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Biochemistry

RTA, a candidate G protein-coupled receptor: Cloning, sequencing, and tissue distribution

(cerebellum/smooth muscle/*mas*)

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ABSTRACT **Genomic and cDNA clones, encoding a protein that is a member of the guanine nucleotide-binding regulatory protein (G protein)-coupled receptor superfamily, were isolated by screening rat genomic and thoracic aorta cDNA libraries with an oligonucleotide encoding a highly conserved region of the M_1 muscarinic acetylcholine receptor. Sequence analyses of these clones showed that they encode a 343-amino acid protein (named RTA). The RTA gene is single copy, as demonstrated by restriction mapping and Southern blotting of genomic clones and rat genomic DNA. Sequence analysis of the genomic clone further showed that the RTA gene has an intron interrupting the region encoding the amino terminus of the protein. RTA RNA sequences are relatively abundant throughout the gut, vas deferens, uterus, and aorta but are only barely detectable (on Northern blots) in liver, kidney, lung, and salivary gland. In the rat brain, RTA sequences are markedly abundant in the cerebellum. RTA is most closely related to the *mas* oncogene (34% identity), which has been suggested to be a forebrain angiotensin receptor. We cannot detect angiotensin binding to the RTA protein after introducing the cognate cDNA or mRNA into COS cells or *Xenopus* oocytes, respectively, nor can we detect an electrophysiologic response in the oocyte after application of angiotensin peptides. We conclude that RTA is not an angiotensin receptor; to date, we have been unable to identify its ligand.**

An interesting recent development in receptor biology has been the realization that many of the receptors interacting with the guanine nucleotide-binding regulatory protein (G protein) signaling system are homologues. The mammalian members of this receptor superfamily described to date by molecular cloning include β-adrenergic (1–3), α-adrenergic (4–6), muscarinic acetylcholine (7–10), serotonin (11–14), dopamine (15), substances K (16) and P (17), luteinizing hormone (18, 19), and rhodopsin (20) receptors. The quintessential feature of these proteins is seven stretches of 20–26 hydrophobic amino acids; these regions, which are the most conserved regions among different receptors, are thought to form α-helices that span the cytoplasmic membrane. Other features common to these proteins include one or more potential N-linked glycosylation sites near the amino terminus, the lack of a discernible amino-terminal signal peptide (except the luteinizing hormone receptor) (18, 19), and several invariant amino acid residues. Another feature of this family is that the coding regions of the genes often lack introns; exceptions to this trend include bovine rhodopsin (20) and D_2 subtype of the dopamine receptor (15). A currently popular model proposes that these proteins have their amino terminus outside the cell and the seven hydrophobic segments spanning the cytoplasmic membrane with the connecting segments alternately extending into the cytoplasm and the extracellular space and the carboxyl terminus inside the cell. The evidence for this structure is largely an extrapolation from results of detailed physical measurements of the related protein bacterial rhodopsin (21); however, limited proteolysis studies with β-adrenergic receptors have resulted in proteolytic fragment patterns consistent with this model (22, 23).

One approach used to identify members of this superfamily is to screen clone banks with oligonucleotides encoding conserved regions of the seven-transmembrane-segment structural motif. We now report the isolation of cDNA and genomic clones encoding a seven-transmembrane helix protein that is expressed in smooth muscle-rich tissues and in cerebellum.* This protein, named RTA (*r*at *t*horacic *a*orta), is most closely related to the *mas* oncogene (24), a putative forebrain angiotensin receptor (25).

METHODS

Construction of a Rat Aorta cDNA Library. The region of the aorta extending from the azygos vein to the diaphragm was removed from 200 freshly killed adult male Sprague–Dawley rats, trimmed of adherent fat and snap frozen in liquid nitrogen. RNA was extracted from 0.5-g samples of tissue by the method of Chirgwin *et al.* (26), enriched for mRNA (27), and used as a template for cDNA synthesis (28, 29). *Sma* I/*Eco*RI adapters (New England Biolabs) were then ligated onto the cDNA and this cDNA was in turn ligated to *Eco*RI-cut λgt10 DNA. The resulting concatamers were packaged *in vitro* and the parental phage (i.e., those without an insert) were eliminated by plating the library on the plaque-suppressive strain *Escherichia coli* C600Δ*hflA150* (30). The resulting library contained ≈1 million independent recombinants.

Oligonucleotide Probes. Two oligonucleotides (5′-ACGGTCAACAACTACTTCCTGCTGAGCCTGGC; 5′-CCGATGATCAGGTCAGCGCAGGCCAGGCTC) were synthesized on a Biosearch 8600 synthesizer and purified. These oligonucleotides anneal to form a 9-base-pair (bp) overlap; double-stranded probe was prepared by the action of the Klenow fragment of DNA polymerase I on the annealed oligonucleotide template in the presence of radiolabeled (^{32}P) deoxynucleoside triphosphates. The resulting 53-mer probe, probe I, which had a specific activity of $\approx 4 \times 10^6$ dpm/pmol, is identical to nucleotides 615–667 of the M_1 subtype of the pig muscarinic acetylcholine receptor cDNA (7).

Library Screening. After amplification of the aorta cDNA library, $\approx 10^6$ clones were screened by standard methods (31). Final wash conditions were 42°C at 50 mM Na^+ for 1 hr. A

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Abbreviation: G protein, guanine nucleotide-binding regulatory protein.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M32098).

rat genomic library, constructed by insertion of partially (*Eco*RI) digested Sprague–Dawley DNA into the vector λ Charon 4A (32) (a gift of T. Sargent, National Institutes of Health), was screened in the same manner. The cDNA or genomic clone inserts were subcloned into a pGEM series (Promega Biotec) plasmid vector for further analysis.

Other methodologic details were as described (33, 34) or are included in the figure legends.

RESULTS AND DISCUSSION

One highly conserved region of the G-protein-coupled receptors described to date is the second putative transmembrane region. To identify previously unknown genes that are members of this superfamily, we used a 53-residue oligonucleotide (probe I) encoding 18 amino acids of transmembrane segment II of the M_1 muscarinic receptor (7) to screen our rat thoracic aorta cDNA library and an existing rat genomic library (32) under conditions of low stringency. The resulting 60 genomic and 10 cDNA clones were grouped by cross-hybridization and restriction mapping and representative clones from each group were subjected to limited DNA sequence analysis. As expected, this screening yielded numerous muscarinic receptor clones as well as clones that do not encode members of this superfamily (data not shown). One group, represented by four cDNA clones and a genomic clone, was found to encode a stretch of amino acids similar to transmembrane segment II of the muscarinic receptors and was thus selected for further study. The amino acid sequence of this protein, as conceptualized from the nucleotide sequence of two cDNAs (pRTA-8 and pRTA-9) and one genomic clone (λ1045) is the subject of Fig. 1.

These clones contain a single extended translation reading frame encoding a protein of 343 amino acids (M_r, 38,352). The first methionine residue is in the context of the Kozak initiation codon consensus sequence (36) and is preceded by an in-frame termination codon. There are no other potential methionine codons in the 230 bp 5′ to the start of the open reading frame. The longest cDNA clone, pRTA-9, is 2.1 kbp and is nearly full length since the predominant RTA mRNA species is ≈2.4 kb (see below).

Expression of either the pRTA-8 or pRTA-9 cDNAs by *in vitro* transcription followed by cell-free translation in the presence of [^{35}S]methionine resulted in a single protein that comigrated with a 36-kDa marker protein on SDS/acrylamide gels. Cotranslational processing occurred during *in vitro* translation in the presence of canine pancreatic microsomal membranes (Promega Biotec), resulting in a shift of the labeled protein band to an apparent size (SDS/acrylamide gel mobility) of 38 kDa (data not shown). The presence of an N-linked oligosaccharide consensus sequence (Asn-Xaa-Thr) near the amino terminus of RTA (amino acids 4–6; Fig. 1) suggests that this decreased mobility may be the result of a core glycosylation event.

A search of reported protein sequences (GenBank/European Molecular Biology Laboratory, release 59; Na-

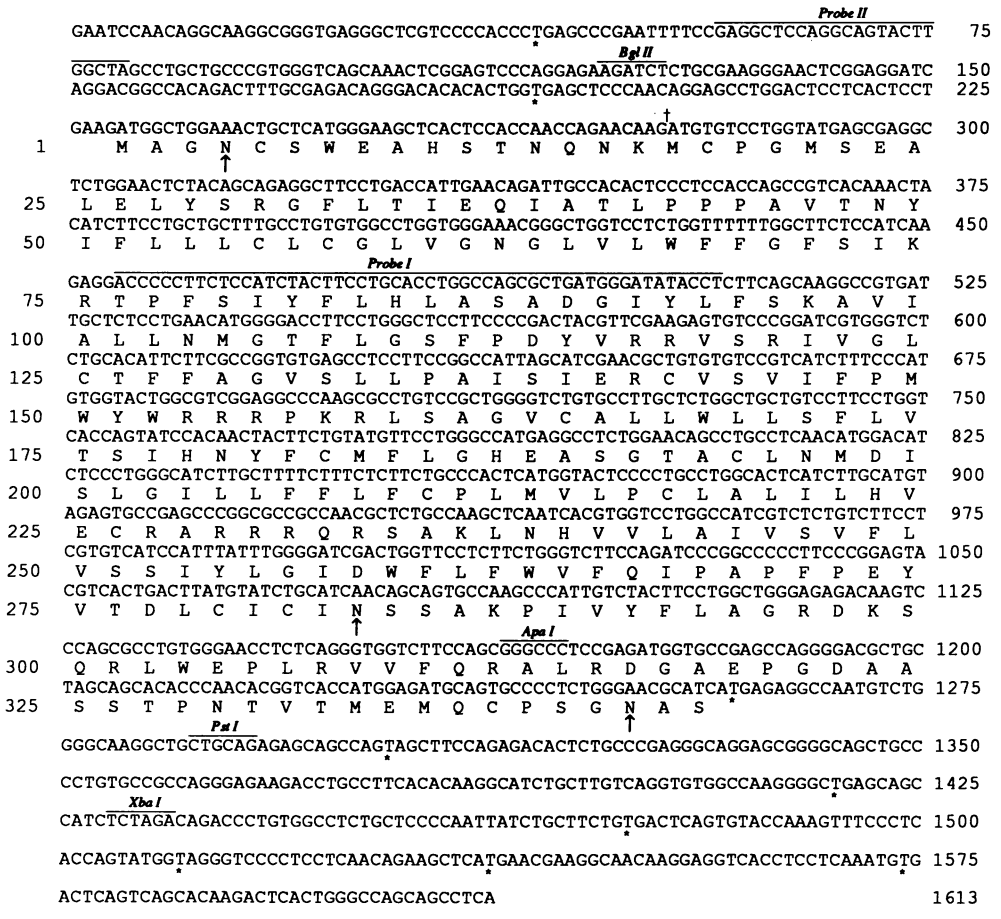

FIG. 1. The amino acid sequence (single-letter code) of the RTA protein as conceptualized from the pRTA cDNAs and the genomic clone λ1045. Both the pRTA-8 cDNA (1.8 kbp) and the pRTA-9 cDNA (2.1 kbp) were sequenced along both strands (nucleotides 220–1613 and 1–300, respectively). A 1.4-kbp *Xba* I fragment of the genomic clone λ1045 generated the identical sequence for nucleotides 278–1435 (see also Fig. 4). Sequencing was carried out by the dideoxynucleotide chain-termination method of Sanger (35) after constructing a series of nested deletions. Predicted in-frame termination codons (★), the point of interruption by the intron (†), and potential N-linked glycosylation sites (↑) are indicated. Horizontal bar indicates regions of the cDNA to which the screening probe (probe I, 43% mismatch) and the "upstream exon" probe (probe II, no mismatch) were directed.

tional Biomedical Research Foundation/Protein Identification Resource, release 22) using the algorithm of Lipman and Pearson (37) revealed that the RTA protein is most closely related to the *mas*-encoded protein (34% identity in a 295-amino acid overlap) and to the muscarinic acetylcholine receptors (e.g., 29% identity to the M_1 subtype in a 198-amino acid overlap), as shown in Fig. 2. The similarity between RTA and *mas* is far more significant because the RTA/*mas* match extends over the full length of these proteins while the RTA/M_1 match covers only transmembrane segments I–V. The RTA protein also contains the Leu^{85}, Asp^{89}, Arg^{141}, Trp^{168}, and Cys^{194} residues that are conserved among known mammalian members of this receptor superfamily (Fig. 2).

The RTA protein contains seven highly hydrophobic regions of ≈24 amino acids each, as shown by hydropathy analysis (data not shown) (38); the predicted seven transmembrane helices are shown in Fig. 2. The RTA protein also lacks an apparent hydrophobic amino-terminal signal peptide, as judged from hydropathy analysis (38). These distinguishing features are shared by many of the known seven transmembrane proteins, most of which have been demonstrated to act as G-protein-coupled receptors; we therefore assign the RTA protein to this superfamily and predict it is a cell-surface receptor that interacts with G proteins.

High-stringency Southern blot analysis and restriction mapping (Fig. 3) of rat genomic DNA revealed that the RTA gene is single copy. We isolated a genomic clone (λ1045) that contained a 9-kbp *Eco*RI fragment that hybridized to both the 53-residue oligonucleotide, probe I, and to the pRTA-8 cDNA. The DNA sequence of a 1.4-kbp *Xba* I fragment of clone λ1045 was determined; nucleotides 204–1361 of this *Xba* I fragment were identical to nucleotides 278–1435 of pRTA cDNA (Fig. 1). The initial 201 nucleotides of the genomic *Xba* I fragment are divergent from the cDNA sequence, contain multiple in-frame termination codons, and are terminated by a C+T-rich region and an AG at nucleotides 202 and 203. This is consistent with the conclusion that this sequence is the splice acceptor site of an intron in the RTA gene (see Fig. 4). The clone λ1045 DNA does not hybridize to an oligonucleotide directed to the 5′-noncoding region of cDNA pRTA-9 (probe II; Fig. 1), and the λ1045 DNA contains 2.5 kbp of genomic DNA sequence 5′ to the RTA exon (data not shown). We therefore conclude that the RTA gene contains an intron at least 2.5 kbp long, located in the region encoding the amino terminus of the RTA protein.

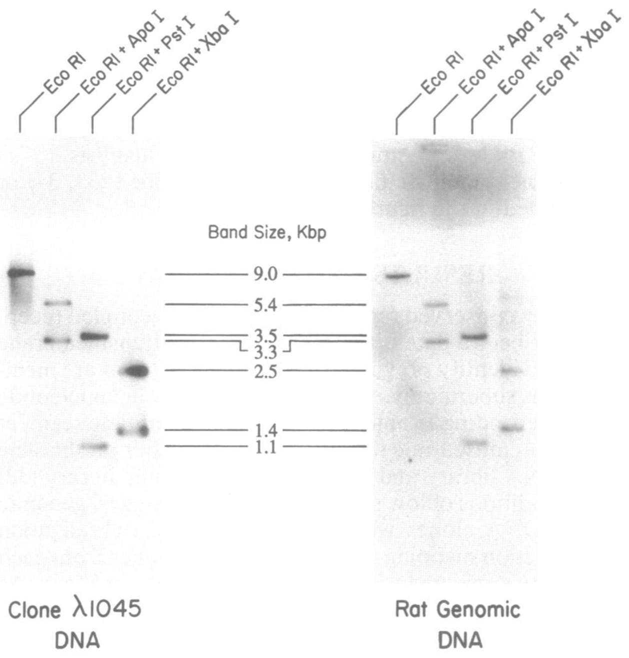

FIG. 3. Genomic clone λ1045 and rat (Wistar Furth) genomic DNA were digested completely with *Eco*RI and then with a second enzyme as indicated. The fragments (1 μg of cloned DNA; 15 μg of genomic DNA) were then subjected to agarose (1%) gel electrophoresis, transferred to a nylon membrane, hybridized to radiolabeled (^{32}P) pRTA-8 cDNA (specific activity, 2×10^8 dpm/μg), and washed at high stringency (final wash conditions, 65°C at 40 mM Na^+). The autoradiogram shown was generated by exposure of the washed membrane to XAR x-ray film (Kodak) for 15 min (cloned DNA) or 3 days (genomic DNA) in the presence of an image intensifying screen. The individual band sizes are indicated and were estimated by comparison to the BRL 1-kbp ladder.

We confirmed the integrity of the λ1045 genomic clone by restriction mapping. When λ1045 DNA or rat genomic DNA was double digested with *Eco*RI and either *Apa* I, *Pst* I, or *Xba* I (each of the latter endonucleases cleaves the RTA cDNA once; see Fig. 1) and hybridized with pRTA-8 cDNA,

```
                                                           I
mas                MDQSNMTSFAEEKAMNTSSRNACLGTSHPPIPIVHWVIMSISPLGFVENGILLWFLCFRMRR   62
                                             :: ........ ..  :.:.::.:::. :...:
RTA      MAGNCSWEAHSTNQNKMCPGMSEALELYSRGFLTIEQIATLPPPAVTNYIFLLLCLCGLVGNGLVLWFFGFSIKR   75
                                                          ::.: ...:: :::  :  ...
m1                       MNTSVPPAVSPNITVLAPGKGPWQVAFIGITTGLLSLATVTGNLLVLISFKVNTEL   56

           *   *    II                                                  III
mas     NPFTVYI-THLSIADISL-LFCIFI-LSIDYALDYE-LSSGH-------YYTIVTLSVTFLFGYNTGLYLLTAISV  127
        .::..:.  ::. ::    ::.  . ...  ...    :.: .       :.  . ... : .. .:. ::.:::.
RTA     TPFSIYF-LHLASADGIY-LFSKAV-IAL-LNMGTF-LGSFP-------DYVRRVSRIVGLCTFFAGVSLLPAISI  139
        .. . :: : ::.:: :    ::...  ..   : :: . ::...      ::: . .....:        ::  ::.
m1      KTVNNYFLLSLACADLIIGTFSMNLYTTY-LLMGHWALGTLACDLWLALDYVASNASVMNL--------LL--ISF  121

         *                           *  IV                         *
mas     ERCLSVLYPIWYRCHRPKHQSAFVCALLWALSCLVTTMEYCMCIDSGEESHS--QSDCR-----AVIIFIAILSFL  196
        :::.::..:.::. .:::. :: :::::: :: :::... ..:.  :.:. :    ..:      .. : ..:: :.
RTA     ERCVSVIFPMWYWRRRPKRLSAGVCALLWLLSFLVTSIHNYFCMFLGHEA-S--GTACL-----NMDISLGILLFF  207
        .:. ::. :. :. .:. : .: . .: ::.::.. .    :  .:  :   .  ...:      .  :..:. .
m1      DRYFSVTRPLSYRAKRTPRRAALMIGLAWLVSFVLWAPAILFWQYLVGER-TVLAGQCYIQFLSQPIITFGTAMAA  196

          V                                         VI
mas      VFTPLMLVSSTILVVKIR-KNTWASHSSKLYIVIMVTIIIFLIFAMPMRVLYLLYYEYWSTFGNLHNISLLFSTI  270
         .: :::....  :....   .. .  .:.::  :..... .::. .. . . ..:.. .  .    . .. :   :
RTA      LFCPLMVLPCLALILHVECRARRRQRSAKLNHVVLAIVSVFLVSSIYLGIDWFLFWVFQIPAPFPEYVTDLCICI  282
         .. :. :.  :   .. :  .: :.
m1       FYLPVTVMCTLYWRIYRETENRARELAAL---                                            225

          VII
mas      NSSANPFIYFFVGSSKKKRFRESLKVVLTRAFKDEMQPRRQEGNG-NTVSIETVV                     324
         ::::.:..::..:..:..:..:.:.::. ::..:. .:  ..... :::..:
RTA      NSSAKPIVYFLAGRDKSQRLWEPLRVVFQRALRDGAEPGDAASSTPNTVTMEMQCPSGNAS               343
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FIG. 2. Comparison of conceptualized RTA, *mas* oncogene (24), and partial M_1 muscarinic receptor (7) amino acid sequences (single-letter code) by the algorithm of Lipman and Pearson (37). Selected conserved amino acid residues (⋆) and the seven transmembrane helices predicted by hydropathy analysis (38) are indicated.

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Xba I
TCTAGAATCTGAGAACCGTGTTAGCATCAGCTGGGGTCAGGGCTCTTTCTGCTCTGGCTTCTGCTGGGTTTGTCA   75
TTCCGTAGACAGGGACCCAGCAGACTCCACCTCCTGGGTAGCTAACAGAAACAGGCCCAGGAACAGGTCTAGCTG  150
AGGGCCAGAGAAGGGCAAGTAGGCTTACACATATGTCCCCTTTGACCTACGAGatgtgtcctggtatgagcgagg  225
                                              M  C  P  G  M  S  E              23
```

FIG. 4. A partial sequence of the RTA gene intron, nucleotides 1–203 of the genomic *Xba* I fragment from clone λ1045. Nucleotides 204–1361 (exon 2) of this *Xba* I fragment were identical to nucleotides 278–1435 of the pRTA cDNA clones (Fig. 1). The remainder of the intron (at least 2.5 kbp long) was not sequenced. The upstream exon(s), as predicted from pRTA-8 and pRTA-9 cDNAs, has not yet been subcloned from a genomic library. Sequencing was performed as described in Fig. 1.

identical patterns were obtained (see Fig. 3). Furthermore, the *Bgl* II site in the 5′-noncoding region of the cDNAs pRTA-8 and pRTA-9 was absent from both the genomic clone λ1045 and the *Eco*RI fragment of rat genomic DNA that was hybridized with pRTA-8 cDNA (data not shown), suggesting that the intron revealed by sequencing of clone λ1045 must also be present in rat genomic DNA.

To determine both the size of the RTA mRNA and the sites of RTA gene expression, we extracted RNA from a variety of rat tissues and detected the RTA RNA species by high-stringency hybridization to radiolabeled (^{32}P) pRTA-8 cDNA (Fig. 5). These data show the predominant species of RTA mRNA is ≈2.4 kb long, while a less abundant 1.4-kb species is also present. Two larger RNAs, migrating at ≈3 and ≈6 kb, are readily detected only in cerebellar RNA extracts. Any of these RNA species are large enough to encode the RTA protein (nominally 1029 nucleotides). We find the RTA mRNA accumulating in tissues that are predominantly smooth muscle, including the vas deferens, uterus, large and small intestines, stomach, and aorta (the source of the cDNA clones). RNA from other tissues, such as liver, kidney, etc., yielded only faint hybridization signals; these may arise from vascular, rather than parenchymal, cells. The only brain region that showed high levels of this mRNA was the cerebellum.

The *mas*-encoded protein was originally conceptualized from the nucleotide sequence of a human genomic clone that was isolated in an oncogene assay protocol (24); it was predicted to be a member of the seven-transmembrane-segment superfamily based on structural similarities with the visual opsins. RNA transcripts that hybridize to the rat analog of the *mas* gene are detected only in the hippocampus and cerebral cortex (39). The expression of the *mas* gene in either cultured NG115-401L cells or *Xenopus* oocytes reportedly confers on these cells the ability to respond with a Ca^{2+} transient when angiotensin peptides are applied to the bathing medium (25). Although this angiotensin receptor-like property of the *mas*-encoded protein is surprising when viewed in the context of the well developed maps of angiotensin binding in brain (40, 41), we tested the closely related RTA to determine whether it is an alternative form of the angiotensin receptor. We injected RTA mRNA (transcribed *in vitro* on either the pRTA-8 or -9 cDNA templates) into *Xenopus* oocytes and assayed changes in membrane potential in response to applied angiotensin peptides. Multiple trials revealed no evidence of angiotensin receptor activity (i.e., angiotensin-induced depolarization to the chloride equilibrium potential; −23 mV), although oocytes injected with liver or brainstem poly(A)$^+$ RNA readily depolarize in response to these peptides (data not shown, but see refs. 42 and 43). To test RTA for angiotensin binding activity, we subcloned a fragment (nucleotides 1–1430) of pRTA-8 cDNA into the expression vector CDM-8 (Invitrogen and ref. 44) and expressed it in COS-7 cells (45); no specific binding of either iodinated or tritiated angiotensin II to crude COS cell membranes was observed (data not shown). In contrast, when the rat M_1 muscarinic receptor cDNA (a gift from T. Bonner, National Institute of Mental Health) was expressed in oocytes and COS cells, these expression systems performed as expected (i.e., carbachol-induced depolarizations and specific [^{3}H]-*l*-quinuclidinyl benzilate binding, respectively; data not shown). We conclude from these studies that the RTA protein is not an angiotensin receptor.

We have attempted to elicit electrophysiologic responses to numerous ligands other than angiotensin II in *Xenopus* oocytes injected with RTA mRNA (data not shown). Our failure to detect a stimulatory ligand in this screening protocol may be attributed to a number of possible factors; for example, the *Xenopus* oocyte may not provide appropriate effector mechanisms to detect a functional response to RTA receptor occupation.

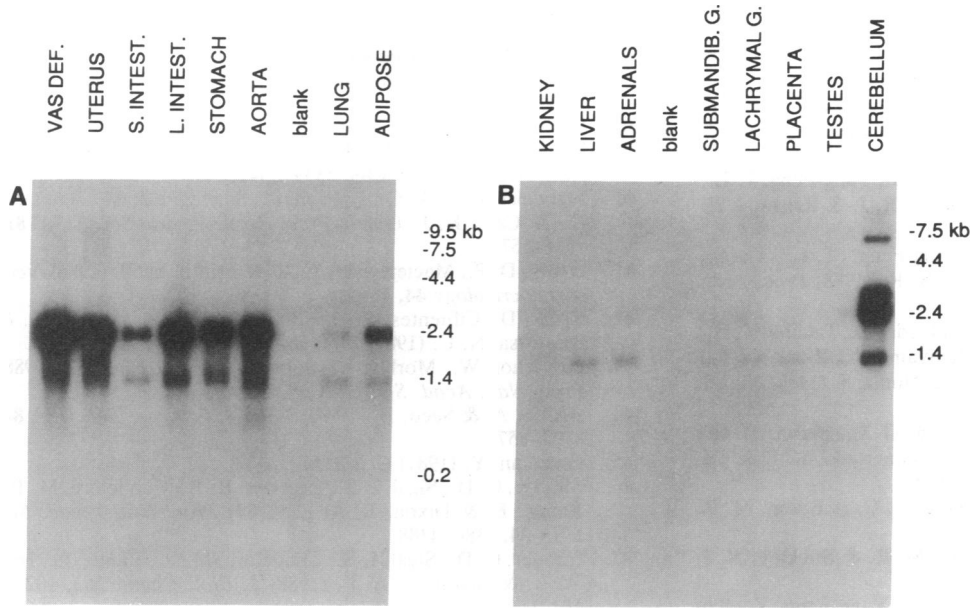

FIG. 5. RNA was extracted from the indicated rat tissues and enriched for poly(A)$^+$ sequences (see *Methods*). The RNA (2 μg each) was subjected to denaturing agarose (1.2%) gel electrophoresis, transferred to a nylon membrane, hybridized to radiolabeled (^{32}P) pRTA-8 cDNA (specific activity, 3 × 10^8 dpm/μg), and washed at high stringency (see Fig. 3). The autoradiogram shown was generated by exposure to XAR x-ray film (Kodak) for 5 days in the presence of an image intensifying screen. Size markers are the BRL poly(A)$^+$ ladder.

The RTA protein does not contain an aspartate residue at a position equivalent to residue 113 in the β_2-adrenergic receptor. This residue, which lies in putative transmembrane segment III, occurs in all biogenic amine receptors described to date and has been implicated in ligand recognition by this subset of the seven-transmembrane-helix superfamily (46, 47). Conversely, the *mas*-encoded protein, the visual opsins, the substance K and P receptors, and the luteinizing hormone receptor do not contain this aspartate residue. On this basis we postulate that the RTA ligand is not a biogenic amine (muscarinic, adrenergic, serotoninergic, histaminergic, or dopaminergic) receptor. By sequence alignment, RTA and the *mas* oncogene are distant from the substance K and P and biogenic amine receptor subgroups. We feel that RTA represents only the second known member of a subgroup of receptors that includes the *mas* oncogene.

Thus, the sequence data for RTA will provide an additional distant data point in computational research based on published receptor protein sequences and may provide a basis for selection of oligonucleotide probes for the detection of additional receptor clones. Furthermore, the tissue distribution data for RTA may allow workers in other laboratories to identify the ligand for RTA based on functional data or model experimental systems that they have developed.

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