Differential Effects of Ureteral Obstruction on Rat Kininogen Gene Family\(^{1,2}\)

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ABSTRACT

The precursors of kinins, K-kininogens and T-kininogens (KG), are encoded by separate genes that display 90% nucleotide sequence homology. Despite their homology, K-KG and T-KG genes are differentially regulated. The K-KG gene is expressed constitutively and encodes high- and low-molecular-weight KG, the precursors of the vasoactive nonapeptide bradykinin. In contrast, the T-KG gene is inducible, and its protein is a potent thiol-protease inhibitor. Given their potential role in the regulation of blood pressure, renal hemodynamics, and the response to inflammation and tissue injury, K-KG and T-KG gene expression in rats subjected to chronic (1 or 5 wk) unilateral ureteral obstruction (UUO), a maneuver that suppresses renal kallikrein synthesis to 25% of controls, has been examined. Northern and slot blots of total liver and kidney RNA were probed with oligonucleotides complementary to either T-KG or K-KG mRNA under high-stringency conditions. Steady-state levels of hepatic T-KG mRNA were increased in the UUO compared with sham-operated rats—2.7-fold at 1 wk and 4.1-fold at 5 wk (\(P < 0.05\)). Western blot analysis revealed that the 68-kd T-KG protein was up-regulated 2.5- to 3-fold in the liver of UUO rats (\(P < 0.05\)). In marked contrast, the abundance of high (2.3-kb)- and low (1.6-kb)-molecular-weight splicing transcripts of hepatic pre-K-KG mRNA was not altered at either time after UUO. The differential expression of T-KG and K-KG genes was also observed in the obstructed kidneys, which displayed a tissue-specific induction of T-KG gene only. Further analysis with a direct radioimmunoassay and immunoblotting indicated that the obstructed kidneys contained sevenfold higher levels of immunoreactive T-KG than sham-operated kidneys (\(P < 0.02\)). Stimulation of T-KG synthesis was not accompanied by any significant changes in either kidney interleukin-6 (an inducer of T-KG transcription) or hepatic interleukin-6 receptor gene expression. It was concluded that UUO exerts differential gene- and tissue-specific effects on the expression of rat KG gene family. The sustained production of high- and low-molecular-weight KG (bradykinin substrates) may help to partially offset the effects of suppressed kallikrein synthesis in the vasoconstricted obstructed kidney. It was speculated that the up-regulation of T-KG synthesis in UUO rats serves to protect the renal cells against cysteine proteases released from obstructed nephrons.

Key Words: Kallikrein-kinin system, kininogen, cysteine protease inhibitors, hydronephrosis, gene expression, interleukin-6

Kininogens (KG) are glycoproteins synthesized mainly in the liver and are the precursors of endogenous kinins. Most mammals, including human, bovine, rat and mouse, express the K-KG gene. K-KG pre-mRNA is differentially spliced into two transcripts that encode for high (H)- and low (L)-molecular-weight KG (1). HKG is the preferred substrate for plasma kallikrein, whereas LKG is preferentially cleaved by tissue kallikrein to yield bradykinin (rat, mouse) or lys-bradykinin (human and cow). Kinins play an important role in the regulation of vascular tone and growth and capillary permeability and pain and modulate coronary, mesenteric, and renal blood flow, as well as urinary water and electrolyte excretion (2).

We have recently reported that experimental hydronephrosis in the rat, resulting from chronic unilateral ureteral obstruction (UUO), was associated with the suppression of the intrarenal kallikrein-kinin system (3). The down-regulation of kallikrein-kinin activity was suggested by a 75% reduction in renal kallikrein expression at both the mRNA and protein level, accompanied by up-regulated kininase II activity in both plasma and kidneys (3). The effects of UUO on plasma and tissue kallikrein substrates (HKG and LKG) are not known. Because the ultimate
effects of UUO on kinin production depend not only on tissue kallikrein but also on the availability of its substrate, the first aim of this study was to determine whether ureteral obstruction affects the expression of K-KG mRNA (HKG and LKG), the precursors of endogenous bradykinin.

The rat is unique in that this species expresses an additional KG, T-KG, which has a molecular weight similar to that of LKG (4). Two closely related T-KG gene products, T1 and TII, have been described (5). Nucleotide sequence comparison has revealed extremely high sequence homology (>90%) between the T-KG and K-KG genes (5,6). Interestingly, despite their extensive sequence homology, the K-KG and T-KG genes display differential responses to a variety of stimuli. T-KG gene expression is induced by inflammation and tissue injury (7,8) and is highly active during early development (9,10). In contrast, K-KG gene expression is constitutive, indicating that the two KG serve different functions. Also, whereas K-KG is cleaved by kallikreins, T-KG is not. Instead, T-KG is hydrolyzed by another member of the kallikrein gene family called T-kininogenase (11), which has recently been cloned from rat kidney and the submandibular gland (12). The physiologic functions of T-KG are not known. T-kinin (Ile-Ser-bradykinin), like bradykinin, is a vasodilator (13). However, it is unclear whether the conversion occurs in vivo. More important is the fact that T-KG itself is a potent inhibitor of lysosomal cysteine proteinases such as cathepsin B (14). Therefore, it has been proposed that T-KG may function as a "healing protein" to reduce the degradative actions of cathepsin-like enzymes released by tissue injury (15). Because prolonged ureteral obstruction causes ischemic and pressure necrosis of renal cells (and presumably the release of lysosomal enzymes), we hypothesized that the T-KG gene is preferentially induced in the setting of obstructive renal injury.

Given the homology in nucleotide sequence between the rat T-KG and K-KG (90% in exon-1 and the 5' upstream region), we used gene-specific oligonucleotides to probe for T-KG and K-KG transcripts. In addition, specific T-KG antibodies were used to detect the translational products of the T-KG gene. The results demonstrate that obstructive renal injury is accompanied by differential and tissue-specific effects on the genes of the rat KG family.

**MATERIALS AND METHODS**

Adult male Sprague-Dawley rats (7 to 9 wk of age, 150 to 200 g) were purchased from Charles Rivers Laboratories (Wilmington, MA) and were allowed free access to standard rat chow (Purina) and water. Young adult rats were studied to avoid the natural increase in hepatic and circulating T-KG levels that is observed during aging in the Sprague-Dawley rat (16). In addition, only male rats were included in the study because female rats are known to produce more T-KG than males (17).

Animals were subjected to either UUO or sham surgery and were studied either at 1 wk (UUO, 1 wk, N = 5; sham, 1 wk, N = 4) or at 5 wk after surgery (UUO, 5 wk, N = 7; sham, 5 wk, N = 5). Complete obstruction of the left ureter was performed as previously described (3). Briefly, with the rat under general anesthesia with pentobarbital sodium (50 mg/kg body wt ip), the left ureter was ligated midway between the bladder and the renal pelvis. In sham-operated rats, the ureter was manipulated but not ligated. None of the rats developed signs of wound or systemic infection. Random urine cultures were obtained periodically by suprapubic needle aspiration and were negative.

**RNA Extraction, Gel Electrophoresis, and Filter Hybridization**

Total tissue RNA was extracted from the liver, kidney, and other tissues by the guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (18). The extracted RNA was quantified spectrophotometrically (Beckman DU 640) by absorbance at 260 nm, dissolved in sterile water, and stored at -80°C until use. For Northern blot analysis, total RNA was pooled for each tissue from four to seven rats per group, and the precipitated denatured RNA was subjected to electrophoresis on a 1.2% agarose gel containing 2.2 M formaldehyde. Equal RNA loading and the integrity of RNA were determined by ethidium bromide staining and visualization of intact 28S and 18S ribosomal RNA. RNA was transferred to a positively charged nylon membrane (Zetabind; AMF Cuno, Meriden, CT) with a vacuum blotter (Vacu gene XL; Pharmacia, Piscataway, NJ) and cross-linked by exposure to UV light for 1 min. Equal transfer of RNA samples was documented by UV transillumination of the membrane at 254 nm. To provide a more quantitative assessment of gene expression, slot blots of total tissue RNA from the individual animals in each group (N = four to five per group) were prepared and hybridized to the oligonucleotide and cDNA probes (see below). Slot blots were performed by dissolving 1 to 4 μg of RNA in 0.5 mL of sterile 25 mM sodium phosphate buffer (pH 7.2) and applying this RNA to a Zetabind membrane prequilled with the same buffer with 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 oligonucleotides complementary to T-KG and K-KG mRNA (6,10). Complementary cDNA for LKG is available (19) but was not used because of the high
sequence homology of K-KG and T-KG. The T-KG oligonucleotide sequence is 5'-GACACAGAGGTCCTCCTGTCCTCTATA-3' and recognizes both TI and TII mRNA; the K-KG oligomer sequence is 5'-CAGACAGFGCTACTGTTGACTA-3' and recognizes both transcripts of the K-KG gene (i.e., HKG and LKG). The oligonucleotides were 3'-end-labeled with [α-32P]dATP with terminal deoxynucleotidyl transferase (TdT) (10,20). The reaction mixture contained 30 ng (1.5 μL) of oligonucleotide, 2.5 μL of RNase-free water, 4 μL of 5× TdT buffer (BRL, Gibco, Grand Island, NY), 10 μL (33 pmol) of [α-32P]dATP (6,000 Ci/mmol; DuPont, NEN Research Products, Boston, MA), and 30 U (2 μL) of TdT (BRL, Gibco). After incubation at 37°C for 1 h, the reaction was stopped by the addition of 10 μL of ice-cold RNase-free water. Unincorporated nucleotides were removed from the reaction mixture with NENSORB-20 cartridges by following the manufacturer's recommendations (DuPont, NEN Research Products). Specific activity was 107 to 2 × 10⁸ cpm/μg of DNA.

Northern and slot blots were prehybridized at 54°C for 4 h in 1 X SSPE (0.15 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA; pH 7.4), 2 X Denhardt's solution, 10% dextran sulfate, 2% sodium dodecyl sulfate, 0.001 M EDTA; pH 7.4), 2 X Denhardt's solution, 10% dextran sulfate, 2% sodium dodecyl sulfate (SDS), and 200 μg/mL of 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 X SSPE/0.5% SDS and 0.2 × SSPE/1% SDS at room temperature (30 min each) and 0.1 × SSPE/0.5% (30 min at 54°C). Signals were detected by autoradiography (XOMAT-AR and XOMAT-RP, Eastman Kodak Co., Rochester, NY) in the presence of one or two image-intensifying screens and quantified by scanning densitometry (Pharmacia LKB, UlstroScan).

Because interleukin (IL)-6 is implicated in the modulation of T-KG gene expression (21) and tissue IL-6 and its receptor are induced by inflammation (22,23), the filters were stripped off the oligonucleotide probe (0.1 X SSPE/0.5% SDS for 60 min at 95°C), and rehybridized with 32P-labeled rat IL-6 (24) and human IL-6 receptor (25) cDNA. Previous studies have shown that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression is not altered by UUO (13,26); therefore, this gene was used as the internal standard.

**Western Blot Analysis of Liver and Kidney T-KG**

The livers and kidneys were removed, rinsed thoroughly in phosphate-buffered saline (PBS), and homogenized in PBS (pH 7.2) on ice. Homogenates were centrifuged at 600g 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 20,000g for 30 min at 4°C. The samples were subsequently assayed for protein concentrations by the method of Lowry et al. (27) with bovine serum albumin (BSA) as a standard.

Aliquots of liver and kidney extracts from each individual rat (50 μg, total protein) were separated by 3 to 12% stacking SDS-polyacrylamide gel electrophoresis (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 to 200,000; Amersham Corp., 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 electrophotically transferred to nitrocellulose in a Genie Electrophoretic Blotter (idea Scientific, Corvallis, OR) at 10 V for 90 min. Transfer buffer is Tris-glycine (25 mM Tris, 0.2 M glycine; pH 8.3) in 20% methanol. Nonspecific binding sites were blocked by incubation of the blots overnight at 4°C in PBS that contained 5% BSA and 0.1% Tween. The blots were washed in PBS-0.1% Tween twice briefly, once for 15 min, and three times 5 min each, all at room temperature. Incubation with the primary antibody (rabbit anti-rat T-KG; 1:10,000) was performed in PBS containing 5% BSA and 0.1% Tween for 1 h at room temperature. The blots were subsequently washed in PBS-0.1% Tween at room temperature in a manner similar to the washes described above. After the washings, the blots were incubated with the secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit immunoglobulin G from Sigma; 1:30,000) for 1 h at room temperature, followed by two 15-min and five 5-min washes in PBS-0.1% Tween. Equal volumes of detection reagents 1 and 2, provided for enhanced chemiluminescence Western blotting (Amersham), were mixed. Detection was accomplished by incubation of the blot with this mixture for 1 min. After the excess fluid was drained, the blot was covered with plastic wrap and exposed to x-ray film (Hyperfilm-ECL; Amersham). The expression of immunoreactive LKG (substrate for tissue kallikrein) was not studied because specific antibodies against rat LKG are not available. Kidney T-KG levels were measured by a direct RIA, as previously described (19,28).

**Data Analysis**

After autoradiography, the intensity of each signal on the Northern and slot blot autoradiograms was measured by optical density recorded on an LKB XL laser densitometer. The abundance of mRNA signals in Northern blots was determined by scanning down
the center of each lane on the autoradiogram and was expressed in densitometry units. The densitometric units were defined arbitrarily as the area under the absorbance curve. Because the RNA samples in the Northern blots were pooled from the individual rats in each group (four to seven per group), comparisons between groups are expressed as fold difference. Further quantitative analysis of mRNA levels for liver and kidney K-KG and T-KG mRNA and for IL-6 and IL-6 receptor mRNA was performed by slot blot hybridization of RNA obtained from individual rats (N = four to five per group). For each tissue sample, three measurements of signal intensity at different dilutions of RNA (1 to 4 μg) were obtained. The dilutions to be analyzed were chosen to ensure that comparisons were performed on the linear portion of the dose response curve. Densitometric values were factored for those of GAPDH.

Western blots of K-KG and T-KG in liver and kidneys contained samples from individual rats. The quantitation of immunoreactive protein signals on Western blots was performed by the densitometric analysis of autoradiograms, as described for the RNA blots. Comparisons between sham and UUO groups were performed by analysis of variance and Scheffe test, and comparisons between the left and right kidneys in each group were performed by paired t test. A P value of less than 0.05 was considered significant. All data are presented as means ± SE.

RESULTS

Effect of Chronic UUO on mRNA Expression of Hepatic T- and K-KG

UUO was associated with differential effects on hepatic KG gene expression. One week after UUO, T-KG mRNA levels were up-regulated 2.7-fold (P < 0.05) and rose to 4.1-fold by 5 wk compared with sham-operated rats (P < 0.02) (Figures 1 and 2). In contrast, K-KG gene expression did not change during the course of UUO. Both HKG (2.3 kilobase [kb]) and LKG (1.6 kb) mRNA were expressed in abundant amounts in the liver of control rats. However, the levels of the two transcripts were not affected by UUO at either 1 or 5 wk. According to Northern blot analysis, the abundance of HKG mRNA was twofold higher than LKG in both sham and UUO groups.

Effect of UUO on Hepatic T-KG

Western blot analysis of liver proteins with the polyclonal T-KG antibody revealed an immunoreactive doublet (68 kd), which corresponds to the known molecular weight of T1 and TII KG. HKG was not detected with this antibody, confirming its specificity for T-KG. Densitometric analysis of the immunoreactive bands showed a 2.5- to 3-fold higher expression...
of T-KG in the livers of UUO than sham-operated rats ($P < 0.05$) at both 1 and 5 wk (Figure 3). The increase was evident in both T1 and TII bands.

**Effect of UUO on Renal T-KG**

Using a direct RIA developed for rat T-KG, we detected immunoreactive KG in kidney extracts. KG levels were sevenfold higher in the obstructed compared with the sham-operated kidneys. The contralateral kidneys also manifested a small but significant increase in KG content compared with sham kidneys (Figure 4). To delineate the identity of the immunoreactive KG species, Western blots of kidney protein extracts were probed with the polyclonal T-KG antibody. The results showed a 68-kd band (doublet) that was fourfold higher in abundance in the obstructed than in the sham kidneys ($P < 0.01$) (Figure 5). To determine whether the increase in T-KG immunoreactivity is due to enhanced intrarenal synthesis, Northern and slot blots of total kidney RNA were hybridized to the T-KG oligonucleotide probe (Figure 6). The results showed that the kidney expresses the T-KG gene and that T-KG mRNA levels

![Figure 3](image3.png)

**Figure 3.** Western blot analysis showing the abundance of hepatic immunoreactive T-KG in UUO and sham-operated rats 1 and 5 wk after surgery. Tissue samples of 50 μg were separated on SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and immuno-oblotted with a polyclonal anti-rat T-KG antibody. After incubation with a secondary antibody, proteins were detected with enhanced chemiluminescence, exposed to an autoradiogram film, and photographed. T-KG levels, determined by densitometric analysis, were 2.5- to 3-fold higher in the livers of UUO versus sham-operated rats.

![Figure 4](image4.png)

**Figure 4.** Renal T-KG contents by direct RIA at 5 wk after UUO.

![Figure 5](image5.png)

**Figure 5.** Western blot analysis of T-KG in the kidney. L, left (obstructed) kidney; R, right (contralateral) kidney. The lower molecular weight bands are degradation products. Two immunoblots were spliced together to allow the visual evaluation of a larger number of samples.

![Figure 6](image6.png)

**Figure 6.** Effect of 5-wk UUO or sham operation on renal T-KG gene expression. Northern blot of total RNA pooled from four to seven rats per group (15 μg/lane) was hybridized to a 32P-labeled T-KG oligonucleotide. L, left (obstructed) kidney; R, right (contralateral) kidney. The lower panel shows UV illumination of the nylon membrane after RNA transfer and confirms the quality of RNA and equal loading. There was a twofold increase in T-KG mRNA in the obstructed kidney compared with other kidneys.
were up-regulated twofold in the obstructed kidney compared with the kidneys of sham-operated rats ($P < 0.05$), findings that are consistent with the results of RIA and Western analysis. Although other tissues (e.g., lung, heart) contained detectable T-KG message, abundance of T-KG mRNA in these tissues was not affected by UUO (not shown).

**Effects of UUO on Kidney IL-6 and Hepatic IL-6 Receptor Gene Expression**

Northern and slot blots of kidney and liver RNA isolated from UUO and sham-operated rats were hybridized to labeled IL-6 and IL-6 receptor cDNA. The results revealed that the message abundance of hepatic IL-6 receptor was not different in the obstructed compared with other kidneys (Figure 7). Similarly, kidney IL-6 mRNA levels were not altered by UUO (Figure 8).

**DISCUSSION**

This study demonstrates that chronic urinary obstruction differentially modulates the expression of KG genes in the rat. Specifically, UUO promotes a tissue-specific induction of the hepatic and renal T-KG gene, resulting in the enhanced accumulation of T-KG mRNA and its 68-kd protein. In contrast, K-KG gene expression and the abundance of hepatic HKG and LKG mRNA are not affected by chronic UUO.

The use of gene-specific oligonucleotide probes permitted us to investigate the differential regulation of two highly homologous KG mRNA transcribed from two different genes. We found that the liver is the main source of KG production, even during conditions of stimulated KG expression. Similar to our findings, Chao et al. (19) studied T-KG gene expression in a model of severe chemical lung injury in the rat and reported up-regulation of T-KG gene expression in the liver, lungs, and kidneys, although the renal levels were reportedly low.

In a previous study in rats subjected to the same experimental protocol outlined here, we demonstrated that chronic UUO for 1 or 5 wk suppresses renal kallikrein gene expression and reduces intrarenal kallikrein levels to 25% of sham-operated kidneys (3). Also, circulating and renal kininase II activity was sixfold and twofold higher in the obstructed as compared with sham-operated rats, respectively (3). The combination of low intrarenal kallikrein and enhanced kinin-degrading activity in the chronically obstructed kidney suggested a state of "intrarenal kinin depletion." This study indicates that the mRNA encoding the kallikrein substrates (HKG, LKG) continue to be produced at control levels in the setting of UUO. Thus, the K-KG gene is not affected by obstructive renal injury, a finding that is at variance with the effect of UUO on the tissue kallikrein gene (3). These and other results (10) documenting dramatic alterations in tissue kallikrein but not its substrate under various physiologic and pathophysiologic conditions suggest that the regulation of the tissue kallikrein-kinin system is mainly exerted at the levels of kallikrein and possibly kininases.

In contrast to the K-KG gene, the T-KG gene is regulated by UUO. The mechanisms responsible for the induction of T-KG gene expression in UUO are not known. It can be argued that UUO may be accompanied by systemic inflammation, and the latter is a common inducer of the T-KG gene (7,8). Against this hypothesis, however, are the following observations. First, there was no evidence of urinary tract or systemic infections. Second, both angiotensinogen and IL-6 receptor genes are known to be induced by inflammation (23,29). Previously, we have shown that hepatic angiotensinogen mRNA is down-regulated at 1 wk and is expressed at control levels at 5 wk after UUO (3). In this study, IL-6 receptor mRNA levels...
were not increased in UUO rats. Thus, even if inflammation was playing a role, it cannot account solely for T-KG induction in UUO.

Because ureteral obstruction results in a striking infiltration of renal tissue with macrophages (30), IL-6 released from infiltrating macrophages or mesangial cells (31) could have mediated the activation of the T-KG gene in the obstructed kidney via specific interactions with the multiple IL-6 response elements in the upstream region of the T-KG gene (21,32). However, we were unable to detect activation of IL-6 mRNA in the obstructed kidney. Furthermore, hepatic IL-6 receptor mRNA levels were not significantly different between UUO and control rats. Although these findings suggest that IL-6 plays little, if any, role in mediating T-KG induction at 7 or 35 days after UUO, we cannot rule out an earlier activation of IL-6 gene expression during the course of UUO.

The physiologic significance of T-KG induction in UUO is not known. T-KG can give rise to the vasoactive peptide T-kinin through the action of high concentrations of trypsin (4). It is not presently known whether this conversion can occur in vivo, because no protease with the correct specificity has thus far been described in serum. Recently, T-kininogenase cDNA was cloned in kidney and submandibular gland (12), raising the possibility of in situ T-KG to T-kinin conversion within the kidney. However, studies in the isolated perfused rat kidney do not agree with this hypothesis (33). The addition of T-KG to the renal perfusate does not alter renal hemodynamics and is not accompanied by changes in intrarenal T-kinin levels. Considering that T-KG is a powerful inhibitor of lysosomal cysteine proteinases (14), it is more likely that the high levels of renal T-KG serve a protective function by neutralizing the activity of lysosomal enzymes released as a result of the cell necrosis and accelerated programmed cell death (apoptosis) that occur in the obstructed kidney (34). This is supported by studies in other organ systems (lung and spinal cord) subjected to chemical or mechanical injury, in which T-KG expression was induced both at the site of injury and in the liver (19,35).

In summary, chronic UUO differentially modulates the KG gene family in the rat. The mRNA transcripts encoding the substrates of plasma kallikrein (HKG) and tissue kallikreins (LKG) are not influenced by UUO. Sustained production of HKG and LKG (bradykinin substrates) may help to partially offset the effects of suppressed kallikrein synthesis in the vasoconstricted obstructed kidney. The physiologic relevance of T-KG induction in response to ureteral obstruction and its relationship to human disease is not clear, because this gene is unique to the rat. However, the differential responses of K-KG and T-KG genes provide a formidable model to study the mechanisms of differential gene regulation in eukaryotes. Indeed, a recently published study (36) demonstrated that the differential expression of K-KG and T-KG genes in the rat liver results from the evolutionary divergence of a few critical nucleotides in the upstream regulatory regions, leading to subtle changes in the binding affinities of a transcription factor(s) to its recognition site, which in turn result in differential expression of the downstream genes.

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