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Molecular cloning and predicted full-length amino acid sequence of the type Iβ isozyme of cGMP-dependent protein kinase from human placenta

Tissue distribution and developmental changes in rat

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In this study we report the isolation and characterization of three overlapping cDNA clones for the type Iβ isozyme of cGMP-dependent protein kinase (cGK) from human placenta libraries. The composite sequence was 3740 nucleotides long and contained 58 nucleotides from the 5'-noncoding region, an open reading frame of 2061 bases including the stop codon, and a 3'-noncoding region of 1621 nucleotides. The predicted full-length human type Iβ cGK protein contained 686 amino acids including the initiator methionine, and had an estimated molecular mass of 77 803 Da. On comparison to the published amino acid sequence of bovine lung Iα, human placenta Iβ cGK differed by only two amino acids in the carboxyl-terminal region (amino acids 105–686). In contrast, the amino-terminal region of the two proteins was markedly different (only 36% similarity), and human Iβ cGK was 16 amino acids longer. In a specific region in the amino-terminus (amino acids 63–75), 12 out of 13 amino acids of the human Iβ cGK were identical to the partial amino acid sequence recently published for a new Iβ isoform of cGK from bovine aorta. Northern blot analysis demonstrated a human Iβ cGK mRNA, 7 kb in size, in human uterus and weakly in placenta. An mRNA of 7 kb was also observed in rat cerebellum, cerebrum, lung, kidney, and adrenal, whereas an mRNA doublet of 7.5 and 6.5 kb were observed in rat heart. Comparison of Northern and Western blot analyses demonstrated that the mRNA and protein for cerebellar cGK increased during the development of rats from 5 to 30 days old, whereas the 6.5 kb mRNA in rat heart declined.

Protein kinase; cGMP-dependent; Molecular cloning; cDNA sequence; Tissue specific expression; (Human placenta)

1. INTRODUCTION

cGK is one of the major receptor proteins for cGMP in addition to certain forms of cyclic nucleotide phosphodiesterases and ion channels [1–4]. Cyclic GMP and cGK play important roles in physiological processes such as relaxation of vascular smooth muscle and inhibition of platelet aggregation [5,6]. In addition, atrial natriuretic factor (ANF) may achieve its hemodynamic effects via activation of cGK [6,7].

Structurally, the most extensively studied form of cGK has been that from bovine lung [8]. However, significant amounts of cGK have been found in smooth muscle [6,9], in epithelial cells [10], in platelets [11], and in rat cerebellar Purkinje cells [12]. The native bovine lung enzyme is a

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homodimer [8,13,14]. Each of the subunits has a catalytic domain in the carboxyl-terminal region and a dimerization domain near the amino-terminus. Each subunit also has an inhibitory domain for the catalytic moiety and two different cGMP-binding sites in the regulatory component located in the midportion of the peptide. Amino acid sequencing of the bovine lung enzyme has shown that it contains 670 amino acids and has a subunit M_r of 76331. Binding of cGMP to the protein effects a conformational change resulting in activation of the enzyme.

As has been the case for several other enzymes, including the cAMP-dependent protein kinase, the cGK has been shown to be a family of closely related enzymes. Two main forms of cGK, a soluble form designated type I [8,15,16], and an intrinsic membrane-bound form designated type II [17], have been identified. Recent data from bovine aorta smooth muscle have shown the existence of two different isozyme forms of soluble type I cGK, designated types Iα and Iβ [16]. The type Iα was shown to be similar to the well characterized bovine lung cGK, while type Iβ was shown to be a novel form of cGK. Types Iα and Iβ had SDS-PAGE M_r = 78000 and 80000, respectively. Partial amino acid sequence analysis of type Iβ showed that it was clearly different from the type Iα isozyme in the amino-terminal region of the protein.

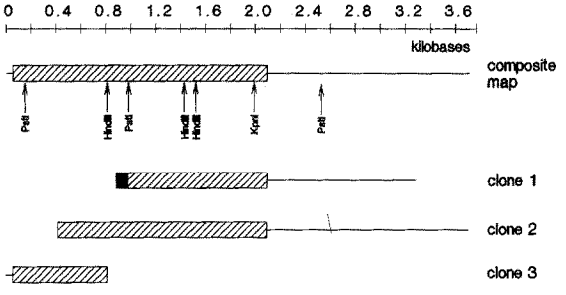

Fig.1. Restriction map of human placenta cGK type Iβ cDNA. Restriction sites are indicated at approximate positions within the composite map. Clones 1, 2 and 3 have been positioned to align identical regions. Hatched bars indicate reading frames, while the black box in clone 2 indicates the 115 nucleotide segment which gave rise to an amino acid sequence with no significant similarity to the bovine type Iα cGK (see text).

No cDNA or genes for cGK from mammalian cells have previously been cloned. Recently, however, the isolation and characterization of several cDNA and genomic clones for cGK from *Drosophila* have been described [18,19].

In this manuscript we demonstrate the isolation and characterization of three human cDNA clones that encode a full-length cGK with amino acid sequence similarities to the bovine type Iβ isozyme. Furthermore, we report the tissue distribution and developmental changes of cGK mRNA and protein levels in rat.

2. MATERIALS AND METHODS

2.1. *Construction of cDNA libraries*

A total of three different human placenta cDNA libraries in λgt11 were screened: (i) a library constructed as described by Gubler and Hoffman [20] using oligo-dT as primer (generous gift from Dr Tanya Z. Schulz, Department of Cell Biology, Baylor College of Medicine, Texas Medical Center, Houston, TX); (ii) an oligo dT-primed library from Clontech, Palo Alto, CA (Cat.no.HL 1008b); and (iii) a library, custom made by Clontech, using specific and random priming.

2.2. *Probe*

The probe used to screen library no.1 was a 684 bp *Eco*RI fragment from a *Drosophila* cDNA for cGK (DG2, TIa) [19]. This *Drosophila* cGK fragment, located between nucleotides F841 and F1592 in the *Drosophila* cGK gene [19], gives rise to an amino acid sequence 74% similar to the ATP-binding and catalytic regions of bovine type Iα cGK (amino acids 351–578). The *Drosophila* probe was radioactively labeled by nick-translation.

2.3. *cDNA screening*

Low stringency screening was performed using a protocol based on that of Rijsewijk et al. [21], and high stringency screening was performed as described by Sandberg et al. [22].

2.4. *Subcloning and sequencing of DNA fragments*

Clones were isolated and digested with various restriction enzymes, ligated into the vector M13 and sequenced by the

Fig.2. Nucleotide and deduced amino acid sequence of human placenta cGK. Nucleotides are numbered starting with 1 at the first nucleotide in the coding region. Amino acids are shown in the three letter code. The stars indicate the first amino acid in the part of the sequence that is common to both Iα and Iβ cGK. The first underlined amino acid (63) differs from that of the published bovine Iβ cGK, while the second (280) and third (290) underlined amino acids represent the two differences between human Iβ and bovine Iα cGK between amino acids 105 and 686.

```
-58   AGGAAGCCTCAAGACGCGGAGCAGCGGCAGGAAGGAGCCCCCGGCAGCCCGGAGGAGCATGGGCACCTTGCGGGATTTACAGTACGCGCTCCAGGAGAAG
                                                                  MetGlyThrLeuArgAspLeuGlnTyrAlaLeuGlnGluLys

 43   ATCGAGGAGCTGAGGCAGCGGGATGCTCTCATCGACGAGCTGGAGCTGGAGTTGGATCAGAAGGACGAACTGATCCAGAAGCTGCAGAACGAGCTGGACAAG
      IleGluGluLeuArgGlnArgAspAlaLeuIleAspGluLeuGluLeuGluLeuAspGlnLysAspGluLeuIleGlnLysLeuGlnAsnGluLeuAspLys

145   TACCGCTCGGTGATCCGACCAGCCACCCAGCAGGCGCAGAAGCAGAGCGCGAGCACCTTGCAGGGCGAGCCGCGCACCAAGCGGCAGGCGATCTCCGCCGAG
      TyrArgSerValIleArgProAlaThrGlnGlnAlaGlnLysGlnSerAlaSerThrLeuGlnGlyGluProArgThrLysArgGlnAlaIleSerAlaGlu

247   CCCACCGCCTTCGACATCCAGGATCTCAGCCATGTGACCCTGCCCTTCTACCCCAAGAGCCCACAGTCCAAGGATCTTATAAAGGAAGCTATCCTTGACAAT
      ProThrAlaPheAspIleGlnAspLeuSerHisValThrLeuProPheTyrProLysSerProGlnSer***LysAspLeuIleLysGluAlaIleLeuAspAsn

349   GACTTTATGAAGAACTTGGAGCTGTCGCAGATCCAGGAGATTGTGGATTGTATGTACCCGGTGGAGTATGGCAAGGACAGTTGCATCATCAAAGAAGGAGAC
      AspPheMetLysAsnLeuGluLeuSerGlnIleGlnGluIleValAspCysMetTyrProValGluTyrGlyLysAspSerCysIleIleLysGluGlyAsp

451   GTGGGGTCACTGGTGTATGTCATGGAAGATGGTAAGGTTGAAGTTACAAAAGAAGGTGTGAAGTTGTGTACCATGGGTCCAGGAAAAGTGTTTGGGGAATTG
      ValGlySerLeuValTyrValMetGluAspGlyLysValGluValThrLysGluGlyValLysLeuCysThrMetGlyProGlyLysValPheGlyGluLeu

553   GCTATTCTTTACAACTGTACCCGGACAGCGACCGTCAAGACTCTTGTAAATGTAAAACTCTGGGCCATTGATCGACAATGTTTTCAAACAATAATGATGAGG
      AlaIleLeuTyrAsnCysThrArgThrAlaThrValLysThrLeuValAsnValLysLeuTrpAlaIleAspArgGlnCysPheGlnThrIleMetMetArg

655   ACAGGACTCATCAAGCATACCGAGTATATGGAATTTTAAAAAGCGTTCCAACATTCCAGAGCCTTCCTGAAGAGATCCTCAGCAAGCTTGCTGATGTCCTT
      ThrGlyLeuIleLysHisThrGluTyrMetGluPheLeuLysSerValProThrPheGlnSerLeuProGluGluIleLeuSerLysLeuAlaAspValLeu

757   GAAGAGACCCACTATGAAAATGGAGAATATATTATCAGGCAAGGTGCAAGAGGGGACACCTTCTTTATCATCAGCAAAGGAACGGTAAATGTCACTCGTGAA
      GluGluThrHisTyrGluAsnGlyGluTyrIleIleArgGlnGlyAlaArgGlyAspThrPhePheIleIleSerLysGlyThrValAsnValThrArgGlu

859   GACTCACCGAGTGAAGACCCAGTCTTTCTTAGAACTTTAGGAAAAGGAGACTGGTTTGGAGAGAAAGCCTTGCAGGGGGAAGATGTGAGAACAGCAAACGTA
      AspSerProSerGluAspProValPheLeuArgThrLeuGlyLysGlyAspTrpPheGlyGluLysAlaLeuGlnGlyGluAspValArgThrAlaAsnVal

961   ATTGCTGCAGAAGCTGTAACCTGCCTTGTGATTGACAGAGACTCTTTTAAACATTTGATTGGAGGGCTGGATGATGTTTCTAATAAAGCATATGAAGATGCA
      IleAlaAlaGluAlaValThrCysLeuValIleAspArgAspSerPheLysHisLeuIleGlyGlyLeuAspAspValSerAsnLysAlaTyrGluAspAla

1063  GAAGCTAAAGCAAAATATGAAGCTGAAGCGGCTTTCTTCGCCAACCTGAAGCTGTCTGATTTCAACATCATTGATACCCTTGGAGTTGGAGGTTTCGGACGA
      GluAlaLysAlaLysTyrGluAlaGluAlaAlaPhePheAlaAsnLeuLysLeuSerAspPheAsnIleIleAspThrLeuGlyValGlyGlyPheGlyArg

1165  GTAGAACTGGTCCAGTTGAAAAGTGAAGAATCCAAAACGTTTGCAATGAAGATTCTCAAGAAACGTCACATTGTGGACACAAGACAGCAGGAGCACATCCGC
      ValGluLeuValGlnLeuLysSerGluGluSerLysThrPheAlaMetLysIleLeuLysLysArgHisIleValAspThrArgGlnGlnGluHisIleArg

1267  TCAGAGAAGCAGATCATGCAGGGGGCTCATTCCGATTTCATAGTGAGACTGTACAGAACATTTAAGGACAGCAAATATTTGTATATGTTGATGGAAGCTTGT
      SerGluLysGlnIleMetGlnGlyAlaHisSerAspPheIleValArgLeuTyrArgThrPheLysAspSerLysTyrLeuTyrMetLeuMetGluAlaCys

1369  CTAGGTGGAGAGCTCTGGACCATTCTCAGGGATAGAGGTTCGTTTGAAGATTCTACAACCAGATTTTACACAGCATGTGTGGTAGAAGCTTTTGCCTATCTG
      LeuGlyGlyGluLeuTrpThrIleLeuArgAspArgGlySerPheGluAspSerThrThrArgPheTyrThrAlaCysValValGluAlaPheAlaTyrLeu

1471  CATTCCAAAGGAATCATTTACAGGGACCTCAAGCCAGAAAATCTCATCCTAGATCACCGAGGTTATGCCAAACTGGTTGATTTTGGCTTTGCAAAGAAAATA
      HisSerLysGlyIleIleTyrArgAspLeuLysProGluAsnLeuIleLeuAspHisArgGlyTyrAlaLysLeuValAspPheGlyPheAlaLysLysIle

1573  GGATTTGGAAAGAAAACATGGACTTTTTGTGGGACTCCAGAGTATGTAGCCCCAGAGATCATCCTGAACAAAGGCCATGACATTTCAGCCGACTACTGGTCA
      GlyPheGlyLysLysThrTrpThrPheCysGlyThrProGluTyrValAlaProGluIleIleLeuAsnLysGlyHisAspIleSerAlaAspTyrTrpSer

1675  CTGGGAATCCTAATGTATGAACTCCTGACTGGCAGCCCACCTTTCTCAGGCCCAGATCCTATGAAAACCTATAACATCATATTGAGGGGGATTGACATGATA
      LeuGlyIleLeuMetTyrGluLeuLeuThrGlySerProProProPheSerGlyProAspProMetLysThrTyrAsnIleIleLeuArgGlyIleAspMetIle

1777  GAATTTCCAAAGAAGATTGCCAAAAATGCTGCTAATTTAATTAAAAAACTATGCAGGGACAATCCATCAGAAAGATTAGGGAATTTGAAAAATGGAGTAAAA
      GluPheProLysLysIleAlaLysAsnAlaAlaAsnLeuIleLysLysLeuCysArgAspAsnProSerGluArgLeuGlyAsnLeuLysAsnGlyValLys

1879  GACATTCAAAAGCACAAATGGTTTGAGGGCTTTAACTGGGAAGGCTTAAGAAAAGGTACCTTGACACCTCCTATAATACCAAGTGTTGCATCACCCACAGAC
      AspIleGlnLysHisLysTrpPheGluGlyPheAsnTrpGluGlyLeuArgLysGlyThrLeuThrProProIleIleProSerValAlaSerProThrAsp

1981  ACAAGTAATTTTGACAGTTTCCCTGAGGACAACGATGAACCACCACCTGATGACAACTCAGGATGGGATATAGACTTCTAATGTATTTCTCTTACCTGCTTC
      ThrSerAsnPheAspSerPheProGluAspAsnAspGluProProProAspAspAsnSerGlyTrpAspIleAspPheEnd

2083  TGCCTTGCTGAAGACAGCTTTTTCTGAGACACAGCTGCCAGCAAACCTGAGGGAAAGAGAGAAGATTAGTGCTCGGGGTCACCATGATGCCTTTGATGGATG
2185  CTGCTCCAGTAACTACAGTGGCATTAGGACTTATCGCTTAGATGACAATAGTGCTCTTTACATGTTTTCTGTTTGAACCTAAAATAGCAGTTGACATGGTGG
2287  TCCTGAAGCAAAGCCTTTCACCAGTAAAGAGATGTTTTCTATTGTTGCAATGACCTTGCTTTGCTCTGATTATAATTTGAAAGACTGTAGGAAACACTTCAA
2389  TGTAGTATAAGAGTCTGTACCTTGCTGGAATATTCAAGAAGATGAAAGAATAATATATTGGGTACAATAGATTACTATGGTACAGAAACTGGGCTATTCCCT
2491  TTCTTCAAGTGAAGGCTGTGGGATCTATTACAGCTGCAGGCCGGTGTATATACCATACAAAAGAGGACCACACATCTGTTGGTCACAGAGTTCATGTCACAC
2593  CAGTGCTAGAAGTTTCATGATTTTATTTCCCAGCAGTGCTGATGACAAGACTGAATGTTACCTTTTCTTTCTGACAGATTTTAAAAATTGATATGATAAAAG
2695  CACAACTGCTATAGATTCTGCTGAGACCTCTCATAGTAGGTATATATGAGTTTTCACAGAAGACTGAAAAATAATGCATGATATTTGTTTGTTTTTTTTGAT
2797  AAATTGGCATGACAGAGTGGGGAAAAAAAGCAATTCACAAAACCATTTCATATTTTTTAAAATATTGTGCTTAAAGATGGTCCTGGAAGTAAATGACTAGCA
2899  GCCAATTGGTTTTACTTAACATACCCTCAAACTGAGGCTTAAAGTATTCCCTTTTATAAAAATAAATGCTTGGGGTAGGGTGGAGTGGGGAGGGATTAAAAC
3001  CCATCCAAAAAATAAATAAAAACTATATAGGTGCTATGTATATCTTTCATCTGTAAATGTCAGTGTCTGAACAGCAACACAAATTCAAATCATTATACGTGT
3103  AGCCAGAAACTCAAGCATTTTCACTAAAGTTATTAAACCAAACTCCTGTCCAATTTGACTTATACAACATAGTCAGTCTAGAGTTGAGAGACAAAGGTAATT
3205  ATAAACCTATTTGAACTAGCTTCTTGTCTTAGGCCTGAACCAAAAAACAACAAACAAACAAAAAACAAGAATGAAAAACAGAAATAAAAGAAGTAGAAAAGA
3307  CAAAGAAAGAAAGCCCAAAGTCAAAGTTGTTAATATTTACAGGTTTACCAGATCTGGAACATTACTTATTTGAGGTCAGAGAACAAAACAAGAACCTGGCCA
3409  GGTGTTGATTACCTTTTAGTGAATAAGCTGAGTCCATATACTTGTCTAACTAAGAAAGCAGTACAGAGGAAAACAGGAACCTGATTTTTTTAAAATAAATTT
3511  TAAATAAAATAGAATTACTACAATTCTGCAATTTCATACTACCTAAAAAAGACTAGATTTGAAAATGTCAAGCTGATTTACTTTATTCACATGGAGAAAAGA
3613  ATCCACAAATTAAACTGAGTCCTTCACTGGCATGCCAGTTGACTATTATTAGCTGTCATAAGTAACCCCG    3682
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dideoxy chain-termination method of Sanger et al. [23]. All regions of the clones were sequenced at least twice. The region between nucleotides 124 and 246 in the composite sequence was difficult to resolve due to GC compressions using ordinary conditions. Therefore, the sequence in this area was verified by automatic sequencing performed by Lark Sequencing Technologies Ltd (Texas Medical Center, 1709 Dryden, Suite 604, Houston, TX). Nucleotide and amino acid sequence data were analysed using the UWGCG program package [24].

2.5. *RNA extraction/Northern blot analysis*

RNA extraction and Northern blots of human tissues were performed as described earlier [25,26]. A 2.2 kb nick-translated cGK cDNA (*Hin*dIII/*Eco*RI fragment, corresponding to nucleotides 1454–3682 in the composite sequence) and a 0.2 kb 5′ *Eco*RI/*Pst*I fragment (corresponding to nucleotides −58 to 129 in the composite sequence) were used for hybridization. For rat tissues, a LiCl-urea method was used to extract RNA [27], and Northern blots were hybridized with a ^{32}P-labeled complementary RNA made by in vitro transcription from a Bluescript plasmid containing the 500 nucleotide *Hin*dIII/*Kpn*I human cGK cDNA insert (corresponding to nucleotides 1454–1937 in the composite sequence) [28].

2.6. *Western blot analysis*

Rat (Sprague-Dawley) tissue extracts were obtained from a 100000 × *g* centrifugation of whole homogenates and were analysed by SDS-PAGE and Western blotting for cGK protein as previously described using a monospecific antibody for cGK and ^{125}I-Protein A [29].

3. RESULTS

3.1. *Isolation and characterization of cDNAs*

We have previously identified and affinity purified cGK protein from human placenta. Based on this information we decided to clone human cGK cDNA from this tissue using a 684 bp *Eco*RI fragment of *Drosophila* cGK cDNA (see section 2) as a probe. The *Drosophila* probe was radioactively labeled and used to screen a human placenta cDNA library (library no.1) under low stringency conditions. Two positive clones, later shown to be identical, were isolated. The inserts from the two positive clones were 2433 bp in length (fig.1, clone 1). Nucleotides 116–1238 of this insert corresponded to nucleotides 936–2058 in the composite sequence (fig.2) and gave rise to a sequence 374 amino acids long. This sequence showed 100% similarity to amino acids 297–670 of the bovine type Iα cGK. The most 5′ 115 nucleotides of the isolated insert, however, gave rise to an amino acid sequence that showed no significant similarity to type Iα cGK (data not shown). No typical exon/intron junction was found to indicate that these 115 nucleotides represented an intron from an unprocessed mRNA [30].

Clone 1 was subsequently digested with restriction enzymes to obtain a 3′ 1.8 kb *Hin*dIII/*Eco*RI fragment (corresponding to nucleotides 1454–3269 in the composite sequence) which was labeled and used to screen another human placenta cDNA library (library no.2) under high stringency hybridization conditions. From this screening, two identical positive clones were identified and isolated (fig.1, clone 2). An insert of 3313 nucleotides was sequenced and shown to contain an open reading frame of 1692 nucleotides (including the stop codon TAA). This coding region gave rise to a sequence 563 amino acids long that was 99.5% similar to amino acids 108–670 of the bovine type Iα cGK. The insert also contained 1621 nucleotides 3′ of the stop codon.

In an effort to isolate the very 5′-end of the cDNA, three specific oligonucleotides were designed which were complementary to nucleotides 745–763, 1306–1324 and 2139–2157, respectively, in the composite sequence. These specific oligonucleotides, together with random oligonucleotides, were used as primers to construct another human placenta cDNA library (library no.3). This library was subsequently screened under high stringency conditions using the 5′ 0.4 kb *Eco*RI-*Hin*dIII fragment (corresponding to nucleotides 370–739 in the composite sequence) from clone 2 as a probe. One positive clone (fig.1, clone 3) with a 0.8 kb insert was detected, isolated

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human type Iß  QSASTLQGEPRTKR-QAISAEP-TAFDIQDLSHVTLPFYPKSPQ  ( 63-104)
bovine type Iß E·····                                        (  1- 13)
bovine type Iα PVP·-HI·---·I·A·G·····Q·YRSFH··RQ-AFRKFI··ER  ( 47- 88)
drosophila     ATRKSG·NFQ·-Q·ALG·····-QSESSLL·E··SF·K·D·DER  (465-506)
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Fig.3. Sequence comparison between human type Iβ cGK, bovine type Iβ, bovine type Iα and *Drosophila* DG2, TIa. Gaps, designated by dashes, are inserted to achieve maximal identity between the proteins. Dots represent identity with the human sequence. Autophosphorylated amino acids in the bovine type Iα sequence are underlined.

and characterized. Sequencing showed that it represented the 5′-end of a type I cGK. In addition to 58 nucleotides 5′ of the start codon ATG, clone 3 contained an open reading frame 751 nucleotides long. The deduced carboxyl-terminal 146 amino acids were identical to the bovine type Iα cGK (amino acids 89–234), but there were substantial differences between the deduced amino-terminal part of clone 3 and bovine type Iα cGK. Only 36% of the amino acids in this region (amino acids 1–104) were identical to the bovine type Iα cGK. However, 12 out of the 13 recently published amino acids from the bovine type Iβ were identical to amino acids 64–75 in the human sequence.

The overlapping regions of clones 1 and 2 (2317 nucleotides) and clones 2 and 3 (383 nucleotides) showed 100% nucleotide similarity making it very likely that these clones were derived from the same gene (fig.1). The composite sequence contained 3740 nucleotides, with an open reading frame of 2061 nucleotides giving rise to a 686 amino acid peptide (fig.2). In the amino acid region 105–686, the human type Iβ cGK was identical to amino acids 89–670 of the bovine type Iα protein, with the exception of two amino acid substitutions. Amino acids 280 (Thr) and 290 (Ser) in the human protein were replaced by Lys and Asn, respectively, in the bovine sequence. Both amino acid substitutions were located in the first cGMP-dependent binding region [8].

However, in the amino-terminal region of the molecules, there were substantial differences between human type Iβ cGK and bovine type Iα, although some areas showed sufficient similarity to indicate a common origin (fig.3).

3.2. *Determination of mRNA sizes and protein levels*

Northern analysis of the mRNA for human cGK (shown in fig.4A) detected a major 7 kb species in human uterus and placenta, although the signal in placenta was much weaker and not visible after photography. The same 7 kb message was identified by two different labeled cDNA probes, either a 0.2 kb 5′ *Eco*RI/*Pst*I fragment (data not shown) expected to be type Iβ specific, or a 2.2 kb *Hin*dIII/*Eco*RI fragment (fig.4A) expected to detect both type Iα and Iβ cGK mRNA. In uterus, a weak 4.2 kb mRNA for cGK was also detected by both probes. Significant levels of cGK protein were

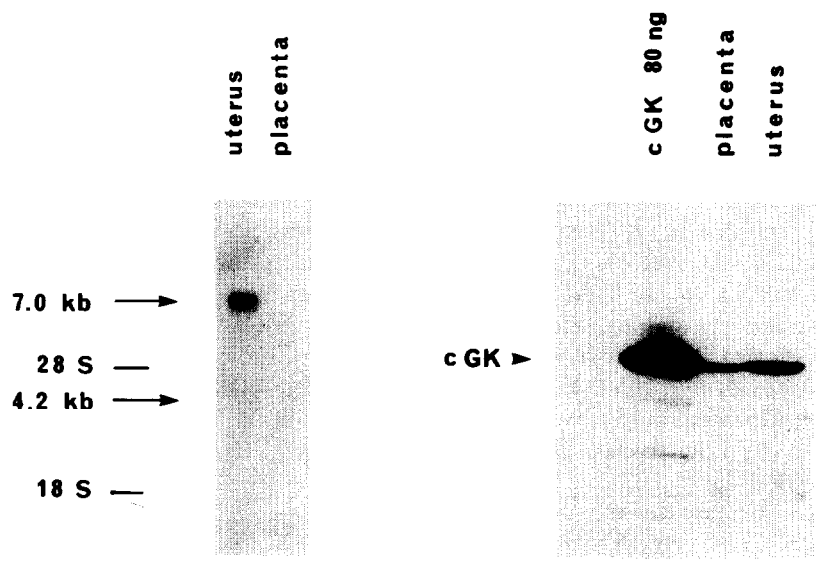

Fig.4. (A) Northern blot analysis for type I cGK in total RNA (20 μg) from human placenta and uterus. (B) Western blot analysis of the amount of cGK protein in human placenta and uterus (300 μg tissue extract each). A purified cGK standard (80 ng) is also shown.

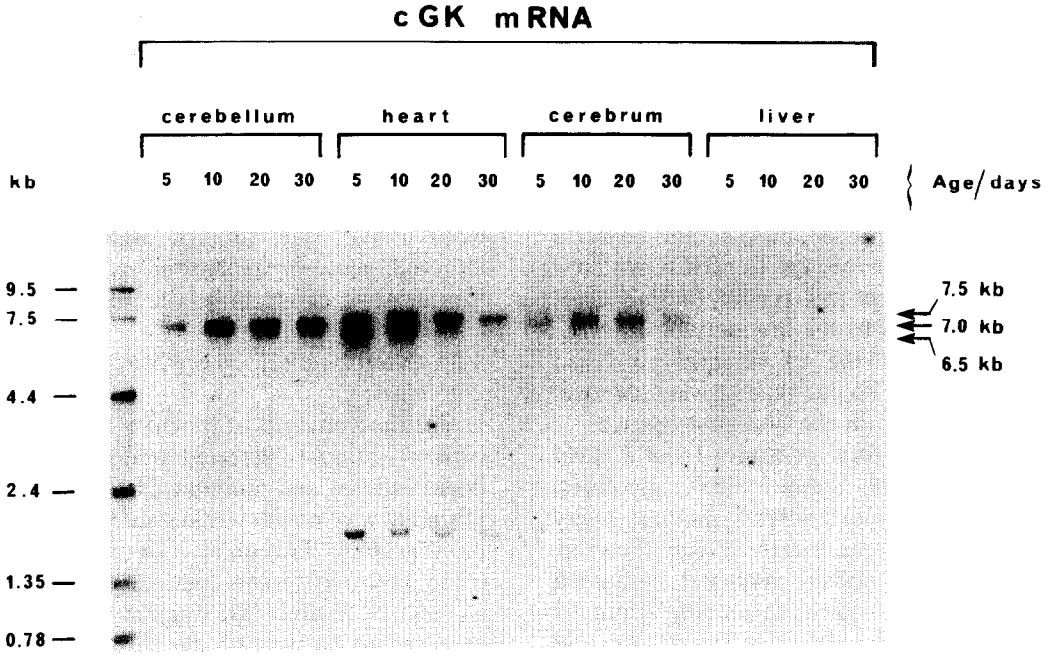

Fig.5. Northern blot analysis of type I cGK mRNA in different tissues from rats at various days of age. Total RNA (10 μg each) was used for analysis. A ^{32}P-labeled RNA ladder used as a standard in determining cGK mRNA size is shown on the left.

found in both uterus and placenta by Western blot analysis (fig.4B).

In order to demonstrate further that the 7 kb mRNA showed other characteristics expected for cGK, Northern (fig.5) and Western (fig.6) analyses of several rat tissues were examined during postnatal development. Northern blots demonstrated a 7 kb mRNA in rat cerebellum and cerebrum, a 7.5 and 6.5 kb mRNA in rat heart, and no detectable mRNA in rat liver. The 7 kb mRNA was also found in rat lung, kidney, and adrenal (data not shown). Western blots showed that cGK protein was evident in all of these tissues, but that the amount expressed in liver was very little in comparison to the other tissues. This agreed with the Northern blots.

A developmental increase in the amount of cerebellar cGK mRNA and protein was observed, in agreement with previous studies [31]. A small increase in cerebrum cGK protein was not correlated with any increase in mRNA in cerebrum. The two heart mRNA forms, which differed from the 7 kb form in brain, showed different developmental declines, that for the 6.5 kb species being faster and more complete (fig.5). This was not accompanied by any obvious decrease in heart cGK protein (fig.6). It is expected that the antibody used in the Western blots can recognize both Iα and Iβ forms of cGK, since this was recently reported by Wolfe et al. [32], who used the same antibody in their studies.

Repassage of total rat cerebellar RNA over a Pharmacia oligo-dT spin column caused a 7-fold enrichment of the major 7 kb mRNA and also minor mRNA forms of 4.2 and 2.5 kb (data not shown), indicating that the latter forms may also represent cGK mRNAs. The small band shown at 1.7 kb in fig.5 did not appear to be enriched in the poly(A)$^+$ RNA.

4. DISCUSSION

This report documents the isolation of cDNA clones encoding a full-length type Iβ cGK from human placenta. Molecular cloning of the human Iβ cGK was performed from placental tissue as a result of our previous knowledge that this tissue contained cGK protein. The fact that a total of ap-

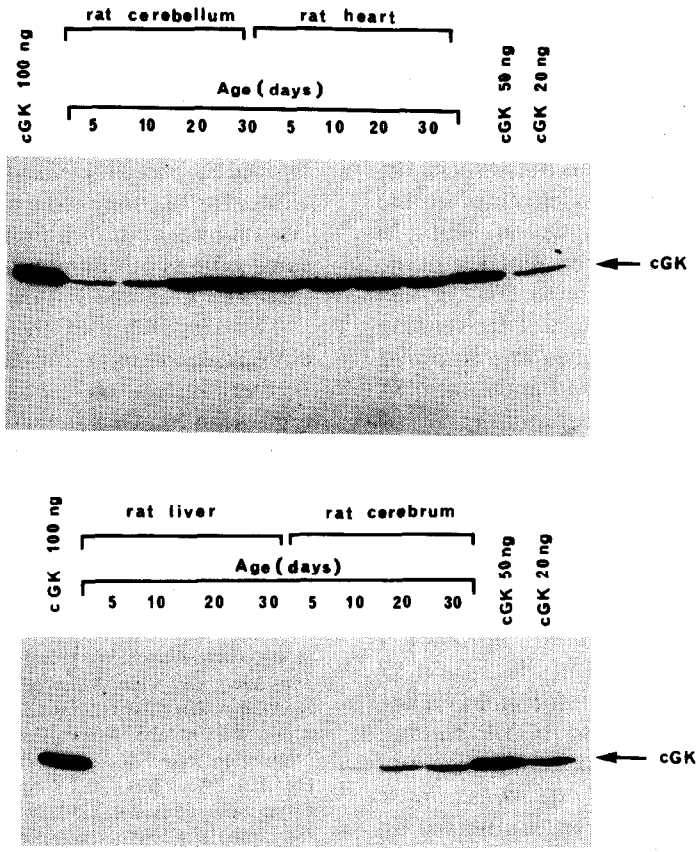

Fig.6. Western blot analysis of cGK protein levels in different tissues (300 μg tissue extract each) of rats at various days of age. Purified cGK standards (100, 50, and 20 ng) are also shown.

proximately three million clones from three different placenta libraries were screened and only three different cDNA clones for cGK were obtained, together with the low cGK mRNA signal in Northern analysis of placenta, indicates that the cGK message is rare in human placental tissue.

The identity of the cDNA clones coding for the Iβ form of the human cGK was verified by comparing the deduced amino acid sequence to the recently published 13 amino acids for bovine type Iβ [16]. Twelve out of 13 amino acids reported for bovine Iβ cGK, whereas only 6 out of 13 amino acids for the same area of bovine Iα showed identity to the deduced amino acid sequence of human Iβ cGK (fig.3). The single amino acid difference (position 63, Gln in human and Glu in bovine) between bovine and human Iβ cGK in this region may be due either to species differences or perhaps to a bovine cGK Iβ Gln^{63}-Glu deamidation conversion prior to amino acid sequencing. Furthermore, the molecular weight of bovine Iβ cGK appeared greater than that of the Iα form on SDS-PAGE [15,32], which corresponds well with our observation that human Iβ is 16 amino acids longer than bovine Iα cGK.

Comparison of the deduced amino acid sequence of the isolated human type Iβ cGK to the known amino acid sequence for the bovine type Iα cGK showed that the carboxyl amino acids (105–686) were 99.7% similar. Only 2 amino acid differences were seen in this region (bovine Iα cGK amino acids 264 (Lys) and 274 (Asn) correspond to human Iβ cGK amino acids 280 (Thr) and 290 (Ser), respectively). In contrast, the amino-terminal 104 amino acids of the bovine type Iα display only 36% similarity to the human type Iβ

form. These data show that there is a clear change in similarity between the Iα and the Iβ forms beginning at amino acid 105. Changes like these typically indicate the involvement of mechanisms such as alternative splicing. The three nucleotides preceding the codon giving rise to amino acid 105 are 'CAG', the most common nucleotides on the 5'-exon of an exon/intron junction [30]. In *Drosophila* there is an exon/intron junction in the cGK DG2 gene making amino acid 506 the last amino acid coded by the first exon of the reading frame. This amino acid corresponds to amino acid 104 in the human type Iβ cGK and amino acid 88 in the bovine type Iα cGK, the last amino acids of the β- and α-specific segments of the molecules, respectively (fig.3). These findings support the possibility that the Iα and Iβ form of cGK are derived from the same gene by use of alternative splicing.

The divergent amino-terminal region of the Iα and Iβ form of cGK corresponds to dimerization and inhibitory domains. Therefore, if Iα and Iβ forms of cGK are 100% similar for amino acids 105–686, which contain the two cGMP-binding sites as well as the catalytic domain, then the dimerization and inhibitory domains must somehow contribute to the differences observed by Wolfe et al. [16,32] in cGMP and cGMP analog binding to types Iα and Iβ.

In the amino-terminal part of the bovine type Iα, four different autophosphorylation sites (Ser-50, Thr-58, Ser-72 and Thr-84) are observed [33]. Of these amino acids, only Ser-50 is conserved in the human type Iβ (fig.3). This is in accordance with the observations of Wolfe et al. [32] that the bovine Iα and Iβ autophosphorylate at different amino acid residues. They also found that autophosphorylation was almost exclusively on serine residues of bovine Iβ. In the amino-terminal part of the human Iβ cGK that has low homology to Iα, there are 6 serine residues (at positions 51, 64, 66, 80, 92 and 102). Based upon our data, it is not possible to predict which of these serine residues can be autophosphorylated.

On Northern blots, human Iβ cGK probes detected a predominant 7 kb mRNA species in the human and rat tissues examined, an exception being specific forms (7.5 and 6.5 kb) in rat heart. The mechanisms giving rise to the different mRNAs are not known. They may be derived from different genes, or from a single gene either by alternative splicing or by use of different polyadenylation site signals. The latter mechanism is the one most commonly used for generating different mRNA species from genes for regulatory subunits of cAMP-dependent protein kinase [34]. None of the human clones for Iβ contained a poly(A) tail at the 3'-end. Assuming that the 7 kb mRNA is derived from the same gene as our human Iβ cDNA clones, approximately 3 kb of the 3'-noncoding region is missing.

The availability of the present human Iβ cGK cDNAs encoding a full-length protein will allow further studies on the structure, function and regulation of cGKs in mammalian cells. Of special interest will be the use of the cDNAs for human Iβ cGK to detect and study the gene(s) for cGK in an attempt to elucidate whether the Iα and Iβ forms are generated by alternative splicing. Such studies are presently in progress in our laboratories.

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