The molecular cloning of a type II regulatory subunit of the cAMP-dependent protein kinase from rat skeletal muscle and mouse brain

(cloning/mRNA/testis)

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ABSTRACT   A cDNA clone for a type II regulatory (R) subunit of the cAMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) was isolated from a rat skeletal muscle library using a specific 47-base oligonucleotide probe. The rat cDNA was 1.2 kilobases (kb) in length and contained an open reading frame of 1.113 kb representing 92% of the coding region of the molecule. Nick-translated rat cDNA was then used to isolate a mouse R^{II} cDNA clone from a brain library that contained an open reading frame of 1.143 kb. Because both cDNAs lacked complete coding sequences, the remainder of the R^{II} coding region was obtained from a 15-kb mouse genomic clone. The mouse R^{II} coding region contains 1.2 kb corresponding to a 400-amino acid protein of 51.141 kDa. The mouse cDNA hybridizes to two mRNA species, a 2.4-kb form that was only observed in testis and a 6.0-kb form found in a wide range of tissues, including testis.

The cAMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) is a key enzyme important in the regulation of metabolism and proliferation of eukaryotic cells (for reviews, see refs. 1–3). There is considerable biochemical evidence to suggest that most, if not all, of the biological effects of cAMP are mediated through the action of the kinase (1–3). The enzyme exists as an inactive tetrameric holoenzyme complex made up of two regulatory (R) and two catalytic (C) subunits. Each R subunit binds two molecules of cAMP, which leads to dissociation of the holoenzyme (4, 5). The free C subunits are then available to phosphorylate target proteins on serine and threonine residues.

Historically, two types of cAMP-dependent protein kinase holoenzyme, termed types I and II, have been distinguished from their order of elution from DEAE-cellulose (6). Examination of these types revealed their difference to be due to distinct R subunits in each form (6). Detailed analysis of both R subunits (designated R^I and R^{II}) showed that they differ in M_r, physiochemical properties, and tissue distribution. One striking difference is that R^{II} can be phosphorylated by the C subunit, whereas R^I cannot (1–3).

Recent studies involving protein sequencing, immunological methods, and cDNA cloning show a multiplicity in C and R subunit isoforms. R^I, with a molecular mass of 49 kDa, as determined by NaDodSO$_4$/PAGE, has been isolated from several species (2). The amino acid sequence of the bovine skeletal muscle R^I has been determined (7), and cDNA clones for this protein have been characterized (8, 9). Recent analysis of cDNA clones indicates a second isoform of R^I that is expressed only in brain (C. Clegg, G. Cadd, and G.S.M.,

unpublished observation). It has been known for some time that at least two forms of R^{II} exist—namely, a 54-kDa form found in most tissues (10) and a 51-kDa form found in brain, granulosa cells, testis, and adrenal tissue (11–15). A partial clone of one 51-kDa R^{II} from rat granulosa cells, which shows some amino acid sequence homology to the 54-kDa R^{II} from bovine cardiac muscle (10), has recently been reported (16). In addition, two genes for the C subunit (designated Cα and Cβ), which code for proteins that are 91% identical but that show different tissue distributions, have been identified (17–19).

In this manuscript the isolation and characterization of cDNA and genomic DNA (from rat and mouse) that code for an R subunit similar to the 54-kDa R^{II} form are described.[‖] The complete predicted amino acid sequence of this form of R^{II} is presented, and the sizes of mRNAs in various mouse tissues are reported.

MATERIALS AND METHODS

Construction of cDNA and Genomic Libraries. A rat skeletal muscle cDNA library was constructed using the plasmids pUC8 and pUC9 as described (20). A phage λgt11 cDNA library from BALB/c mouse brain was constructed as described (21). Mouse genomic DNA partially digested with Mbo I and enriched for fragments of 15 kilobases (kb) by size fractionation on agarose gels was ligated into phage EMBL 3 and packaged (22).

Screening of cDNA and Genomic Libraries. Ampicillin-resistant colonies were grown on nitrocellulose filters, and this procedure was followed by cell lysis and DNA covalent binding (23). These filters were prehybridized for 4–16 hr at 37°C in prehybridization buffer [6× standard saline citrate (SSC)/50 mM Na$_2$HPO$_4$, pH 6.7/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/0.1% Ficoll/0.1% NaDodSO$_4$/200 μg of herring sperm DNA per ml]. Hybridization was done at 37°C overnight using the hybridization buffer, which was the same as the previous buffer except that it lacked 0.1% NaDodSO$_4$. The nitrocellulose filters were washed at 37°C for 1 hr in 6× SSC/0.1% sodium pyrophosphate before washing at 42°C (low stringency) or 49°C (higher stringency) for 3 min.
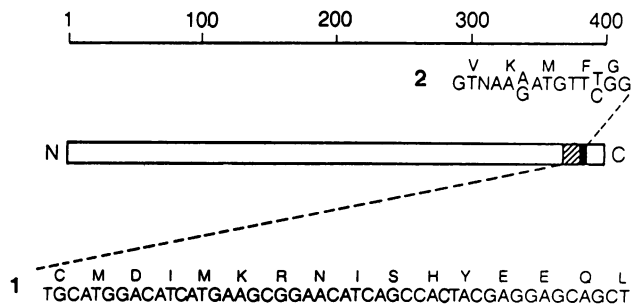
FIG. 1. The strategy for cloning rat skeletal muscle R^{II}. A 47-base oligonucleotide that represents residues 370–385 of the bovine sequence (sequence 1) was synthesized and used for primary screening of the library. A second nucleotide (sequence 2) of mixed degeneracy representing residues 386–389 was used to screen positive colonies. N, amino terminus; C, carboxyl terminus.

Phage plaques were transferred to nitrocellulose filters, denatured, and prehybridized at 42°C in prehybridization buffer for 4–16 hr. Hybridization was done in the hybridization buffer containing 50% (vol/vol) formamide at 42°C with nick-translated rat or mouse R^{II} cDNA used as a probe. The filters were washed with 2× SSC/0.1% (wt/vol) sodium pyrophosphate at 65°C for 15 min followed by several washes in 0.5% NaDodSO$_4$/10 mM Tris chloride, pH 7.5/2.5 mM EDTA/0.1% (wt/vol) sodium pyrophosphate at 45°C.

Subcloning and Sequencing of DNA Fragments. The rat skeletal muscle *Eco*RI/*Sal* I cDNA insert RSK 1 (Fig. 2) was subcloned into M13 phage, and both strands were sequenced. The mouse brain *Eco*RI cDNA of MB 1 was initially subcloned into pUC8 for restriction mapping and then into M13 phage for sequencing. The mouse genomic *Sal* I/*Eco*RI DNA fragments of MG 1 were subcloned into pUC18, pUC19, and Bluescribe M13$^+$ for restriction analysis and sequencing. Nucleotide sequencing was by the dideoxy chain-termination method of Sanger *et al.* (24).

RNA Blot Analysis. Samples of RNA were denatured in 20 mM 4-morpholinepropanesulfonic acid (Mops), pH 7.0/1 mM EDTA/5 mM sodium acetate/2.2 M formaldehyde/50% (vol/vol) formamide at 55°C for 10 min. The samples were loaded onto a 1% agarose gel and run in the same buffer without formamide. The gel was then blotted onto nitrocellulose, baked at 80°C for 2 hr, and hybridized 16–20 hr. Filters were washed in 6× SSC/0.1% (wt/vol) pyrophosphate at 37°C for 1 hr before higher-stringency washing with 1× SSC/0.1% (wt/vol) NaDodSO$_4$ at 55°C for 15 min.

RESULTS

Isolation and Characterization of DNA. To isolate cDNA clones for R^{II} a specific 47-base oligonucleotide probe was synthesized corresponding to amino acids 370–385 of the

bovine heart R^{II} (Fig. 1). This region was chosen because it contained several amino acids of low-codon degeneracy and was in a segment of R^{II} not homologous to R^{I} (10). For degenerate amino acids codon-bias tables were used to predict the third-base position. The oligonucleotide probe was phosphorylated with [γ-^{32}P]ATP and used to screen a rat skeletal muscle library under low-stringency hybridization conditions. Sixteen positive colonies were isolated. These were rescreened with a second oligonucleotide probe (probe 2) from the bovine R^{II} sequence (Fig. 1). Four colonies hybridized to both oligonucleotides.

Restriction analysis of one plasmid, RSK 1, (Fig. 2A) showed that it contained an insert of 1.2 kb. This was subcloned into M13 vectors and sequenced. Plasmid RSK 1 contained an open reading frame for 1113 bases that coded for a type II regulatory subunit (Fig. 3), as determined by homology to known protein sequences (10). The 5' end of plasmid RSK 1 began at amino acid 27 of the published bovine cardiac sequence (10).

A mouse brain cDNA library was probed with nick-translated RSK 1, and under stringent washing conditions four cDNA clones were isolated. One clone hybridized to an oligonucleotide representing bases 1–50 of plasmid RSK 1. This mouse cDNA (MB 1) was 1.5 kb in length (Fig. 2B), and, when sequenced, was found to have an open reading frame for 1143 bases (Fig. 4). As expected, the rat and mouse cDNAs were highly conserved with 91% nucleotide sequence identity. Unfortunately MB 1 protein did not contain the initiator methionine, and by comparison with the bovine sequence (10) was missing ≈20 amino acids of the coding region (Fig. 2B).

The amino-terminal region of the mouse R^{II} gene was finally obtained by isolating a genomic clone using nick-translated MB 1 as a probe. Thirty positive clones were isolated and further screened with the 50-base oligonucleotide representing the 5' region of RSK 1. Six clones hybridized to both probes, and restriction mapping indicated that they all contained the same 15-kb fragment (MG 1) containing the 5' region of the R^{II} gene (Fig. 2C). The strategy to locate the remaining 5' coding region (57 bases) within MG 1 (15 kb) took advantage of the *Sal* I site previously discussed (Fig. 2C). Restriction mapping with *Eco*RI and *Sal* I produced several fragments, but only two fragments hybridized to probe MB 1 and related oligonucleotides. Both were subcloned and partially sequenced. The larger fragment, MG 2 (5.3 kb), contained the ATG initiation codon and bases 1–87 of the R^{II} coding region (Fig. 4). The smaller *Eco*RI/*Sal* I genomic DNA fragment, MG 3 (0.5 kb), contained a sequence in which the first 167 bases were identical to those of MB 1 (Fig. 4). The identity ended at the sequence GGAAGT, consistent with a consensus splice donor sequence (25). These results showed that the first coding exon of the mouse R^{II} gene, which encodes amino acids 1–84, had been isolated.

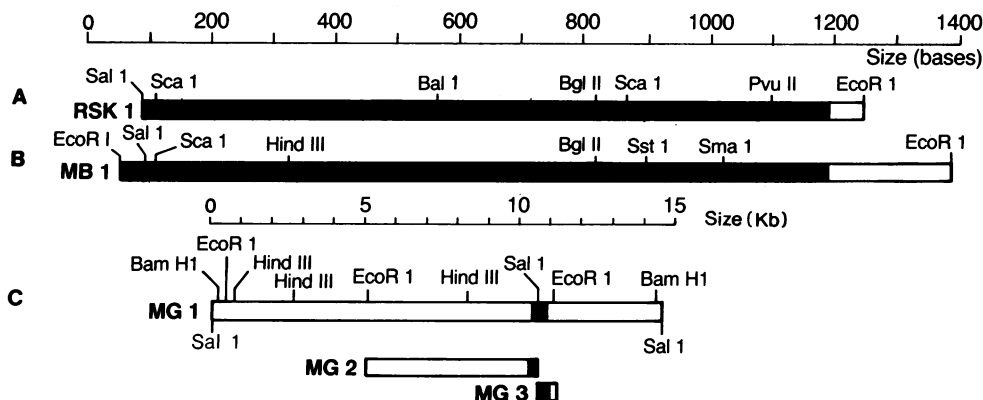FIG. 2. Restriction maps of R^{II} cDNA and genomic clones. Restriction sites are indicated at approximate positions within each DNA as is the protein-coding region. The rat cDNA RSK 1 (A) and mouse cDNA MB 1 (B) are positioned to align homologous regions. The mouse genomic fragment MG 1 (C) shows the restriction sites used to generate the two fragments MG 2 and MG 3 that contained the first coding exon of the mouse R^{II} gene.

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TTC GCG GTG GAG TAC TTC ACA CGC CTG CGC GAG GCC CGC CGC CAG GAA TCA GAC TCG TTC ATC GCC CCC        69
Phe Ala Val Glu Tyr Phe Thr Arg Leu Arg Glu Ala Arg Arg Gln Glu Ser Asp Ser Phe Ile Ala Pro
                                                                      Thr         Val Ser

CCG ACG ACC TTT CAC GCG CAG GAG TCC AGC GGG GTC CCC GTC ATC GAG GAG GAC GGG CAG AGT GAA TCG        138
Pro Thr Thr Phe His Ala Gln Glu Ser Ser Gly Val Pro Val Ile Glu Glu Asp Gly Gln Ser Glu Ser
                  Thr             Ala                                 Glu     Asp

GAC TCG GAC GAT GAG GAT CTG GAA GTT CCG ATT CCA GCA AAA TTT ACT AGA CGA GTA TCA GTC TGT GCA        207
Asp Ser Asp Asp Glu Asp Leu Glu Val Pro Ile Pro Ala Lys Phe Thr Arg Arg Val Ser Val Cys Ala
          Glu     Ala                     Val     Ser

GAA ACG TTT AAC CCT GAT GAA GAA GAA --- GAT AAT GAT CCA AGG GTG GTT CAC CCA AAA ACC GAC GAG        273
Glu Thr Phe Asn Pro Asp Glu Glu Glu --- Asp Asn Asp Pro Arg Val Val His Pro Lys Thr Asp Glu
                              Glu

CAG AGG TGC AGA CTT CAG GAA GCC TGT AAA GAC ATT CTG CTT TTC AAA AAC CTG GAT CAG GAA CAG CTT        342
Gln Arg Cys Arg Leu Gln Glu Ala Cys Lys Asp Ile Leu Leu Phe Lys Asn Leu Asp Gln Glu Gln Leu

TCT CAA GTT CTG GAC GCC ATG TTC AAA AGG ATA GTC AAA ACT GAC GAG CAT GTC ATT GAC CAA GGA GAT        411
Ser Gln Val Leu Asp Ala Met Phe Lys Arg Ile Val Lys Thr Asp Glu His Val Ile Asp Gln Gly Asp
                              Glu Lys

GAT GGA GAC AAC TTT TAT GTC ATA GAA AGG GGA ACC TAT GAC ATT TTA GTA ACA AAG GAT AAT CAA ACA        480
Asp Gly Asp Asn Phe Tyr Val Ile Glu Arg Gly Thr Tyr Asp Ile Leu Val Thr Lys Asp Asn Gln Thr

CGA TCT GTT GGT CAG TAT GCA AAC CGT GGC AGT TTT GGA GAA CTA GCC CTG ATG TAC AAT ACC CCG AGA        549
Arg Ser Val Gly Gln Tyr Ala Asn Arg Gly Ser Phe Gly Glu Leu Ala Leu Met Tyr Asn Thr Pro Arg
                          Asp

GCT GCT ACC ATT GTG GCC ACC TCA GAC GGC TCC CTT TGG GGA TTG GAC CGG GTG ACT TTT AGG AGA ATC        618
Ala Ala Thr Ile Val Ala Thr Ser Asp Gly Ser Leu Trp Gly Leu Asp Arg Val Thr Phe Arg Arg Ile
                  Ile             Glu

ATA GTG AAG AAC AAT GCA AAG AAG AGG AAG ATG TTC GAA TCG TTT ATT GAG TCT GTA CCG CTC TTT AAA        687
Ile Val Lys Asn Asn Ala Lys Lys Arg Lys Met Phe Glu Ser Phe Ile Glu Ser Val Pro Leu Phe Lys

TCA CTA GAG ATG TCA GAA CGA ATG AAG ATT GTG GAT GTG ATC GGG GAA AAG ATC TAT AAG GAT GGA GAG        756
Ser Leu Glu Met Ser Glu Arg Met Lys Ile Val Asp Val Ile Gly Glu Lys Ile Tyr Lys Asp Gly Glu

CGA ATA ATC ACT CAG GGT GAA AAA GCC GAC AGC TTT TAT ATT ATA GAG TCT GGA GAA GTG AGC ATC TTG        825
Arg Ile Ile Thr Gln Gly Glu Lys Ala Asp Ser Phe Tyr Ile Ile Glu Ser Gly Glu Val Ser Ile Leu
        Ala

ATT AGA AGC AAG ACT AAA ACG AAC AAG AAC GGC GGG AAC CAG GAG GTT GAG ATT GCC CAC TGC CAT AAG        894
Ile Arg Ser Lys Thr Lys Thr Asn Lys Asn Gly Gly Asn Gln Glu Val Glu Ile Ala His Cys His Lys
                  Ser

GGG CAG TAC TTT GGA GAA CTT GCC CTG GTA ACC AAC AAG CCA AGA GCT GCT TCT GCT TAT GCG GTT GGA        963
Gly Gln Tyr Phe Gly Glu Leu Ala Leu Val Thr Asn Lys Pro Arg Ala Ala Ser Ala Tyr Ala Val Gly
                                                                                    Gly

GAC GTC AAA TGC TTA GTC ATG GAT GTT CAA GCA TTT GAG AGG CTT CTG GGC CCC TGC ATG GAC ATC ATG        1032
Asp Val Lys Cys Leu Val Met Asp Val Gln Ala Phe Glu Arg Leu Leu Gly Pro Cys Met Asp Ile Met
                                                                      *****************

AAG AGG AAC ATC TCA CAT TAC GAA GAA CAG CTG GTG AAG ATG TTT GGC TCC AAC TTG GAT CTA TTG GAC        1101
Lys Arg Asn Ile Ser His Tyr Glu Glu Gln Leu Val Lys Met Phe Gly Ser Asn Leu Asp Leu Leu Asp
************************************************ ******************            Met
                1                                      2

CCC GGG CAG TAG ATGTGATGAATCTCGGAGCCTTCTCAGTGTGATACCAAATCCTTCCAGTCAGCCACAAGAACACACCCAGAAAC        1188
Pro Gly Gln
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AGACACGACAGAACTGCGCCTGCTGCTGTCTCTGCTGCTGCCATCGCTGTGGTAAAGGGCACTTA   1253

FIG. 3.   The nucleotide sequence of rat skeletal muscle R^II. The nucleotide sequence of RSK 1 (1253 bases) is presented with the predicted amino acid sequence. At sites of amino acid divergence from mouse the corresponding mouse residue is presented below in italics. A single gap (---) was introduced for maximum homology to MB 1. The sites of hybridization of oligonucleotides 1 and 2 to RSK 1 are indicated by (***).

Determination of mRNA Size. RNA blots were probed with R^II cDNA to determine the size and tissue distribution of R^II mRNA in the mouse (Fig. 5). Nitrocellulose filters containing total RNA from various tissues were probed with either nick-translated, 734-base-pair, Sal I/Bgl II RSK 1 fragment or EcoRI insert of MB 1; both probes gave similar results. Two species of R^II RNA were identified migrating at 2.4 kb and 6.0 kb, respectively (Fig. 5A). The 2.4-kb species was detected only in testis, where it was present in large amounts as compared with the 6.0-kb form observed in other tissues (Fig. 5A). In an attempt to determine which mRNA species was present in mouse brain, poly(A)^+ RNA was probed with nick-translated MB 1 (Fig. 5B). As shown in Fig. 5B, only the 6.0-kb RNA species could be detected, suggesting that mouse

brain mRNA contains 4.5 kb of untranslated regions. Furthermore, the 2.4-kb species appears to be a testis-specific form, unless it is expressed in other tissues at levels too low for detection by RNA transfer.

DISCUSSION

In this report the isolation of cDNA clones for a type II regulatory subunit of the cAMP-dependent protein kinase from mouse brain and from rat skeletal muscle is described. We believe that the cDNA isolated from mouse brain is the homologue of the rat R^II cDNA cloned from skeletal muscle. Their nucleotide sequences are ≈91% identical, with most differences occurring at the third-base position in the codons.

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ATG AGC CAC ATC CAG ATC CCG CCG GGG CTC ACG GAG CTG CTG CAG GGC TAC ACC GTG GAG GTT GGC CAG      69
Met Ser His Ile Gln Ile Pro Pro Gly Leu Thr Glu Leu Leu Gln Gly Tyr Thr Val Glu Val Gly Gln
                                                                                    Leu Arg

CAG --- CCG CCC GAC CTC GTC GAC TTC GCG GTG GAG TAC TTC ACA CGC CTG CGC GAG GCC CGC CGC CAG     135
Gln --- Pro Pro Asp Leu Val Asp Phe Ala Val Glu Tyr Phe Thr Arg Leu Arg Glu Ala Arg Arg Gln
       Arg                                    Asp                               Ser Arg

GAA TCA GAC ACG TTC ATC GTC TCC CCG ACG ACC TTT CAC ACG CAG GAG TCC AGC GCA GTC CCC GTC ATC     204
Glu Ser Asp Thr Phe Ile Val Ser Pro Thr Thr Phe His Thr Gln Glu Ser Ser Ala Val Pro Val Ile
Ala         Thr Pro Pro Ala Ala Pro     Ser Gly Ser Gln Asp Phe Asp Pro Gly     Gly Leu     Ala

GAG GAG GAC GGG GAG AGT GAC TCG GAC TCG GAA GAT GCC GAT CTG GAA GTT CCG GTT CCT AGC AAA TTT     273
Glu Glu Asp Gly Glu Ser Asp Ser Asp Ser Glu Asp Ala Asp Leu Glu Val Pro Val Pro Ser Lys Phe
Asp Ala Val Ala Asp     Glu     Glu Asp     Glu ---     Asp         Ile     Gly Arg

ACT AGA CGA GTA TCA GTC TGT GCA GAA ACG TTT AAC CCT GAT GAA GAA GAG GAG GAT AAC GAT CCA AGG     342
Thr Arg Arg Val Ser Val Cys Ala Glu Thr Phe Asn Pro Asp Glu Glu Glu Glu Asp Asn Asp Pro Arg
Asp                                      Tyr                               Thr

GTG GTT CAT CCC AAA ACT GAT GAG CAG AGA TGC CGG CTT CAG GAA GCT TGT AAA GAT ATT CTT CTT TTC     411
Val Val His Pro Lys Thr Asp Glu Gln Arg Cys Arg Leu Gln Glu Ala Cys Lys Asp Ile Leu Leu Phe
    Ile                     Gln

AAA AAC CTT GAT CAG GAA CAG CTT TCT CAA GTT CTG GAT GCC ATG TTT GAA AAG ATT GTC AAA ACT GAC     480
Lys Asn Leu Asp Gln Glu Gln Leu Ser Gln Val Leu Asp Ala Met Phe Glu Lys Ile Val Lys Thr Asp
            Pro                                                   Arg Thr         Val

GAG CAT GTC ATT GAC CAA GGC GAC GAC GGG GAC AAC TTT TAT GTC ATA GAA CGG GGA ACC TAT GAC ATT     549
Glu His Val Ile Asp Gln Gly Asp Asp Gly Asp Asn Phe Tyr Val Ile Glu Arg Gly Thr Tyr Asp Ile

TTA GTA ACG AAA GAT AAT CAA ACG CGT TCT GTT GGT CAG TAT GAC AAC CGT GGC AGT TTT GGA GAA CTA     618
Leu Val Thr Lys Asp Asn Gln Thr Arg Ser Val Gly Gln Tyr Asp Asn Arg Gly Ser Phe Gly Glu Leu
                                                            His

GCT CTG ATG TAC AAT ACC CCG AGA GCT GCT ACC ATC ATC GCC ACC TCA GAA GGC TCC CTT TGG GGA TTG     687
Ala Leu Met Tyr Asn Thr Pro Arg Ala Ala Thr Ile Ile Ala Thr Ser Glu Gly Ser Leu Trp Gly Leu
                                             Val

GAC CGG GTG ACT TTT AGA AGA ATC ATA GTG AAA AAC AAT GCA AAG AAG AGG AAG ATG TTT GAA TCA TTT     756
Asp Arg Val Thr Phe Arg Arg Ile Ile Val Lys Asn Asn Ala Lys Lys Arg Lys Met Phe Glu Ser Phe

ATT GAG TCT GTT CCA CTC TTT AAG TCA CTA GAG ATG TCA GAA CGA ATG AAG ATT GTG GAT GTG ATC GGG     825
Ile Glu Ser Val Pro Leu Phe Lys Ser Leu Glu Met Ser Glu Arg Met Lys Ile Val Asp Val Ile Gly
                              Leu             Val

GAA AAG ATC TAT AAG GAC GGA GAG CGA ATA ATC GCA CAG GGT GAA AAG GCC GAC AGC TTC TAT ATC ATA     894
Glu Lys Ile Tyr Lys Asp Gly Glu Arg Ile Ile Ala Gln Gly Glu Lys Ala Asp Ser Phe Tyr Ile Ile
                Val                             Thr

GAG TCT GGG GAA GTG AGC ATC TTG ATT AGA AGC AAG ACC AAG TCA AAC AAG AAT GGA GGG AAC CAG GAG     963
Glu Ser Gly Glu Val Ser Ile Leu Ile Arg Ser Lys Thr Lys Ser Asn Lys Asn Gly Gly Asn Gln Glu
                                        Lys             Val         Asp     Glu

GTC GAG ATT GCC CAT TGC CAT AAG GGG CAG TAC TTC GGA GAA CTT GCC CTG GTC ACC AAC AAA CCC AGA    1032
Val Glu Ile Ala His Cys His Lys Gly Gln Tyr Phe Gly Glu Leu Ala Leu Val Thr Asn Lys Pro Arg
                Arg

GCT GCT TCC GCT TAC GGC GTT GGA GAT GTC AAA TGC TTA GTT ATG GAT GTT CAA GCA TTT GAG AGG CTT    1101
Ala Ala Ser Ala Tyr Gly Val Gly Asp Val Lys Cys Leu Val Met Asp Val Gln Ala Phe Glu Arg Leu

CTG GGC CCC TGC ATG GAC ATC ATG AAG AGG AAC ATC TCA CAT TAC GAA GAA CAG CTG GTG AAG ATG TTT    1170
Leu Gly Pro Cys Met Asp Ile Met Lys Arg Asn Ile Ser His Tyr Glu Glu Gln Leu Val Lys Met Phe

GGC TCC AAC TTG GAT CTG ATG GAC CCC GGG CAG TAG ATGTGATGAATCTTGGAGCCTTCTCAGTGTGATACCAAAACCT    1249
Gly Ser Asn Leu Asp Leu Met Asp Pro Gly Gln
        Ser Met         Ile

TCCAGTCAGCCACAAGAACACACACAGAAAACAGACACGACAGAACCGCTCCTGCTGCTGTCTCCGCCGCTGCCATTGCTGTGGTTAAGGG    1340

CATTTAGAAATCTTGAAAGTCAGCACTGAAAGATGGATGGAGGTTCCACCCACACCCACCCTCCACTTTGCTTCTGAACGCCGTCATTAGA    1431

CCACTTATGTCATGAGTACTCCAACCCAGGTTTCACATTTGGTTCAGAATGTCTCTCCAGCAATTTAAGTGCCTGATTCAAAGTCCAAAAA    1522

AAAACCTTAAG
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FIG. 4. The nucleotide sequence of mouse brain R^{II}. The nucleotide sequence of the composite mouse brain R^{II} (MB 1 plus coding sequence from MG 2) is presented with the predicted amino acid sequence. At sites of divergence from bovine R^{II} (11) the corresponding bovine residue is presented below in italics; a gap (---) is introduced to maximize homology.

The degree of identity may be slightly altered when nucleotide sequence data become available for the 5' region of the rat cDNA. There are 21-amino acid differences between the rat and mouse R^{II} proteins, of which 13 represent classical conserved changes (Fig. 3). The degree of identity between both proteins is 96%, which is, as expected, higher than for their nucleotide sequences.

The predicted amino acid sequences of the mouse brain and rat muscle R^{II} subunits were very similar to that of bovine cardiac R^{II} (10) with 85% identity overall. Recently Hemmings *et al.* (26) have reported the isolation of a partial cDNA clone of 467 bases from the porcine cell line LLC-PK$_1$, which is more homologous to the bovine heart R^{II} than to the rat or mouse sequences reported here. The differences between all

four R^{II} sequences are highlighted in Fig. 6, which identifies a region of 38-amino acid residues (43–81) in mouse and rat that shows little homology to either the bovine or porcine sequences. This lack of homology from previously published R^{II} sequences (10, 26) was surprising. It is probable that the data represent real sequence differences for several reasons: (*i*) all pieces of DNA have been sequenced on both strands, and no sequence homologous to bovine R^{II} appears, (*ii*) the rat and mouse cDNAs are very similar in this region (Figs. 3 and 4), and (*iii*) the mouse cDNA and genomic DNA sequences are identical. Furthermore, an oligonucleotide representing bases 1–50 of the porcine R^{II} cDNA (corresponding to bases 128–178 in mouse) does not hybridize to either the rat or mouse cDNAs (J.D.S., unpublished obser-
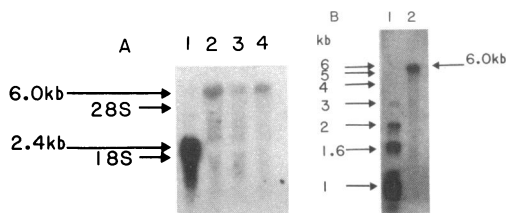
FIG. 5. Hybridization analysis of R[II] mRNA. (*A*) RNA blot analysis of mouse total RNA (20 μg) from testis (lane 1), kidney (lane 2), liver (lane 3), and heart (lane 4) were electrophoresed under denaturing conditions on a 1% agarose gel and blotted to nitrocellulose. The nitrocellulose was hybridized to nick-translated MG 1 insert, and the size of the R[II] mRNA was determined by its mobility in relation to the 18S and 28S ribosomal RNA species or a set of ^{32}P-labeled markers from Bethesda Research Laboratories. (*B*) RNA blot analysis of mouse brain poly(A)$^+$ RNA (17 μg). Lane 1 contains size markers indicated in kb.

vation). Multiple R[II] genes or differential splicing of a single gene could explain these differences. A comparison of the pig and mouse nucleotide sequence reveals a diminished identity of 64% to base 254 in mouse, whereas the remainder shows an 87% identity that approaches the similarity between the rat and mouse cDNAs (91%). It is intriguing to note that the first intron in the coding sequence begins at the 3' boundary of the divergent region as shown in Fig. 6.

Three forms of R[II] have been identified from rat granulosa cells (15), of which one has been cloned (16). This form, R[II]-51 (16), was previously called the neural form or R[II]B, as it was first identified when immunoprecipitated from brain extracts (11, 13, 14). Other investigators have reported at least six forms of R[II], excluding autophosphorylated species, from various mammalian tissues (27). These authors suggested that most of the R[II] heterogeneity could be localized to the amino-terminal 10,000–20,000 portion of the proteins (27).

There are two distinct mRNA species for R[II] with apparent sizes of 2.4 kb and 6.0 kb, but the smaller form was detected only in testis. The two different sizes might result from preferential use of differing poly(A) addition sites in processing, a phenomenon that has been reported for the granulosa R[II] molecule (16).

Testis is the tissue with the highest R[II] mRNA levels as determined by solution hybridization (G.S.M., unpublished observation); all this mRNA seems to be the 2.4 kb species (Fig. 5A). This mRNA species can also be detected in rat and bovine testis (J.D.S., unpublished observation). The testis-specific 2.4-kb mRNA species hybridizes to both mouse and rat cDNAs and to a 50-base-pair oligonucleotide synthesized to bases 128–178 of the mouse cDNA. This eliminated the possibility that the testis mRNA was binding to sequences present in the antisense strand of the R[II] cDNAs and suggests that the 2.4-kb mRNA is a tissue-specific transcript for mouse R[II].

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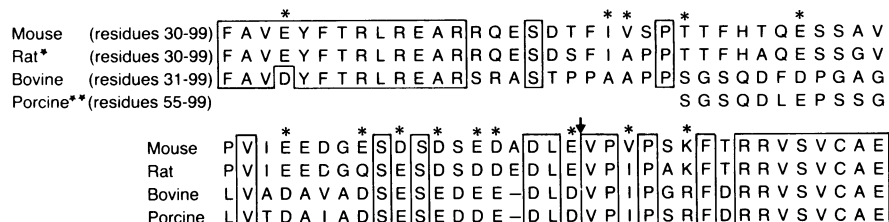

FIG. 6. Sequence differences among mammalian R[II] molecules. The predicted rat and mouse amino acid sequences (residues 30–99) are compared with corresponding regions of the bovine protein (10) and porcine R[II] (26). The sequences were aligned to maximize identity with the mouse; the boxed areas represent common amino acids. Conserved amino acid replacements (★) are also indicated. * and ** denote partial sequences that are aligned according to mouse R[II] numbering. ↓, 3' boundary of the first coding exon in the mouse gene.