses more than one LMW KG gene (5, 6). The rat is the only species known to express the T-KG gene. This gene is expressed in a tissue-specific manner and is induced by inflammation (7, 10). More recently, the hepatic T-KG gene was also found to be developmentally regulated; its expression is highest during the immediate postnatal period and decreases significantly thereafter (20). The rat K-KG gene, on the other hand, is the counterpart of human and bovine

LMW KG, the substrate for tissue kallikreins, and the precursor of the vasoactive peptide bradykinin (5). This gene does not encode an acute-phase protein and is not up-regulated in response to tissue injury (10).

The most important finding of the present study is that despite substantial nucleotide sequence homology between the two KG genes, only the T-KG gene is subject to developmental regulation, whereas the gene encoding K-KG does not appear to be under significant developmental control. Importantly, much of the developmental changes in T-KG occurred around the time of birth, suggesting that perinatal events may be involved in this effect. Stress appears to be a common inducer of T-KG gene expression; tissue injury and inflammation are powerful inducers of T-KG (7, 15). Preliminary results from our laboratory also showed a significant up-regulation of hepatic T-KG but not K-KG gene expression in response to chronic unilateral ureteral obstruction in the rat (manuscript in preparation). Whether the stress of delivery (hypoxia and acidosis) plays a role in mediating the perinatal changes in T-KG gene expression remains to be determined. The presence of multiple GRE in the T-KG upstream region also suggests that the perinatal surge in glucocorticoid secretion may be involved in stimulating T-KG expression at the transcriptional level (20).

Control of gene transcription is mediated by protein-protein and protein-DNA interactions that, for the most part, take place in the 5' upstream region of the target gene (21–23). Comparison of T- and K-KG genes reveals that the two genes have 90% sequence homology in their 1st exons and in the 5' flanking regions; the homology decreases to about 50–60% toward the 3' regions (6). A TATA box-like sequence (a consensus sequence present about 25–35 bp from the start site of most genes that accounts for a major part of basal promoter activity) is not found in the 5' flanking regions of either T- or K-KG genes. Other common promoter consensus sequences (CCAAT and GC boxes) are also absent. Rather, the two genes contain AT-rich sequences (AT content is higher in T-KG) about 28 bp from the transcription initiation site. Eight GRE are present in their upstream regions. Reporter gene transfection studies revealed that one of these GRE located at −119 to −124 (from transcription start site) is able to confer full inductivity to exogenous glucocorticoids (24).

It is possible that, given the appropriate developmental stimulus (*e.g.* labor or birth), *de novo* expression or activation of sequence-specific DNA-binding protein(s) occurs. To promote differential cell-specific (hepatocyte) expression of T- and K-KG genes, however, one must assume that the induced transcription factor(s) is extremely specific for its cognate *cis*-acting elements in that it recognizes even a single base difference between the upstream regulatory regions of the two genes. An example of this would be in the consensus sequence for the glucocorticoid receptor present at nucleotides −65 to −69. This sequence matches the putative GRE (5'-TGTTCT) in the T-KG gene. In the K-KG gene, the C in the latter sequence is replaced by T. Alternatively, because the T and K genes diverge in sequence in the 3' portions of their sequence, it is conceivable that the transcriptional regulation is mainly exerted in that portion of the gene rather than at the 5' end. The developmental expression of KG genes may also be regulated at the transcriptional level via a temporally programmed demethylation of CpG bases (25). Several liver-specific genes are subject to demethylation after their activation in the fetal liver, including the rat phosphoenolpyruvate carboxykinase and albumin genes (26, 27). It is believed that these genes are first activated by DNA methylation-independent mechanisms; this is followed by gene demethylation, which now allows the transcription to proceed independent of the initial differentiation factors. Further studies are needed to explore all these possibilities.

The Western blotting results demonstrate that the fetal liver is capable of translating the KG message into its proper molecular mass protein and secreting it into the circulation. The physiologic

significance of the postnatal surge in hepatic T-KG synthesis is not known. However, given that T-KG is an inhibitor of lysosomal cysteine proteinases (cathepsins) (12), it seems likely that the high tissue and circulating levels of antiprotease activity serve a protective role in the newborn animal. In this regard, it has been proposed that a low pH environment generated in a restricted space outside the cell may activate these proteinases, which in turn can activate latent collagenases, thereby playing an indirect role in degrading the extracellular matrix (28).

The present study is the first to describe the developmental expression of the K-KG gene that encodes for the kallikrein substrate. We detected K-KG mRNA sequences in the near-term fetal and neonatal liver. Unlike T-KG, however, K-KG mRNA levels did not change appreciably with maturation. Unfortunately, the lack of antibodies against rat K-KG prevented us from evaluating the expression of the K-KG protein. We have recently demonstrated that the fetal and newborn kidneys express the true tissue kallikrein gene and that renal kallikrein synthesis increases 6-fold during the transition from newborn to adult life (13). Similar findings were reported for kallikrein mRNA in the submandibular gland (29). Thus, considering the constant levels of K-KG expression during maturation, it appears that the rate of kallikrein synthesis is probably most important in determining the final activity of the kallikrein-kinin system during normal growth.

In conclusion, the present study shows that the fetal rat liver expresses the kininogen genes. However, only the T-KG is subject to developmental control and is up-regulated in the immediate postnatal period. In contrast, the K-KG gene is expressed in a constitutive manner and is not induced by maturation. Studies of the developmental expression of kininogen genes will not only enhance our understanding of the role of kinins during early life, but will likely provide a suitable model for the study of differential gene regulation in eukaryotes.

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