

Structure of the Gene Encoding Human Casein Kinase II Subunit β *

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Casein kinase II (CKII) is a ubiquitous serine/threonine protein kinase with numerous key functions in cell metabolism and growth. The human CKII has a tetrameric structure; two catalytic subunits (α and α') form the holoenzyme together with two presumably regulatory subunits (β). The gene encoding CKII subunit β was isolated from human genomic DNA and analyzed for its primary structure using exclusively nonradioactive procedures. The gene was found to span 4.2 kilobase pairs and to be composed of seven exons. Exon sizes range from 76 (exon 5) to 329 base pairs (bp) (exon 1), intron sizes from 145 (intron V) to 965 bp (intron II). All exon-intron junctional sequences conform to the canonical GT-AG rule. Primer extension analysis determined three transcription initiation sites, at 951, 919, and (minor) 840 bp upstream of the translation start site. The translation start is located early in the second exon; exon 1 is untranslated. The 3'-cleavage/polyadenylation signal sequence (AATAAA) is in the last exon at position 4173 bp relative to the first transcription initiation site. The coding sequence for CKII β comprises 648 nucleotides identical to the published CKII β -cDNA sequence (Jakobi, R., Voss, H., and Pyerin, W. (1989) *Eur. J. Biochem.* 183, 227–233). The upstream promoter region of the CKII β gene contains multiple potential gene regulatory sequence elements, noticeable DNA structures, and the characteristics of a housekeeping gene (more than one transcription initiation site, lack of a TATA-box, presence of a CpG island, occurrence of multiple GC boxes and of nonstandard positioned CCAAT boxes). The CKII β gene promoter shares common features with that of mammalian protein kinases and is closely related to the regulatory subunit gene promoter of cAMP-dependent protein kinase.

Casein kinase II (CKII)¹ is a protein kinase (EC 2.7.1.37) particularly prominent due to the role (or possible role) it serves in the regulation of metabolic pathways, signal transduction, replication, transcription, and translation (1–3). Cur-

rently, special attention is being paid to CKII's ability to phosphorylate growth regulatory proteins. This includes oncogene products such as Myc (4), Myb (5, 6), Fos (7), adenovirus E1a (7), SV40 large T antigen (8), human papilloma virus E7 (9, 10), and the tumor suppressor protein p53 (11) as well as factors involved in the expression of these such as the serum-responsive factor (12). The observed rapid and transient increase in activity following treatment of cells with mitogenic stimuli, such as serum (13), epidermal growth factor (14–16), insulin (16, 17), or insulin-like growth factor I (17), and the inhibition of mitogenic stimulation through pretreatment of cells with CKII antisense oligodeoxynucleotides (18) indicate that these phosphorylations may be terminal steps in the propagation of mitogenic signals and thus CKII be one of the signal-transducing protein kinases.

Human CKII is composed of the three subunits, α , α' , and β , with molecular masses of approximately 43, 38, and 28 kDa, respectively (19). These form an $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, and/or $\alpha'\beta_2$ tetrameric holoenzyme. Like CKII from other sources (1–3), subunits α and α' are the catalytic subunits identified by affinity labeling and catalytic activity (19–23), sequencing, and sequence identities with other protein kinases (24–28). Subunit β serves regulatory functions. However, unlike regulatory subunits of other protein kinases (1), subunit β seems rather to play a stimulatory role. Addition of isolated subunit β markedly increases the kinase activity of isolated gel-extracted subunits α and α' (22, 23). In cultured cells, the above mentioned increase of cytosolic CKII activity following treatment with growth factors has been ascribed to changes in subunit β due to hyperphosphorylation (15). Hyperphosphorylation occurs at serine and threonine residues implying that it cannot stem from direct phosphorylation through receptor kinase(s) because these are tyrosin-specific (30) but from intermediary serine/threonine kinase(s) conceivably related to those growth factor-stimulated protein serine/threonine kinase activities analyzed recently (31, 32). The cdc2 kinase has been identified as an enzyme hyperphosphorylating CKII β subunit from *Xenopus laevis* ovary (33).

The amino acid sequence of human CKII subunit β has been deduced from cDNA clones isolated in several laboratories from different sources, i.e. HeLa cells (34), HepG2 cells (35), and T-cells (28). The sequences are identical. The human CKII β nucleotide sequence shows a similarity of 75% and 90% to that of *Drosophila melanogaster* (25) and mouse (36), respectively. The amino acid sequence has a similarity of 88, 99, and 97% to that of *D. melanogaster* (25), mouse (36), and bovine (Ref. 24; obtained by direct protein sequencing), respectively. Thus, the β subunit of CKII is a highly conserved protein.

Here we describe the isolation and sequencing of a genomic DNA clone of human CKII subunit β . The sequenced DNA comprises the entire gene and several kilobase pair-long

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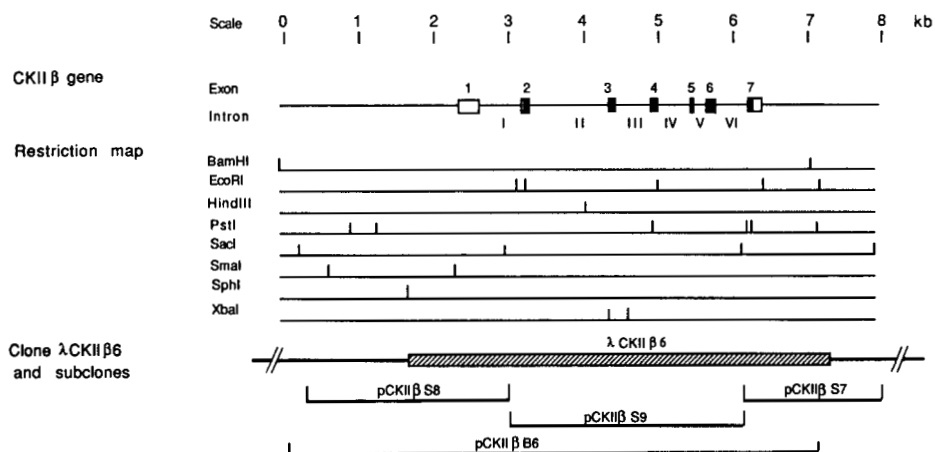
The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X57152.

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¹ The abbreviations used are: CKII, casein kinase II; kb, kilobase pair(s); bp, base pair(s); SDS, sodium dodecyl sulfate.

FIG. 1. Physical map of the human CKII β gene and subcloning strategy. The structure of the gene is schematically represented by the bar at the top of the diagram. Exons are indicated by boxes numbered 1 to 7; untranslated regions are shown in plain coding regions by filled boxes. Intron regions are numbered I to VI. The restriction map shows cleavage sites for endonucleases *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Sac*I, *Sma*I, *Sph*I, and *Xba*I. The phage clone λ CKII β 6 is represented to scale by the bar as are the subclones pCKII β -S7, -S8, and -S9 as well as pCKII β B6; hatched box indicates sequenced region.



stretches of both the 5'- and the 3'-flanking region. The 4.2 kb-CKII β gene consists of seven exons with the translation start situated in the second exon, the first remaining untranslated. The characteristics of the 5'-flanking region plus that of exon 1 render the CKII β gene a housekeeping gene and reveal common features to promoters of other mammalian protein kinase subunits.

EXPERIMENTAL PROCEDURES

Construction of a Human Genomic DNA Library—High molecular DNA was isolated from human white blood cells by cell lysis with detergent (Sarkosyl), incubations with proteinase K and RNase A, phenol/chloroform extractions, and ethanol precipitation according to standard protocols (38). The DNA was partially digested with *Sau*3A and size-fractionated by sucrose gradient centrifugation (10–40%). Fractions containing DNA between 15- and 25-kb fragment size were pooled, dialyzed with 10 mM Tris-HCl, pH 8, 1 mM EDTA, precipitated by ethanol, and redissolved in 10 mM Tris-HCl, pH 8, 1 mM EDTA (final DNA concentration was 0.5 μ g/ml). The DNA was ligated within *Bam*HI/*Hind*III predigested λ DASHII DNA, *in vitro* packaged, and propagated in *Escherichia coli* LE 392 (Stratagene).

Nonradioactive Screening of Human Genomic DNA Library—Non-radioactive nucleic acid methodology based on digoxigenin labeling and immunological detection was used in all described hybridization experiments. For probe preparation, 1 μ g of a 537-bp *Eco*RI/*Pst*I fragment of human CKII β subunit cDNA (34) was digoxigenin-labeled (Boehringer Mannheim) by the random priming method (37) using hexanucleotides in a labeling mixture of 100 μ M dATP, 100 μ M dCTP, 100 μ M dGTP, 65 μ M dTTP, 35 μ M digoxigenin-dUTP, and 2 units of Klenow enzyme. The library was screened by filter hybridization with the digoxigenin-labeled probe. Hybridization was at 68 $^{\circ}$ C for 6 h in 5 $\times$ SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 1% blocking reagent (Boehringer Mannheim). Subsequently, the filters were washed twice for 5 min at room temperature in 2 $\times$ SSC, 0.1% SDS, and twice for 15 min at 68 $^{\circ}$ C in 0.1 $\times$ SSC, 0.1% SDS. Immunological detection was performed with polyclonal sheep anti-digoxigenin fab fragments conjugated to alkaline phosphatase according to the kit manual (Boehringer, Mannheim). Reactive phage clones were plaque-purified and the phage DNA was isolated from plate lysates (39).

Restriction Analysis and Subcloning of λ DNA—The clones were characterized by restriction digestion with *Bam*HI, *Hind*III and *Sac*I, followed by nonradioactive Southern blot analysis as described above. The genomic clone λ CKII β 6 (see Fig. 1) was chosen for further more detailed analysis. A 7.1-kb *Bam*HI fragment of λ CKII β 6 was subcloned into plasmid pUC19 (38) to generate pCKII β B6, as well as three *Sac*I fragments of 2.0, 2.8, and 3.0 kb to yield plasmid pCKII β S7, -S8, and -S9, respectively. All plasmid clones were isolated in both orientations. For establishing a restriction map, the isolated inserts were digested by *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Sac*I, *Sma*I, and *Sph*I.

Sequence Analysis of Human CKII β Clones—A set of suitable overlapping plasmid clones was generated in both orientations from plasmid subclones pCKII β S7 and pCKII β S9 (see Fig. 1) by unidirectional nested deletion strategy (40) using exonuclease III and S1 nuclease. The GC-rich region of the gene was sequenced by walking

primer strategy using either subclone pCKII β S8 or pCKII β B6 as well as the genomic clone λ CKII β 6 directly as a template (41). Oligonucleotides for walking primer sequencing were synthesized with the Gene Assembler Plus (Pharmacia LKB Biotechnology Inc.) and fluorescently labeled as described previously (42). All DNA sequencing was performed on the European Molecular Biology Laboratory (EMBL) automated fluorescent DNA sequencer (43) or its commercial version A. L. F. from Pharmacia using a modified double-stranded dideoxy sequencing protocol for T7 DNA polymerase with fluorescently labeled primers as described previously (44), including 7-deaza-dGTP routinely to eliminate gel compressions in GC-rich regions (45). Sequences were compiled and aligned using fragment assembly programs of the University of Wisconsin Genetic Computer Group DNA sequence analysis package (Madison, WI).

Fluorescently Labeled Primer Extension Reactions on an Automated DNA Sequencer—Total RNA from human lymphocytes and JEG3 chorion carcinoma cells (American Tissue Culture Collection) were isolated by the guanidine isothiocyanate method (46). Primer extension analysis was performed by modification of standard protocols (38) and adapted to fluorescent on-line detection using the EMBL automated DNA sequencer. Three pmol of the fluorescently labeled primer *EXT1 (5'-*GGCGACCGCTACAGCGCAGGCC-3') complementary to CKII β nucleotide sequence region 248–269 (see Fig. 2) were mixed with 25 μ g of total RNA (yeast tRNA in control reactions) in 12 μ l containing 50 mM Tris-HCl, pH 8.5, 10 mM MgCl₂, 40 mM KCl, and 1 mM dithiothreitol, 25 units RNase inhibitor, and 500 μ M dNTPs, heated for 10 min at 65 $^{\circ}$ C, and slowly cooled to 42 $^{\circ}$ C. Then 40 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) were added and incubated for 60 min at 42 $^{\circ}$ C. Reactions were stopped with 5 μ l of deionized formamide containing 5 mg/ml dextran blue, heat-denatured, and one-third of the sample was loaded on a 5% polyacrylamide sequencing gel together with a known sequencing ladder as a size standard.

RESULTS AND DISCUSSION

Identification and Sequence of the Human CKII β Subunit Gene—A human leukocyte genomic DNA library prepared by using a λ replacement vector was screened with a 537-bp *Eco*RI/*Pst*I cDNA fragment of CKII β representing 80% of the coding region (34). Out of 6 $\times$ 10⁵ screened clones, a clone was identified, λ CKII β 6, containing the complete gene of CKII subunit β plus approximately 7 and 4 kb of 5'- and 3'-flanking regions, respectively (Fig. 1). Subcloning of λ CKII β 6, restriction analysis by Southern blot hybridization with various defined probes corresponding to regions throughout the CKII β -cDNA (34), and partial sequencing allowed construction of a restriction map and determination of exon/intron organization of the complete CKII β gene. Accordingly, the coding sequence for the mRNA of the CKII β protein is divided into seven exons (Fig. 1).

The complete sequence of the CKII β gene and adjacent 5'- and 3'-regions, altogether roughly 6 kb (Fig. 1), was then determined by automated fluorescent DNA sequencing of

deduced amino acids sequence is shown below the nucleotide sequence in single-letter code. The oligonucleotide sequence used for primer extension is *underlined*, and the three indicated transcription start sites (1, 33, and 113) are marked by *boldface letters*. *Underlined* also are the inverted AAT repeat in intron II and the putative polyadenylation signal AATAAA.

elegans cAMP-dependent protein kinase has 8 (63) and 20 kb (64), respectively.

As summarized in Fig. 3, the sizes of exons were found to be 329, 83, 103, 116, 76, 190, and 231 bp for exon 1, 2, 3, 4, 5, 6, and 7, respectively; those of the introns 610, 965, 568, 445, 145, and 326 bp, respectively. The exon/intron boundaries have the consensus sequence $-5'$ GTGAG- $\overline{\text{C}}$ AG3'/. Hence the junctions conform to the general rule with GT at the splice donor site and AG at the splice acceptor site (47). In addition, the splice donor sites appear to be highly conserved for the next three nucleotides as well, showing the consensus sequence GTGAG in five out of six introns. This consensus sequence seems also to be the preferred splice sequence in

mammalian protein kinase subunit genes (58–61) and to occur even in the kinase genes of *C. elegans*, the genetic structure of which deviates frequently from those of mammals. The sequence is found several times in its CKII α gene (62) and CKII β gene² as well as the genes for the regulatory and the catalytic subunit of cAMP-dependent protein kinase (63, 64) and constitutes, with one exception (64), every first junction in these genes. The intron splice phases of the human CKII β gene, on the other hand, appear rather randomly emerged; all possible types are realized (48), i.e. type 0 (intron between codons) for introns II and IV, type I (intron interrupts codon between 1st and 2nd base) for introns III and V, and type II (intron interrupts codon between 2nd and 3rd base) for intron VI.

Intron I interrupts the corresponding 5'-untranslated part of the CKII β -mRNA sequence. Thus, not all of the exons contain translated sequences. The information of the CKII β protein is coded in exons 2–7. The start codon ATG is at position 12 of exon 2. An untranslated exon 1 is rarely found among protein kinase genes and kinase regulatory subunit genes (58, 59, 61–64). It has been described only for the regulatory subunit gene of porcine cAMP-dependent protein kinase (60). Here, the translation start codon is similarly located (see below).

Nature of the Transcribed RNA Message—The coding sequence of the human CKII subunit β comprises 648 nucleotides, including the stop codon. This is exactly the size found also with CKII β -cDNA analyses in human (34, 35) and mouse (36) cells. There is also perfect sequence matching between our cDNA data (34) and the genomic data shown here. Apart from minor differences, the same is true with the cDNA sequence reported for HepG2 cells (35).

To determine the site(s) of transcription initiation, primer extension experiments were performed. Using a fluorescently labeled primer (22-mer; EXT1) complementary to region 248–269 in exon 1 (see Fig. 2), the extension products of human total RNA preparations (lymphocytes; chorion carcinoma cell line) were analyzed by on-line detection on the EMBL automated DNA sequencer. Sizing of products was by comparison to bands generated by dideoxy sequencing of M13mp18 DNA or clone pCKII β S8 (see Fig. 1) with EXT1 primer. Three prominent transcription start sites were determined; two major extension products of 248 and 216 bases in length and one minor product of 136 bases were obtained (Fig. 4). The 5'-end of the longest product is therefore located 951 bases upstream of the first letter of the translation initiation codon (AUG) and was assigned number 1 (G) in the genomic DNA sequence of CKII β . The positions of the two others are at

² C. S. Rubin and E. Hu, personal communication.

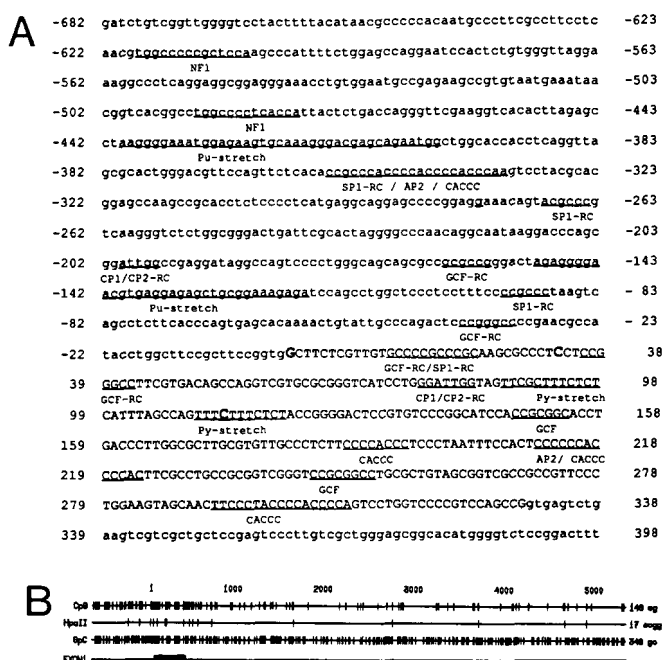


FIG. 5. Nucleotide sequence of the 5'-end region of the human CKII β gene. A, promoter elements, transcription factor binding sites, and unusual DNA structure regions. Putative control element consensus sequences (binding sites for transcription factors SP1, CP1/CP2, GCF, AP2, NF1-CS2/CS3), purine stretches (*Pu*), and pyrimidine stretches (*Py*) are underlined. Untranscribed 5'-region and intron I sequence given in lowercase letters, exon 1 sequence in capital letters, and transcription start sites in boldface letters. Nucleotide numbering refers to first transcription initiation site. B, CpG island. Vertical bars indicate CG nucleotide pairs (1st row), HpaII restriction sites (2nd row), and GC nucleotide pairs (3rd row), respectively. Location of exon 1 is shown to scale at bottom line.

TABLE I

Common features in the promoter regions of mammalian protein kinase genes and kinase regulatory subunit genes

Compared are the sequences of the first 400 bp of the 5'-flanking regions of the following genes: human casein kinase II subunit β , CKII β (see Fig. 5); regulatory subunit of porcine cAMP-dependent protein kinase, PKA-RI (60); catalytic subunits α and β of murine cAMP-dependent protein kinase, PKA-C α and PKA-C β (61); human EGF receptor, EGFR (58); and human insulin receptor, INSR (59).

Feature	Gene					
	CKII β	PKA-RI	PKA-C α	PKA-C β	EGFR	INSR
TATA box	—	—	—	—	—	—
Multiple transcription initiation sites	+	+	+	+	+	+
CCAAT box	—	—	—	—	—	—
At standard position	—	—	—	—	—	—
At nonstandard position	2	2	2	2	—	—
GC content	64%	64%	76%	74%	71%	79%
GC box	3	3	3	5	2	7
Interrupted 5'-untranslated region	+	+	—	—	—	—

sites 33 (C) and 113 (C), respectively (see Fig. 2). No products were detected in control experiments where yeast tRNA was substituted for the analyzed human RNA.

The presence of the three transcription initiation sites defines that three sizes of mRNA can form, 1128, 1096, and (minor) 1018 bp in length, plus a poly(A) tail in each case. Northern blot analyses of RNA preparations from human tissue (placenta) and cultured cells (HeLa, HepG2) indeed reveals the presence of mRNAs corresponding to these lengths (34, 35). In addition to these and in contrast to our observations with placenta and HeLa RNAs (34), two minor CKII β -mRNA species with 3.0 and 4.0 kb in length were reported in HepG2 cells, compatible with the described 1.8-kb 3'-untranslated sequence of the HepG2-cDNA and its suspected differential splicing (35). However, none of our genomic CKII β clones provides any evidence for the existence of such a sequence. Genomic DNA shows (i) no indication of poly(A) stretch(es) in the 3'-flanking region following the terminating *Hae*III restriction site (GGCC) at position 4190, whereas two poly(A) stretches are present in HepG2-cDNA, (ii) no sequence homology to any part of the additional HepG2-cDNA sequence 3' from the *Hae*III site, and (iii) no consensus splice sequence (GT_A^GAG) to facilitate alternative splicing. In addition, poly(A) stretches seem to be normally absent when alternative splicing in the 3'-region is observed (65).

Putative Regulatory Sequence Elements Identify the CKII β Gene as a Housekeeping Gene—In order to identify sequence elements that might be involved in the control of CKII β gene expression, the 5'-flanking region and the untranslated exon 1 were analyzed by computer programs for the presence of known regulatory sequences. A TATA box (TATAAA), which is generally located at a position around -30 relative to the RNA start site in protein coding RNA polymerase II-transcribed genes (49), was found nowhere in the entire region (Fig. 5A). Thus, the CKII β gene promoter belongs to the subclass of TATA-less RNA polymerase II promoters which are found in many housekeeping genes and cellular oncogenes (49, 61, 66-68). These genes have CCAAT sequences remote from the standard positions between -60 and -80 (49, 50). The CKII β gene possesses, in accordance with these, two CCAAT boxes as reverse complements (GATTGG) at positions -201 and 78 (Fig. 5A). CCAAT boxes are known to specifically bind transcription factors CP1, CP2, and also NF1 (50).

The 5'-flanking region and exon 1 turned out to be strikingly rich in C and G (content 64%). These constitute a CpG island over a stretch of roughly 1000 bp, i.e. CG dinucleotides are clustered in this area (Fig. 5B). Consistently, there is also clustering of *Hpa*II restriction sites within this area termed HTF island (*Hpa*II tiny fragment fraction; Ref. 51). The interchanged dinucleotide, GpC, shows random distribution throughout the whole gene. CpG islands are typical features of housekeeping genes (51). Such islands are kept demethylated in cells and, because DNA methylation suppresses transcription of inactive genes (51), are thus kept transcribable and readily repairable and constant over long evolutionary periods. Since CKII β has been found in all cell types of eucaryotes studied so far (1-3), and since the CKII β -cDNA sequences are extremely similar in evolutionary distant organisms (25, 34, 36), it is not surprising to find this housekeeping character in the structure of the CKII β gene.

A true GC motif observed was the hexanucleotide sequence GGGCGG, the binding sequence for one of the earliest identified transcription factors, the SP1 (52). Four such sequence elements were found as reverse complements, located at po-

sitions -355, -263, -94, and 16 (Fig. 5A). Furthermore, the heptanucleotide element, C_G^CGGG_G^CCCC, was localized six times, at positions -161 (reverse complement), -39 (reverse complement), 19 (reverse complement), 34 (reverse complement), 148, and 244. This element binds factor GCF, a negative regulatory protein (53). TATA-less promoter regions can exhibit transcriptional enhancer activity when at least two activating factors such as SP1 can bind (69) and repressor activity when inhibitory factor GCF binds (53). One might speculate that this applies also for the regulation of the cellular level of transcription of the human CKII β gene.

Beside these G-C composed boxes, a number of additional putative control elements were identified. Among these was the element CACCC, described as a determinant of tissue-specific transcription of the β -globin gene (54). This pentanucleotide was found in the form of repeats four times; a strong repeat at -350, others at 191, 214, and 294 (Fig. 5A). Within the repeats, two putative AP2 binding sites were discovered at positions -348 and 212. These are known to be accessible to activation by cAMP and by protein kinase C (55). The most remote putative expression control motifs identified in our analysis were binding sites for NF1-CS2 (TGGNNNNNNGCCA) and NF1-CS3 (TGGNNNNNNNGCCA) as reverse complements at positions -618 and -491, respectively. These proteins can act as transcription enhancers (56).

In addition to these protein binding motifs, the 5'-region also turned out to contain unusual DNA structures. Apart from the already mentioned strong CACCC repeats, two purine-rich regions were found starting at -440 and at -150, respectively (Fig. 5A). Two such regions at similar locations are also present in the regulatory subunit gene of cAMP-dependent protein kinase (60). Furthermore, two pyrimidine(CT)-rich regions appear within exon 1, at positions 87 and 110, respectively. This structure is found also in the epidermal growth factor receptor gene (58).

Repetitive DNA in the CKII β Gene—A complete Alu repeat was identified in intron II with a similarity of 81% to the published Alu consensus sequence (57). The reversely oriented repeat stretched from position 1252 to 1530 (see Fig. 2). Other known repetitive DNA sequences were not seen within the CKII β gene.

The Human CKII β Gene Promoter Shares Common Features with Mammalian Protein Kinase Genes and Genes of Kinase Regulatory Subunits—Are the observed CKII β gene regulatory features unique among genes of the mammalian protein kinase family? The comparison of the first 400 bp of the six promoter sequences given in Table I reveals that several common features are shared. These include lack of TATA box, multiple transcriptional initiation sites, lack of CCAAT box at standard position but presence of two CCAAT boxes in serine/threonine kinase and their regulatory subunit genes at nonstandard positions (CKII β , PKA-RI, PKA-C α , and PKA-C β), unusually high GC content of promoters (64-79%) including presence of CpG islands (data not shown), and several GC boxes (two to seven). Interestingly, the tyrosine kinase genes (epidermal growth factor receptor and insulin receptor) lack CCAAT boxes within the first 400 bp of their promoters. These genes code for protein kinases that are not constitutively expressed and, therefore, in contrast to the listed serine/threonine kinases, are rather non-housekeeping genes.

It should be stressed that the promoter of one of the recorded genes has a particularly high similarity to CKII β , the regulatory subunit of cAMP-dependent protein kinase. The promoters of these two genes share all of the listed

features including an untranslated exon 1 and an identical GC content of 64%. Moreover, the translation start is found in both genes at similar sites, i.e. early in the second exon, after 7 and 12 bp in the PKA-RI and CKII β gene, respectively. Since additional similarities between these two genes are found upstream, particularly two purine-rich stretches at same locations (see Fig. 5), this might indicate a close relation in the control of regulatory subunit genes of protein kinases.

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