Structural organization of the rat branched-chain 2-oxo-acid dehydrogenase kinase gene and partial characterization of the promoter-regulatory region

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The gene encoding the rat branched-chain 2-oxo-acid dehydrogenase kinase (EC 2.7.1.115) has been isolated and partially characterized. The entire gene, including the promoter-regulatory region, spans 6 kb and contains 11 exons. The 5'-untranslated region comprising 264 bp is interrupted by intron 1 which is 581 bp in size. The complete in-frame sequence of intron 7 encodes the 49 amino acid insert previously reported to be present in the larger isoform of the rat kinase (Harris, Popov, Shimomura, Zhao, Jaskiewicz, Nanaumi and Suzuki (1992) *Adv. Enzyme Regul.* **32**, 267–284). Sequencing of the 679 bp of the 5'-flanking region showed the absence of a canonical TATA box,

similar to other branched-chain 2-oxo-acid dehydrogenase-complex genes. Several candidate *cis*-acting elements are present. These include CAAT boxes, Sp-1-binding sites, GCN-4 sites, CCAAT enhancer binding-protein sites (C/EBP) and glucocorticoid-responsive element (GRE) sites. Also present are a pair of direct repeats of unknown function. The luciferase-reporter assay showed that promoter activity is markedly higher in normal rat kidney (NRK-52E) cells than in rat hepatoma (FTO-2B) cells, and that the 5'-flanking region between bases –449 and +264 is both necessary and sufficient for basal transcription of the kinase gene.

INTRODUCTION

The mammalian mitochondrial branched-chain 2-oxo-acid dehydrogenase complex (EC 2.7.1.115) catalyses the oxidative decarboxylation of the branched-chain 2-oxo-acids derived from leucine, isoleucine and valine [1]. This multienzyme complex is a macromolecule comprising three catalytic components, a thiamine pyrophosphate dependent decarboxylase, or E1 (branched-chain 2-oxo-acid decarboxylase) with two E1 α ($M_r = 45\,500$) and two E1 β ($M_r = 37\,500$) subunits; a transacylase or E2 (dihydrolipoyl transacylase), which contains 24 lipoate-bearing polypeptides (monomer $M_r = 46\,500$); and a dehydrogenase, or E3 (dihydrolipoyl dehydrogenase), which is a flavoprotein of homodimeric structure (monomer $M_r = 55\,000$) [2,3]. In addition, the branched-chain 2-oxo-acid dehydrogenase complex contains two regulatory enzymes, a specific kinase and a specific phosphatase, which control enzyme activity through reversible phosphorylation (inactivation)–dephosphorylation (activation) of E1 [4]. The branched-chain 2-oxo-acid dehydrogenase complex is organized around the E2 cubic core ($M_r = 1.1 \times 10^6$), to which E1, E3, the kinase and the phosphatase are attached through ionic interactions [2,5].

The regulation of the activity of the branched-chain 2-oxo-acid dehydrogenase complex through a kinase-mediated phosphorylation has been extensively studied. Rats fed low protein diets showed low hepatic enzyme complex activity [6–8]. This is accompanied by reduction in the activity state (the percent of total enzyme in the dephosphorylated or active form) of the enzyme complex compared with fed rats [6–8]. Starvation and diabetes stimulate activity of the branched-chain 2-oxo-acid dehydrogenase complex in skeletal muscle by increasing the proportion of active or dephosphorylated enzyme complex [9,10]. Administration of glucagon or adrenaline was shown to result in a 4-fold increase in the percentage of active or dephosphorylated enzyme complex in the liver of rats fed a low protein diet [11].

Similar effects on the activity state occur with insulin and cyclic AMP, but to a lesser extent [11].

The rat branched-chain 2-oxo-acid dehydrogenase kinase has been purified [12–14] and cloned [15]. Surprisingly, the deduced amino acid sequence exhibits extensive similarity to prokaryotic histidine protein kinases and few similarities to mammalian serine-threonine protein kinases. Despite this similarity, the conserved histidine residues in the rat kinase do not mediate the phosphorylation of E1, indicating that the rat kinase is not, functionally, a histidine protein kinase [16]. The kinase activity in the liver of rats fed 0% protein diets increases 3- to 4-fold compared with rats on a 50% protein diet [17]. The increase in kinase activity is accompanied by a corresponding elevation in the levels of kinase protein and mRNA. The same study [17] also shows that the kinase protein and mRNA levels are low in the liver and high in the kidney and heart of rats fed a normal diet. The data support the thesis that the kinase is subject to nutritional control and tissue-specific expression. This provides an effective mechanism to modulate the flux of branched-chain amino acids through the branched-chain 2-oxo-acid dehydrogenase complex.

To facilitate investigation into the molecular mechanisms regulating the expression of the kinase, we have isolated the gene encoding the rat branched-chain 2-oxo-acid dehydrogenase kinase. Here we report the genomic organization of the coding region. We have also sequenced and partially characterized the promoter-regulatory region of the rat kinase gene.

MATERIALS AND METHODS

Screening of rat P1 genomic library

A sense primer, 5'-GCTGAAATCCGGAGAAAACC-3', corresponding to bases 145–164 of the rat branched-chain 2-oxo-acid dehydrogenase kinase sequence [15], and an antisense primer,

Abbreviations used: E1, branched-chain 2-oxo-acid decarboxylase; E2, dihydrolipoyl transacylase; E3, dihydrolipoyl dehydrogenase; RACE, rapid amplification of cDNA ends; INR, initiator element; DMEM, Dulbecco's modified Eagle's medium.

The nucleotide sequence of the promoter-regulatory region of the rat branched-chain 2-oxo-acid decarboxylase kinase gene has been deposited with the GenBank®/EMBL Data Bank under the accession number U36774.

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5'-CCAGCACTGAAGTCAGTAT-3', coinciding with bases 286–267, were utilized to screen a P1 genomic library by PCR. These primers amplified a 142 bp fragment from the rat genomic DNA, indicating that they encompass an uninterrupted exonic region. A top-down pyramid approach [18] was adopted in that the P1 clones were organized into 300 primary pools with each pool containing 400–500 clones. For screening purposes, the primary pools were combined into 30 secondary pools of 10 primary pools each, and the secondary pools into 6 tertiary pools of 5 secondary pools each. The tertiary pools that showed a specific amplification of the 142 bp fragment were used as tracers to identify the parental secondary pools for further PCR screening. The same procedure was repeated to select the primary pools for further screening, using the positive secondary pools as a guide. P1 clones containing the rat kinase genomic inserts were isolated from the positive primary pools by filter hybridization [19] using the 142 bp fragment as a probe. Positive clones confirmed by PCR were purified and the cultures were stored as frozen glycerol stocks.

P1 plasmid preparation

Positive P1 clones containing the gene insert were grown overnight at 37 °C to stationary phase in Luria–Bertani medium with added kanamycin (25 µg/ml). Mini (40 ml) or large (2 liters) preparations of media were inoculated with the overnight culture at 1/30 dilution. The inoculated cultures were shaken for 1.5 h and isopropyl β-D-thiogalactoside was added to a final concentration of 0.5 mM to induce the replication of P1 plasmids (from 1 copy/cell to approx. 20 copies/cell). After 6 h of isopropyl β-D-thiogalactoside induction, the P1 plasmid was isolated by alkaline lysis [19]. For the mini DNA preparation, RNA contamination was eliminated by RNase A (50 µg/ml) digestion at 37 °C for 30 min, followed by precipitation of the plasmid with polyethylene glycol. For the large preparation, the P1 plasmid was purified and concentrated by centrifugation in CsCl gradients.

Isolation of total RNA and Northern blot analysis

Sprague–Dawley rats maintained on a normal chow were sacrificed by decapitation. Tissues were removed from the rat, rinsed with PBS [18] and frozen in liquid nitrogen. Frozen tissues were ground using a mortar and pestle chilled by liquid nitrogen. Total RNA was extracted from the ground tissues with acid guanidinium thiocyanate/phenol/chloroform as described previously [20]. Northern blot analysis of total RNAs from various tissues was carried out also as described previously [21].

S1 nuclease mapping of the transcription start site

A 502 bp antisense single-stranded DNA probe was synthesized by asymmetric PCR of the region from bases +286 into intron 1 (3' → 5'), with a 1.2 kb fragment of the rat kinase gene as template. The antisense cDNA probe was hybridized with 6 µg of poly(A)⁺ RNA isolated from rat heart. The hybridization was performed at 37 °C overnight in the presence of 80 % formamide [19]. The RNA/DNA heteroduplex was digested with S1 nuclease, followed by phenol/chloroform extraction. The digested reaction was electrophoresed on a 6 % sequencing gel.

Primer extension analysis

An end-labelled antisense primer 5'-AGAGAGAGTGAG-TGGACAGGGTTTTCTCCG-3' (bases +183 to +154)

(2 × 10⁶ c.p.m.) was annealed to 100 µg of rat kidney total RNA or yeast transfer RNA (negative control) in a final volume of 10 µl. The mixture was heated at 70 °C for 10 min, cooled slowly to 50 °C (in approx. 15 min) and then chilled on ice. To the mixture was added the first-strand buffer (final concentration 50 mM Tris/HCl, pH 8.3, 75 mM KCl and 3 mM MgCl₂), 10 mM dithiothreitol, 1 µl RNasin (Promega) and 0.5 mM each of the four dNTPs in a final volume of 20 µl. After preincubation at 42 °C for 2 min, 400 units of SuperScript RNase H⁻ reverse transcriptase (GIBCO-BRL) were added to the mixture and the reaction was continued for 2 h at 42 °C. At the end of incubation, the sample was denatured in 0.1 M NaOH with heating at 100 °C for 3 min, and neutralized with 0.5 M HCl. To the sample was added 0.3 M sodium acetate, pH 5.2, followed by the precipitation of DNA with three vol. of 100 % ethanol. The pellet was washed with 70 % ethanol and dissolved in 12 µl of H₂O with the addition of 6 µl of the sequencing stop solution (stock concentration 10 mM NaOH/95 % formamide/0.05 % Bromophenol Blue/0.05 % Xylene Cyanol). The extended product was electrophoresed on a 6 % DNA sequencing gel in 8.3 M urea along with two known sequencing ladders.

Construction of promoter–luciferase vectors

A medium-length promoter–luciferase construct (clone –33, with the 5'-end terminating at nucleotide –33) was prepared by amplification of the promoter region using the sense primer 5'_P: 5'-ACTGGGCACACACAGCC-3' (bases –33 to –17), and the antisense primer 3'_{Po}: 5'-TGTCGCGCTTCTTTGTTG-3' (bases +264 to +247), with the positive P1 clone as a template. The amplified product was purified by agarose-gel electrophoresis, phosphorylated with the T4 kinase and then ligated into the plasmid containing a luciferase cDNA (pBS-LucPA). The orientation of the promoter insert was determined by nucleotide sequencing.

For the construction of the luciferase vector containing longer promoter regions, an intermediate plasmid, pBS-KP, was generated as follows. An *EcoRI* (2 kb) or a *PstI* fragment from the P1 clone that hybridized with the 5'-end of the rat kinase cDNA was ligated into the pBluescript vector pBS (SK⁻) to produce pBS-RI and pBS-*PstI* clones. A 1.1 kb fragment, corresponding to a promoter region (bases –449 to intron 1), was obtained by *EcoRI* and *BamHI* digestion of the pBS-RI plasmid; and a 150 bp fragment containing a promoter region (intron 1 to +264) and an 8-bp 5'-flanking sequence of the luciferase vector was produced by *BamHI*–*PstI* digestion of the promoter–luciferase construct –33. The two fragments were ligated simultaneously into the pBS (SK⁻) plasmid linearized by the *EcoRI* and *PstI* digestion to result in the pBS-KP vector.

To produce the 1.7 kb promoter construct (clone –1700, with the 5'-end terminating at nucleotide –1700), a 1.3-kb *PstI*–*EcoRI* fragment (bases –1700 to –449), excised from the pBS-*PstI* plasmid by *PstI* digestion, was blunt-ended with T4 DNA polymerase, followed by *EcoRI* digestion. The fragment along with the *EcoRI*–*PstI* fragment (bases –449 to +264) from the pBS-KP plasmid were ligated into the pBS-Luc PA vector previously digested with *SmaI* and *PstI*.

To generate nested, deleted promoter constructs, the intermediate pBS-KP plasmid was linearized with *EcoRI* and digested with exonuclease III for 2–8 min. The digested DNA was blunt-ended with S1 nuclease, followed by the Klenow reaction. The various deleted DNAs were treated with *PstI* and then separated on 1 % agarose. DNA fragments smaller than 1 kb were excised from the agarose gel, extracted with Qiax resin (Qiagen,

Chatsworth, CA, U.S.A.) and ligated into the pBS-Luc PA vector previously digested with *Sma*I and *Pst*I.

Cell culture and luciferase reporter assays

Rat hepatoma cells FTO-2B (a gift from Dr. R. Hanson, Case Western Reserve University, Cleveland, OH, U.S.A.) were grown in 50% Dulbecco's modified Eagle's medium (DMEM) and 50% F-12 medium supplemented with 5% bovine serum and 5% fetal calf serum. Normal rat kidney/epithelial-like cells NRK-52E (supplied by Dr. R. A. Star, Department of Internal Medicine at this institution) were grown in DMEM with 5% bovine serum. Cells were harvested by treatment with trypsin, and transfected with the promoter-luciferase vector by electroporation, as described previously [22]. The luciferase-reporter assay was carried out with a cotransfected β -galactosidase as the internal standard also as described in [22].

RESULTS AND DISCUSSION

Isolation and characterization of the rat branched-chain 2-oxo-acid dehydrogenase kinase genomic clones

We approached the cloning of the rat kinase gene by screening a rat P1 genomic library. The library consists of 12000–15000 clones with an average insert size of approx. 75 kb. Thus, the total size of the P1 library covers the rat genome (about 3×10^9 bp) 2–3 times. A top-down pyramid screening by PCR was carried out (see the Materials and methods section). The primers used allowed the amplification of a 142 bp continuous exonic region corresponding to bases 145–286 of the rat kinase cDNA sequence [15]. Six positive P1 plasmid clones were isolated. Four of them were characterized, and each clone was found to contain the entire branched-chain 2-oxo-acid dehydrogenase gene. As shown in Figure 1, upper panel, the rat kinase gene contains 11 exons and spans approx. 6 kb. Table 1 depicts the complete exon-intron boundaries of the gene. Nucleotide sequences at the 5' donor and the 3' acceptor sites of all introns

conform to the gt-ag rule [23]. The sizes of exons range from 69 bp (exon 3) to 1026 bp (exon 11). The longest intron of 1.1 kb in size occurs between exons 8 and 9. The entire mitochondrial targeting pre-sequences of 30 amino acid residues are encoded by exon 2.

DNA sequencing of all 11 exons disclosed several differences between the genomic and the published cDNA sequences [15] of the rat kinase. There is a single C nucleotide deletion at base 448 and a single C nucleotide insertion at base 491 of the reported cDNA sequence, when the latter is compared with the genomic sequence of exon 4. The deletion causes a frame shift after Phe-80, and the insertion restores the coding frame at and after Val-95 of the genomic sequence. The fidelity of the genomic sequence was confirmed by nucleotide sequencing of a cDNA amplified from a rat kidney cDNA library [16]. A second variation in the amino acid sequence is at codon 192, where the published cDNA sequence shows TCG encoding a serine, but the corresponding codon in exon 8 is TGC, which codes for a cysteine. The accuracy of the genomic codon was also confirmed by sequencing of the amplified cDNA. Other differences between the reported cDNA and the present exonic sequences are in the 5' and 3' untranslated regions of the mRNA. These include two single-base changes: G (base 1 in the cDNA) to C, and A (base 2167) to C; and four single-base deletions (underlined) relative to the genomic DNA: GCC (bases 1422 and 1423 of the cDNA), ATG (bases 1925 and 1926), TGC (bases 2042 and 2043) and ATG (bases 2146 and 2147).

Determination of the transcription-initiation site

Initial S1 nuclease mapping was performed using a 502 bp single-stranded DNA probe corresponding to the region from base +286 into intron 1 (3' to 5') (Figure 2) that contained the initiation methionine. The results showed a putative 5'-untranslated region with transcription initiation at base +175 (Figure 2). However, the later rapid amplification of cDNA ends (RACE) experiments indicated that there is a 581 bp intron immediately upstream of base +175, and that the 5'-untranslated region is longer than 175 bp, as originally determined (results not shown). The precise transcription-initiation site was determined by primer extension using an end-labelled primer corresponding to bases +154 to +183 of the genomic sequence (Figure 2). Figure 3 shows that the primer extension product is 183 bp in length and the 5'-end is base +1. The data combined with the results of RACE, which delineate the 5'-end of exon 1, indicate that this exon contains 89 bp. The transcription-initiation site was confirmed by S1 nuclease mapping using appropriate probes (results not shown). The results, taken together, establish that the kinase gene contains a relatively long 264 bp 5'-untranslated region, which is interrupted by intron 1 of 581 bp in size.

Alternate splicing of the rat kinase gene

Two cDNAs for kinase isoforms of different size have been isolated from a single rat heart library [24]. The sequence of the longer cDNA is identical with the shorter cDNA reported previously [15], with the exception of an in-frame insertion of 147 bp. This results in the addition of 49 amino acids to between Lys-184 and Pro-185 (Table 1) of the previously reported shorter kinase isoform. An examination of the 49 amino acid insert present in the larger isoform [24] showed that the inserted sequence corresponds precisely to an unspliced intron 7 of the rat kinase gene (Figure 1, lower panel). The physiological significance of this apparent alternate splicing of the kinase gene is presently unknown, although the reverse transcription PCR of mRNA showed that the two isoforms are differentially expressed in

Figure 1 Structure and the sequence of intron 7 of the rat branched-chain 2-oxo-acid dehydrogenase kinase gene

Upper panel: exonic-intronic organization. The kinase gene contains 11 exons and is about 6 kb in length. Exons 1–11 are represented by solid rectangular bars and the scale of DNA is shown at the bottom of the Figure. Two inverted solid triangles indicate the initiation and termination sites of translation. Lower panel: nucleotide and deduced amino acid sequences of intron 7. The amino acid sequence is deduced from the open-reading frame corresponding to the entire intron 7 and is identical with the sequence of the 49 amino acid insert present in the previously described larger kinase isoform [24].

Table 1 Nucleotide sequences flanking exon–intron boundaries of the rat branched-chain 2-oxo-acid dehydrogenase kinase gene

The nucleotide sequences were determined from a PI-kinase clone. Exon and intron sequences are presented in upper case and lower case letters respectively. Numbers above the sequence refer to base co-ordinates for the full-length kinase cDNA (this study), and those in parentheses below are amino acid positions in the pre-kinase. Amino acid residues –1 to –30 constitute the complete mitochondrial targeting sequence.

Exon	Intron Size	Exon	Exon	Exon Size
	1			
	GGGGGAGGGCGA.....CGGACCCGGGCCAGCGTCT	1		89
89		90		
GGCCTCGCGtaagtggactaactcgtagta...../581bp/...ccttcactactccaatagATGGAAGCT		2		370
459		460		
GCAGAGAAGgttcgcacatagcacgatacac...../100bp/....agtgcatcttcccttagCCCTCAGTC		3		69
AlaGluLys (35)		ProSerVal (36)		
528		529		
CACCTTCTGgtaaggtctctcttcttcttct...../76bp/...ttccctctttttcttctacagAAAAGTGGT		4		111
HisLeuLeu (58)		LysSerGly (59)		
639		640		
CTGCATGTGgtggtgtaaggccgaggggacctt...../250bp/..ccatactttactccatacagCACGAGCTA		5		48
LeuHisVal (95)		HisGluLeu (96)		
687		688		
TTCCTCCGgtgagggccgggtgagtaaggt...../147bp/..cataactctgtgacctgacagATCAAGGAC		6		120
PheProPro (111)		IleLysAsp (112)		
807		808		
CACATAGAGgtcagaggcaacagagaatgg...../80bp/...ctgaggctcaccctttatagGATGAAAAG		7		99
HisIleGlu (151)		AspGluLys (152)		
906		907		
GAAGACAAGgtggagctgagacccgagaatg...../147bp/...cccttctcttctcctgccagCCTGATTTT		8		74
GluAspLys (184)		ProAspPhe (185)		
980		981		
GATTTTGCCAGgtgagactagaatggtggggg...../1.1Kb/....gcccttctatccaagagACGCCTGTGC		9		219
AspPheAlaAr (209)		gArgLeuCys (210)		
1199		1200		
CTCATCATCAGgtgaggggtgcccgccggga...../350bp/...ttctcaacatcctgccagGATCTCAGAC		10		159
LeuIleIleAr (282)		gIleSerAsp (283)		
1358		1359		
CCTATGCATGGgtaagatcccacctgcccacat...../83bp/.....cctgtccactgctcccagCTTTGGCTTC		11		1026
ProMetHisGl (335)		yPheGlyPhe (336)		
2310		2388		
TTCAGAATCTGA.....AATAACCCTCCCTGCTGTTTAGGACCTAAGGGCTTGCTTGGTGGGA...GCTTT				
PheArgIle*** (382)				

various tissues, with the smaller isoform predominating in the kidney [24]. It remains to be determined whether the larger isoform is enzymically active. An efficient *Escherichia coli* expression system for the rat branched-chain 2-oxo-acid dehydrogenase kinase in the presence of overproduced chaperonins GroEL and GroES has been described recently [16]. This

expression system should facilitate enzymic studies of the recombinant larger kinase isoform.

Sequence analysis of the promoter-regulatory region

Using specific primers, the 5'-flanking region of the rat kinase

C/EBP
 -679 CCCACAACCT TTCTGTAGGA CCCTAGCACA GCAAGCAGAC CCAACCGTC TTCTCTCTCA
 GCN-4
 -619 GAGTAGATGC AGATTTTATT TTGAAAACAC TCCCACCTC CACCCGGGGC ACAGGAAGT
 C/EBP
 -559 CACCCTAGTC ATATTAAGTT GCAGGCAGGT AGAAATAGTA ACTCTCTGT CAATTCACA
 GRE
 -499 CGCAACACTG TTCTAGACAG TTTACATAGA TAAATTTGCA TAATCTACAG AATTCGAAGA
 CCAAT box
 -439 TAAATACCTT TTTATTTTGC CAATAGCTAA TTACAGCATT GAGAAATTGG CTGACTTCCC
 -379 TTGGTGTTTA AGGGAGCTGA GCTAGAGTTT GAAACCAGGC AGACCTTGAA TGGGCTAGAA
 DR-1 DR-1
 -319 GCTTGAATG ATTGGACCTT AAGAAGAAGG CTGGGCCCTA GCAAGCGCAA GAAGAAGGCT
 -259 GGTCTTATT TGTACACATT TGAGGATTTT TGGTGCACTT TAGCATTTT CTCACAGGGA
 Sp1
 -199 CCTTATTTT TACAGTGAA GACAGAGTCG ATATAGACTA CTGATAACTT CGGGCGGGG
 GCN-4
 -139 TGGTAGGGT GCTGGCAGAG TCAACTACT CATCTGAAGG GGTAGGGGAT ACCCTAAAGA
 -79 ACAGGAACGT ACTCGAGTTG TGGTCAAACT ACAACTCCCA GGAGGCACATG GGCACACACA
 +1 Sp1
 -19 GCCCGCGCA TGTACACGC GGGGAGGGG AAGCGGGCT CGGCCAGCGG GGCGGTCGA
 +89 Intron1
 +42 CTGCTCCCGC GGTTCGCTC GGACCCGGG CCAGCGTCTG GCCTCGCGT aagtggacta
 actcgtagta actcacacag accccctccg ggggacactc gacagcacag gtcttcagcc
 tccacctctg atggcagtc gcatgggccc gggctggcac ctaccatag ggggggaccc
 tggccagact aagtgcacca gcagaggggg agctccggtg gggaactctg ggaccgcagt
 tctgtcacg aaccagttc tcccggtcgc tattcttgta ggggggacgc agggcagctc
 tcaaaagctc aggtccctca acgccaagta ggagtcagat ccagccctctg aaaaggggtg
 ttcagaacct gaggaggctg gggagaggtt ttatgggtag aatgaactgg agattactcc
 acccgtttcc tctagtgctc acacaagcct ttatggtgtt ctacatagaa gactagaaca
 cctggaggtg gttgtccagc ctgcgctgta ttgtatctcg ggagatgggt tttgaaggg
 ctggacaagg tctattgggt tacaacttga gaaatagtgc aagccatttg gatttggatc
 CCAAT box +90
 cttaaaatct ccttcaccta ctccaataga TGAAGCTGC TGTGGCGGC CCGAGCATGT
 +121 CAGCATCTC GAGGGAGCCA CATCCCTGAA ATCCGGAGAA AACCTGTCC ACTCACTCTC
 +181 TCTCTACTCC TGCTTTGGGG TCACCGTTGC CTGTCGAGCC CTTAGTCCCA TAGTATCACC
 start codon
 +241 CTAGTTCAAC AAAGAAGCGG GACAATGATA CTGACTTCAG TGCTGGG

Figure 2 Nucleotide sequence analysis of the promoter-regulatory region of the rat kinase gene

The promoter-regulatory region of the kinase gene is numbered from -679 to -1. The transcription start site is designated as base +1. The complete sequence of intron 1 (581 bp), which interrupts the 5'-untranslated region, is shown in lower case. The following perfectly matched *cis*-acting elements are individually marked and underlined: Sp1-binding sites (Sp1), glucocorticoid-responsive element (GRE), CCAAT enhancer binding-protein sites (C/EBP), GCN-4 and direct repeats (DR). The initiation ATG codon (bases +265 to +267) is indicated in bold-faced letters.

gene was sequenced. Figure 2 shows the sequence of 679 bp 5' of the transcription initiation site +1, 581 bp intron 1 and a 287 bp portion of exon 1 including the translation initiator codon ATG. This region contains candidate *cis*-acting elements that completely match the reported sequences. These include: one GRE motif with the sequence ACTGTTCT [25] at bases -486 to -493, two CAAT box motifs CCAAT [26] at bases -416 to -420 and in intron 1, two Sp1-binding sites with the core sequence GGGCGG [27] at bases +31 to +36 and bases -142 to -147, two GCN-4 sites with the motif G/AAGTCA [28] at bases -563 to -558, bases -122 to -117, and two C/EBP (SV-40) sites with the sequences TTCTGTA and TTCCACA [29] at bases -500 to -506 and bases -664 to -670 respectively. In addition, a pair of direct repeats, AAGAAGAAGG, of unknown function are located at bases -257 to -271 and bases -285 to -299.

It is of interest that a canonical TATA box [32] is absent at bases -20 to -30 relative to the transcription start site, similar to E1 α [22], E1 β [33] and E2 [34] genes of the human branched-chain 2-oxo-acid dehydrogenase complex. The initiator element (INR), PyAPyTCPyPyPy (with Py denoting pyrimidine bases) [35], is also absent at the transcription initiation site of the branched-chain 2-oxo-acid dehydrogenase kinase gene. The

Figure 3 Primer extension of rat branched-chain 2-oxo-acid dehydrogenase kinase gene

An end-labelled antisense primer, 5'-AGAGAGAGTGAGTGGACAGGGTTTCTCCG-3' (bases +183 to +154), was annealed to 100 mg each of yeast transfer RNA (lane 1) or total RNA prepared from the rat kidney (lane 2). Primer extension was carried out at 42 °C for 2 h using SuperScript reverse transcriptase. At the completion of the reaction, the sample was subjected to electrophoresis on a 6% sequencing gel in the presence of 8.3 M urea. Radiolabelled bands were scanned by storage-phosphor autoradiography. The arrow indicates the primary extension product (lane 2) which is 183 bp in length as determined by comparing to the two sequencing ladders shown on the left.

INRs may be important for both promoter strength and for determining the actual transcription site in TATA-less genes [35]. The mechanism for transcription initiation by RNA polymerase II in TATA⁻/INR⁻ gene promoters is unknown. Nonetheless, the lack of both TATA and INRs coincides with the occurrence of multiple transcription start sites observed in previous [22] studies. The results support the role of these two elements in determining the precise transcription-initiation site [35]

Characterization of the promoter-regulatory region

The promoter activity of the kinase gene was studied by the luciferase-reporter assay using FTO-2B (rat hepatoma) cells or NRK-52E (rat kidney/epithelial-like) cells as the host. Promoter constructs containing different lengths of the 5'-flanking region were transfected into host cells by electroporation. Luciferase reporter activity was normalized to cotransfected β -galactosidase to correct for variation in transfection efficiencies. Promoter constructs studied contained an identical 3'-end at base +264 and varying 5'-ends upstream of the transcription-start site (Figure 4a). As shown in Figure 4b, clone -449 (with the 5'-end terminating at base -449) exhibits highest luciferase activity in FTO-2B cells. Lower luciferase activity was observed with clones -1700, -128 and -255. Clones -114 and -33 and the pBST-Luc plasmid (negative control) showed background activity (Figure 4b). Approx. 19-fold higher luciferase activity was obtained with NRK cells (Figure 4c) compared with FTO-2B. Clone -449 exhibited slightly lower (80%) luciferase activity than clone -1700 in NRK cells. Transfection of clones -255, -128, -114 and -33 and the pBST-Luc plasmid into NRK cells produced 50% of background luciferase activity (Figure

Figure 4 Promoter activity of the 5'-flanking region measured by the luciferase-reporter assay

DNA fragments representing various lengths of the promoter-regulatory region of the rat kinase gene were ligated into the pBS-Luc PA plasmid containing the luciferase reporter cDNA. (a) The constructs are designated according to position of the 5'-terminal base of the DNA fragment. The 3'-end of all constructs is base +264. The broken, open box depicts intron 1 (581 bp) that interrupts the 5'-untranslated region (264 bp). In (b) and (c) luciferase activity was

Figure 5 The branched-chain 2-oxo-acid dehydrogenase kinase mRNA level in different rat tissues

For Northern blot analysis, 20 μ g of total RNAs from the brain, eye, heart, small intestine, kidney, liver, lung, skeletal muscle, spleen and testis of rats fed a normal diet were electrophoresed and transferred to nylon membrane. The membrane was hybridized with the rat kinase cDNA probe. Ethidium bromide staining of 28 S and 18 S rRNA showed that approx. equal amounts of total RNAs were applied to the agarose gel.

4c). The results indicate that the promoter-regulatory region between bases -449 and +264 is both necessary and sufficient for basal transcription of the rat kinase gene in FTO-2B and NRK cells.

Regulation of the kinase gene expression

As described above, the rat branched-chain 2-oxo-acid dehydrogenase kinase is differentially expressed in heart, liver and kidney [17]. We have confirmed and extended these studies to other tissues of rats maintained on a normal diet. Figure 5 shows that the mRNA level of the rat kinase is high in heart, kidney and skeletal muscle, intermediate in spleen, lung and small intestine, low in brain, eye and testes and is found in trace amounts in liver. Changes in kinase activity are correlated with differences in kinase mRNA and protein content [17], however, it is unknown whether these changes are mediated at the transcriptional level. Liver is the major site where the branched-chain 2-oxo-acid dehydrogenase complex is subjected to regulation by altering the activity state of the enzyme complex [4]. In rats fed a 0% protein diet for 14 days, the activity state of the enzyme complex is drastically reduced [6–8]. The decrease in the activity state results from increases in the protein and mRNA levels of the branched-chain 2-oxo-acid dehydrogenase kinase [17]. In contrast, the activity state of the hepatic branched-chain 2-oxo-acid dehydrogenase complex is increased in rats treated with glucagon and adrenaline [11], suggesting that the expression of the kinase is repressed. The isolation of the promoter-regulatory region presented in this study will facilitate studies of the mechanisms by which the expression of the kinase gene is regulated in different tissues by nutritional and hormonal stimuli.

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measured in cell extracts and expressed as percent activity of clone -1700. To correct for variation in transfection efficiency, luciferase activity was normalized to cotransfected β -galactosidase. The 100% activities in (b) FTO-2B and (c) NRK cells are 2.9×10^4 and 5.4×10^5 relative light units/ A_{570} respectively. The A_{570} was obtained from β -galactosidase assays after incubation of the reaction mixture for 1 h at 37 °C. The pBS-Luc PA plasmid served as a negative control. Error bars are standard errors of the mean ($n = 6-9$).

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