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MOLECULAR CLONING, cDNA STRUCTURE AND TISSUE-SPECIFIC EXPRESSION OF THE HUMAN REGULATORY SUBUNIT RIβ OF cAMP-DEPENDENT PROTEIN KINASES

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SUMMARY: Complementary DNA clones for the regulatory subunit RIβ of cAMP-dependent protein kinases were isolated from a human testis cDNA library using a mouse RIβ cDNA probe. One clone 2.4 kilobases (kb) in length contained an open reading frame of 1137 bases, and encoded a protein of 379 amino acids (excluding the initiator methionine). The human RIβ protein was one amino acid shorter than the corresponding protein in mouse and rat. The nucleotide similarity to mouse and rat sequences was 85.6 % and 84.8 %, respectively, while the amino acid similarity was 97.6 % and 97.3 %, respectively. Northern blot analyses revealed a 2.7 kb mRNA in human tissues and a 2.8 kb mRNA in mouse tissues. Both mouse and human RIβ mRNA were found to be expressed in most tissues, and not restricted to brain and testis as reported by others.

Cyclic AMP exerts its effects on the function and metabolism of eucaryotic cells through the activation of cAMP-dependent protein kinase (EC 2.7.1.37). The cAMP-dependent protein kinase in its inactive form is a tetramer of two regulatory (R) and two catalytic (C) subunits (1), which dissociates upon binding of two cAMP molecules to each of the R subunits (2,3). The free activated C subunits phosphorylate specific substrate proteins on serine and threonine residues and thereby alter their function or activity (4). Multiple isoforms of both the R and C subunits have been identified, and they are called RIα (5-10), RIβ (11,12), RIIα (13-17), RIIβ (18-20), Cα (21-26), Cβ (23,24,26-28) and Cγ (26). With the exception of RIβ, the human cDNA sequences for all the regulatory subunits have been described (7,10,17,19). The RIβ subunit has been cloned from mouse brain and rat brain, and its mRNA expression has been reported to be brain and testis specific (11,12,29-31). In human, the RIβ mRNA has been detected in the colonic cancer cell line HT-29 (32). In the present study, we report the molecular cloning, cDNA structure and amino acid sequence of the human RIβ. Furthermore, we demonstrate that the RIβ mRNA is expressed in most human and mouse tissues.

MATERIALS AND METHODS

cDNA cloning: A human testis cDNA library in lambda gt11 was purchased from Clontech, Palo Alto, CA, U.S.A. (Cat # HL 1010). Phage plaques were transferred to nitrocellulose filters, denatured, baked, and prehybridized at 68°C for 4 hours in 6 X SSC and 2 X Denhardt's solution. Hybridization was carried out in the same solution at 68°C with a labeled mouse RIβ cDNA probe, kindly provided by Dr. G. Stan-

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The abbreviations used are: cAMP, cyclic adenosine 3',5'-monophosphate; cDNA, complementary DNA; RI, regulatory subunit of the type I cAMP-dependent protein kinase; RII, regulatory subunit of the type II cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; kb, kilobase; SDS, sodium dodecyl sulphate; SSC, standard saline citrate (0.15 M NaCl, 15 mM sodium citrate, pH 7.0).

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ley McKnight, Dept. of Pharmacology, University of Washington, Seattle, WA 98195, U.S.A. (11). The filters were washed twice in 1 X SSC, 0.5 % SDS for two hours at 68°C, dried, and autoradiographed (Kodak XAR-5).

Nucleotide sequencing: Overlapping clones for use in DNA sequencing were produced by the Cyclone I Biosystem (International Biotechnologies, Inc., New Haven, CT, U.S.A. (Cat # 77200))(33). Nucleotide sequencing was performed by the dideoxy chain termination method (34) using the modified T7 DNA polymerase ("Sequenase" from United States Biochemical Corporation, Cleveland, OH, U.S.A. (Cat # 70700))(35).

Computer analysis: Nucleotide sequence data were analyzed using the GCG computer package from the University of Wisconsin Genetics Computer Group (36).

Preparation of total RNA and Northern blot analysis: Human tissue samples from lung, jejunum, colon, ovary, fallopian-tube, testis, prostate, lung, spleen and kidney were obtained from The Surgical Department, Rikshospitalet, Oslo, Norway. Total RNA from human brain was purchased from Clontech, Palo Alto, CA, U.S.A. (Cat # 64020). Mouse tissue samples from lung, colon, brain, ovary, testis and prostate were obtained from BALB/c mice (females, 12-weeks-old and males, 5-weeks-old). All tissue samples were as soon as possible frozen in liquid nitrogen, and stored at -70°C for subsequent RNA extraction. Extraction of total RNA was performed by the guanidine isothiocyanate method (37). Twenty μg of RNA from each sample was denatured in 50 % (v/v) formamide, 6.0 % (v/v) formaldehyde followed by 15 min on ice, and resolved on a 1.5 % agarose gel containing 6.7 % (v/v) formaldehyde and 20 mM sodium phosphate, pH 7.0. Equal loading and the quality of the RNA were verified by ethidium bromide staining the gels before blotting onto nylon membranes (Biotrans, ICN, Irvine, CA, U.S.A.). The membranes were baked at 80°C for 1 hour, prehybridized in 5 X Denhardt's solution, 5 X SSC, 50 mM sodium phosphate buffer, pH 6.5, 0,1 % (w/v) SDS, 250 μg/ml salmon sperm DNA, and 50 % (v/v) formamide at 42°C, and hybridized using similar conditions in a solution containing the labeled probe. The membranes were washed four times in 2 X SSC, 0.1 % (w/v) SDS at room temperature for 5-10 minutes, followed by two washes using 0.1 X SSC, 0.1 % (w/v) SDS at 50°C. Autoradiography was performed at -70°C using Amersham Hyperfilm MP.

Labeling of probes: Probes for screening were labeled using α-[^{32}P]dCTP (PB.10205, specific activity 3000 Ci/mmol, 10 mCi/ml, Amersham, Buckinghamshire, U.K.) and a nick translation kit (N.5000, Amersham). Probes for Northern analysis were labeled using α-[^{32}P]dCTP and a multiprime DNA labeling kit (RPN.1601, Amersham).

RESULTS AND DISCUSSION

Using a 1.5 kb mouse RIβ cDNA as a probe (11), one million recombinant clones from a human testis cDNA library were screened. Three cDNA clones of 2.1 kb, 1.6 kb and 2.4 kb (clone 15, 20, 36, respectively), were isolated. All clones started at the same nucleotide in the 3'-end (fig. 1). Nucleotide sequencing revealed that all three clones represented partial clones for the human RIβ. The longest clone, 2.4 kb in length (clone 36), was almost a full-length clone starting at the G in ATG start codon, compared with mouse and rat RIβ (11,12)(fig. 2 and 3). The cDNA sequence predicted an open reading frame of 1137 bases, coding for a protein of 379 amino acids (excluding the initiator methionine) and a 3' nontranslated region of 1222 bases. The location of two potential polyadenylation site signals are indicated in fig. 2. The second signal, ATTAAA, at the end of the sequence was also found in the rat RIβ sequence (12) 24 nucleotides 5' of the poly A tail. Our sequence ended 12 nucleotides 3' of the potential polyadenylation site, and was lacking the poly A tail. Two out of the three independently isolated clones (clone 15 and 20) contained a T in position 841, whereas the longest clone (clone 36) had a C in this position. Thus the sequence in figure 2 reads T at nucleotide 841. The mouse RIβ sequence had a T at the corresponding position while the rat sequence contained a C.

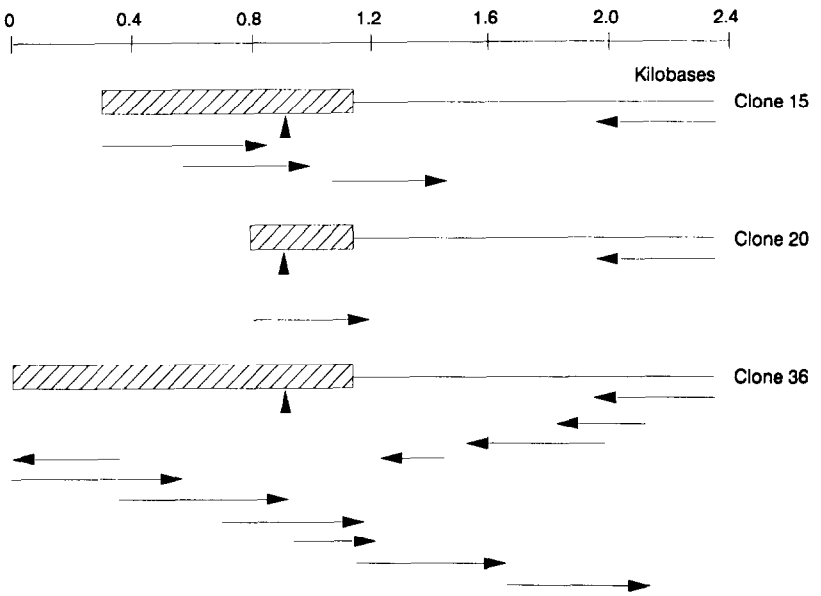

FIGURE 1. Restriction map and sequencing strategy for the human RIβ cDNA. Clones 15, 20 and 36 have been positioned to align identical regions. The hatched bars indicate coding regions and black lines indicate 3' nontranslated regions. The scale, in kilobases, starts at the first nucleotide of the coding region. Arrows below each clone indicate the direction and extent of the DNA sequencing. The PstI restriction site is indicated by an arrow-head.

The regulatory subunits from several mammals have previously been isolated and the interspecies differences are small both at the nucleotide and amino acid level. In the case of human RIβ, the cDNA sequence appeared to encode a protein of 379 amino acids, one amino acid shorter than the mouse RIβ, rat RIβ, human RIα, and mouse RIα proteins (fig. 3). The similarity between human and mouse/rat sequences were 85.6 % / 84.8 %, on the nucleotide level within the coding region and 97.6 % / 97.3 %, on the amino acid level (fig. 3). In contrast, the similarity between mouse and rat RIβ was 95.7 % on the nucleotide level and 99.7 % at the protein level (12). Compared to human and mouse RIα (7, McKnight, unpublished data) the similarities were also high; 70.0 % and 73.6 %, respectively, at the nucleotide level, and 88.6 % and 89.7 %, respectively, at the amino acid level (fig. 3).

The dissimilarities between the RI subunits were mainly located in the amino terminal region of the proteins (fig. 3), containing the dimerization domain. Furthermore, in human RIβ an amino acid deletion was located at amino acid 98 which was within the pseudosubstrate binding site of RIα (fig. 3)(38). Only the RII subunits have been thought to have a site of phosphorylation. Substrates for the C subunits have two basic residues, usually arginines, preceding the serine to be phosphorylated; Arg-Arg-X-Ser(P). In RIα, when Ala^{97} at the hinge region (Arg^{94}-Arg-Gly-Ala) was replaced with Ser (38), an autophosphorylation site was introduced. Due to the amino acid deletion and the Arg^{98} substitution, the human RIβ had the sequence Arg^{97}-Arg-Leu-Ser. Since glycine and leucine both are classified as amino acids with nonpolar side chains, the RIβ protein may be autophosphorylated at serine 100. Futhermore, these amino acid changes could alter the binding affinity of RIβ to C.

Northern blot analysis of total RNA from various human tissues revealed one mRNA band 2.7 kb in length for RIβ (fig. 4). Weak cross-hybridizing to the RIα mRNA bands of 1.5 kb and 3.0 kb (7), could be seen. The intensity of the mRNA bands demonstrated relatively equal levels of expression in the human

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1    GGCCTCCCCGCCCGCCTGCCCCTCGGAGGAGGACGAGAGCCTGAAGGGCTGTGAGCTGTACGTGCAGCTGCACGGGATCCAGCAGGTCCTCAAAGACTGT
       AlaSerProProAlaCysProSerGluGluAspGluSerLeuLysGlyCysGluLeuTyrValGlnLeuHisGlyIleGlnGlnValLeuLysAspCys

101  ATCGTCCACCTCTGCATCTCCAAGCCCGAACGCCCCATGAAGTTCCTCCGGGAGCACTTCGAGAAGCTGGAGAAGGAAGAAAACAGGCAGATTTTGGCGCGG
       IleValHisLeuCysIleSerLysProGluArgProMetLysPheLeuArgGluHisPheGluLysLeuGluLysGluGluAsnArgGlnIleLeuAlaArg

203  CAAAAGTCAAACTCACAGTCGGACTCCCATGATGAGGAGGTGTCGCCCACCCCCCCGAACCCTGTGGTGAAGGCCCGCCGCCGACGGAGGCTGAGTGCCGAG
       GlnLysSerAsnSerGlnSerAspSerHisAspGluGluValSerProThrProProAsnProValValLysAlaArgArgArgArgArgLeuSerAlaGlu

305  GTGTACACCGAGGAGGACGCCGTGTCCTACGTCAGGAAGGTGATTCCCAAGGACTACAAAACCATGACTGCGCTGGCCAAGGCCATCTCCAAGAACGTGCTC
       ValTyrThrGluGluAspAlaValSerTyrValArgLysValIleProLysAspTyrLysThrMetThrAlaLeuAlaLysAlaIleSerLysAsnValLeu

407  TTCGCTCACCTGGATGACAACGAGAGGAGTGACATATTCGATGCCATGTTCCCTGTCACTCACATCGCTGGGGAGACTGTTATACAGCAAGGGAATGAAGGA
       PheAlaHisLeuAspAspAsnGluArgSerAspIlePheAspAlaMetPheProValThrHisIleAlaGlyGluThrValIleGlnGlnGlyAsnGluGly

509  GACAACTTCTATGTCGTTGATCAAGGGGAAGTGGATGTGTACGTGAACGGAGAGTGGGTGACCAACATCAGCGAGGGAGGCAGCTTCGGGGAGCTGGCGCTC
       AspAsnPheTyrValValAspGlnGlyGluValAspValTyrValAsnGlyGluTrpValThrAsnIleSerGluGlyGlySerPheGlyGluLeuAlaLeu

611  ATCTACGGCACCCCCAGGGCTGCGACCGTGAAAGCCAAGACGGACCTCAAGCTCTGGGGGATCGACCGGGACAGCTACCGGCGCATCCTTATGGGCAGCACG
       IleTyrGlyThrProArgAlaAlaThrValLysAlaLysThrAspLeuLysLeuTrpGlyIleAspArgAspSerTyrArgArgIleLeuMetGlySerThr

713  CTGAGGAAACGCAAGATGTACGAGGAGTTCCTCAGCAAGGTCTCCATCCTAGAGTCCCTGGAGAAGTGGGAGCGTCTGACCGTGGCGGATCGGCTGGAGCCC
       LeuArgLysArgLysMetTyrGluGluPheLeuSerLysValSerIleLeuGluSerLeuGluLysTrpGluArgLeuThrValAlaAspArgLeuGluPro
                                        *
815  GTCCAGTTTGAAGATGGAGAGAAAATTGTGGTCCAGGGAGAGCCTGGGGACGACTTTTACATCATCACGGAGGGCACCGCGTCCGTGCTGCAGCGCCGGTCC
       ValGlnPheGluAspGlyGluLysIleValValGlnGlyGluProGlyAspAspPheTyrIleIleThrGluGlyThrAlaSerValLeuGlnArgArgSer

917  CCCAATGAGGAGTACGTGGAGGTGGGGCGCCTGGGACCCTCTGACTACTTCGGGGAGATTGCACTGCTGCTGAACCGGCCCCGGGCGGCCACTGTCGTGGCC
       ProAsnGluGluTyrValGluValGlyArgLeuGlyProSerAspTyrPheGlyGluIleAlaLeuLeuLeuAsnArgProArgAlaAlaThrValValAla

1019 CGGGGGCCCCTCAAGTGTGTGAAGCTGGACCGGCCCCGCTTCGAGCGTGTGCTGGGGCCCTGCTCTGAGATCCTCAAGAGGAACATTCAGCGTTACAACAGC
       ArgGlyProLeuLysCysValLysLeuAspArgProArgPheGluArgValLeuGlyProCysSerGluIleLeuLysArgAsnIleGlnArgTyrAsnSer

1121 TTCATCTCCCTCACCGTCTGAGCACACGTCCCGCCCTGCAGCCCCAGCTCCCCAGTGTGGTGGCCGTGCCTGCTCGTCTGTGTCGGGGGCCCGGGAGCCGCT
       PheIleSerLeuThrValEnd

1223 GTGTGAGGTGTGGGCCGGGTGGGGCTGGGTCCCGGCAGCGTGAGGACTGCCCCTTCCCCGGACTCACTTTTTGGAATAAATGATCACCTTGTGCATTTCCAA
1325 ATCAAAGGACAAGCGGACAAAATGCATCCCAAGATCAAGGAAGGGACAGGCCAGCTTCCTCCCCACACGCCTCCCCGGCTGCCTCTGTGGGCTTCTCCTGGG
1427 GGGCCCACCCCACCCCTGCCAGTCTCCTGGAGATGCTTGAGGATCGGTCCTCCCCAGAACCAGGCCAGGACGTTGCCCCTGGCGGCTGGTGACCCTGTGAGG
1529 TCAGGTCCCCCAGATTGAGGTCTGAGTGTGGGCAAGTGTGTCAAAAGGGGCTGCCCCCCAGGAGATGAGGCTGAGAGCAGGGAGTTGAGGCCGAAGAAGTCA
1631 AGGCCCCTCCCGCAAATGTGTACCCCTGCCCGCGCCACTGCACCCCGCCGCACCCCCACCTCCCCGGGGGCCCTGCTGCGGATCGCGGAGTGGGAGAGTCTC
1733 TGAGCTATGAGATTGATCTTGCCCCTAATTGGAGAGGAAGCCGGGCGCCAAGACACACGGGGCTCCTGCCTTGGGAGCCAGGGCCGCGGCCGCAGGTAGACC
1835 CCAGTAGGGGGGGCCGGGCTCGAAGTTCCTTTGGGAGGGGCTGGCGGGACTCCAGCAGGCCGTCCTCACCTTTCTTAGAAAGTCCACCCAGGGCAAGTTGAT
1937 GTTGGGGGAAAGCAGAAGTCAAGCCAGCCGCGGCCCCACACGCCCCGACCCGCTCAACCGACTTGTCCCTTAAATGTGTCTTGGATCCCGCAGTGATGACGT
2039 GAGCCAGCCAGGCCCGAAAGGGTGAGGCCAGTGCAGAGAAGCTTCCCAGGGGATTCCTGGTTCCCCCGAAAGACAAGCGAGGTCATTGCAGTTCACCCGATG
2141 TTGCTCCTGTCCCGTGCGTCCCGGGGAGGCTGTCCTTGGTCCGCATGGCTCGTTGCAGCCCCTCCCCTGCTGGCGGTTACGATGCGTGGGGTCCCCCTCCCC
2243 ACCCAGCCCCGGCACCGTCGCCGTGTCCCGCCTGTGCCTGTGACGTCCCTGTGGACCTGTGAGCCATCCCCCCCTTATCTCTGCTCTGAATACTGCATGAAG
2345 CATTAAACGTGCAATGAAG
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FIGURE 2. Nucleotide sequence of the cDNA encoding the human RIβ, and the corresponding amino acid sequence. Nucleotides are numbered starting with 1 at the first nucleotide in the coding region. The amino acids are shown in three letter code. Two potential polyadenylation site signals (AATAAA, ATTAAA) are underlined. The star (•) indicates the nucleotide represented by a T in two clones (clone 15 and 20) and by a C in one clone (clone 36).

tissues investigated, although, a stronger signal was found in brain (fig. 4). In mouse, RIβ has earlier been detected only in brain and testis (11,12,29,30). To compare the observed expression of RIβ mRNA in human tissues to mouse tissues, a filter with total RNA from different mouse tissues was prepared and hybridized with a labeled mouse RIβ probe. A 2.8 kb mRNA for RIβ was detected in all mouse tissues

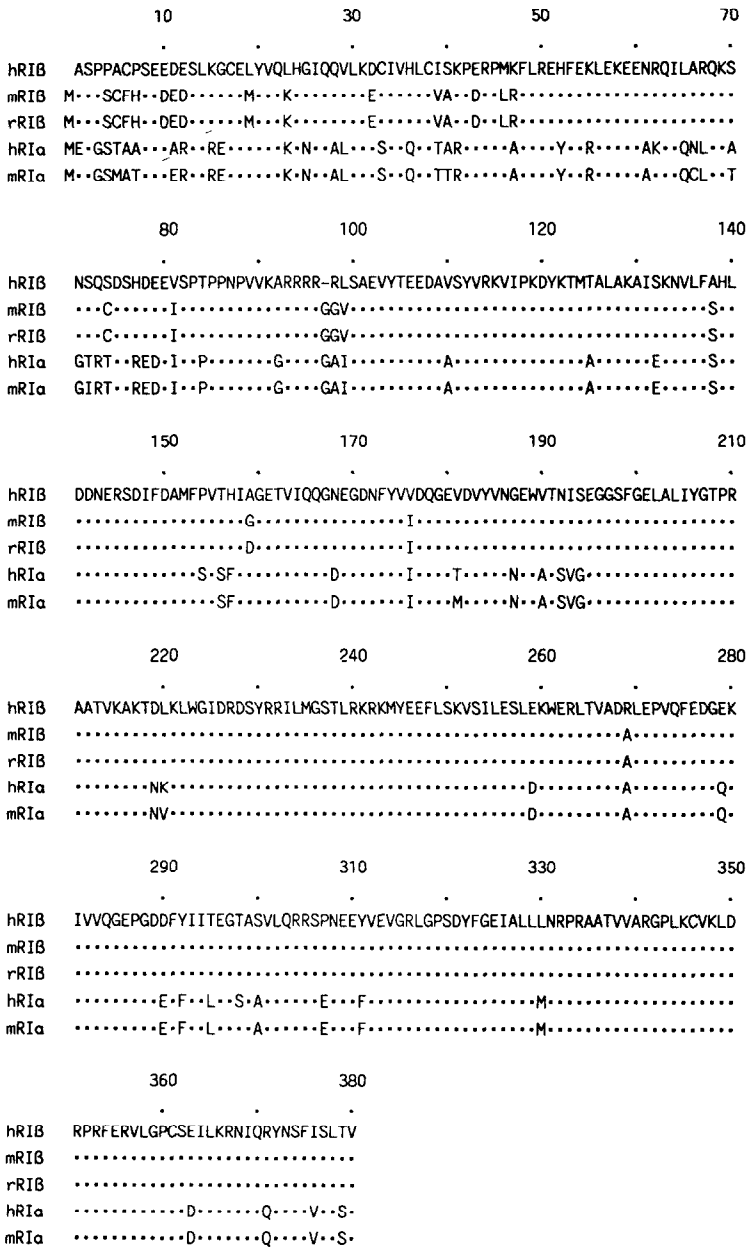

FIGURE 3. Comparison of the deduced amino acid sequence of human RIβ (hRIβ) with the amino acid sequences of mouse RIβ (mRIβ), rat RIβ (rRIβ), human RIα (hRIα) and mouse RIα (mRIα). Dots represent identity with the human RIβ amino acid sequence. The dash (-) represents an amino acid deletion in human RIβ.

investigated except liver. After a short exposure, RIβ was seen only in brain (fig. 5, upper panel), but after longer exposures, signals were observed also in mouse colon, ovary, testis and prostate (fig. 5, lower panel). This shows that RIβ mRNA expression is not restricted to brain and testicular tissues as earlier reported (11,12,29,30).

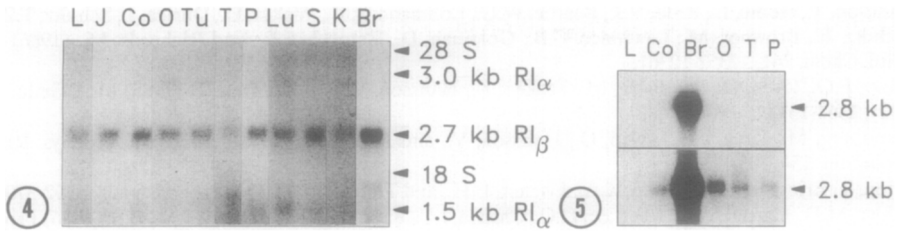

FIGURE 4. Expression of the RIβ mRNA in various human tissues. Total RNA was isolated from different human tissues, electroforesed, and blotted onto a nylon membrane. The filter was hybridized with a [^{32}P]-labeled human RIβ cDNA probe corresponding to 906 bases of the coding region (EcoRI/PstI fragment of clone 36, fig. 1). Human tissues are indicated as follows: L, liver; J, jejunum; Co, sigmoid colon; O, ovary; Tu, fallopian-tube; T, testis; P, prostate; Lu, lung; S, spleen; K, kidney; Br, brain. The size of the RIβ mRNA have been estimated to be 2.7 kb.

FIGURE 5. Expression of the RIβ mRNA in various mouse tissues. Total RNA was isolated from different mouse tissues, electroforesed, and blotted onto a nylon membrane. The filter was hybridized with a [^{32}P]-labeled mouse RIβ 1.5 kb cDNA probe. Mouse tissues are indicated as follows: L, liver; Co, colon; Br, brain; O, ovary; T, testis; P, prostate. The size of the RIβ mRNA has been estimated to be 2.8 kb. Upper panel: 6 days exposure using Kodak regular screens; lower panel: 7 days exposure using Kodak super rapid screens.

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