BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **240,** 183–188 (1997)
ARTICLE NO. RC977631

Molecular Cloning of a Novel Human Receptor Gene with Homology to the Rat Adrenomedullin Receptor and High Expression in Heart and Immune System

Jörg Hänze, Katalin Dittrich, Jörg Dötsch, and Wolfgang Rascher
Department of Pediatrics, University of Giessen, Feulgenstrasse 12, D-35385 Giessen, Germany

Received September 25, 1997

A novel human gene (named hrhAMR) encoding a seven transmembrane receptor which shares a homology of 73% on the amino acid level with the rat adrenomedullin (AM) receptor has been isolated by a polymerase-chain-reaction (PCR) cloning strategy. Southern- and Northern-blot analysis suggest a single hrhAMR gene, which is highly expressed in heart, skeletal muscle, immune system, adrenal gland and liver, an expression pattern being quite different to that found in rat tissues with the homologue rat probe. Because of its homology with the rat AM receptor, we speculate that potential ligands may be members of the calcitonin-gene-related-peptide/ amylin / adrenomedullin peptide family.

Several genes encoding G-protein associated receptors have been isolated by PCR cloning procedures based on sequence homologies especially pronounced in the transmembrane regions (1). Following this strategy, the aim of this study was to isolate a potential human adrenomedullin receptor gene using primers which are homologue to a rat adrenomedullin receptor sequence (2).

Adrenomedullin (AM), is a 52 aminoacid peptide originally isolated from a human phaeochromocytoma by a bioassay which measured cAMP increase in platelets (3). It shares some sequence homology with calcitonin gene related peptide (CGRP) (4) and amylin (5) which also have a six amino acid ring structure and amidated c-termini. Beside the adrenal gland endothelial cells (6), vascular smooth muscle cells (7) and some neuronal cells (8) have been demonstrated to synthesize AM. Among the actions exerted by AM vasodilation in lung (9), kidney (10) and and bronchien (11) have been observed. Furthermore it interacts with various systems influencing body fluid homeostasis. Centrally, it inhibits drinking (12), peripherally it inhibits aldosterone secretion (13) and ANP gene expression in cardiomyocytes (14), and in the kidney natriuretic properties have been described (15). Several studies observed AM actions which seem to be mediated via CGRP receptors (16, 13), a phenomen which was studied in more detail on the cellular level in the human SK-N-MC cell line (17). However, in rat the first gene encoding a specific adrenomedullin receptor was characterized by expression studies, and showed specific adrenomedullin binding with an Kd of 8.2 nM and a specific cAMP response, whereas CGRP was without any effect (2). In bovine aortic endothelial cells specific adrenomedullin binding has been shown to be coupled to cytosolic Ca^{++} and cAMP increase (18). Different EC50 values of the respective dosis effect relation suggest that both intracellular signallings are triggered by at least two receptors. In human so far no adrenomedullin receptor encoding gene has been characterized which is an important preposition for the elucidation of adrenomedullin receptor function by molecular biological methods for elucidation of intracellular second messenger pathways and the developing of therapeutic applicable pharmacological agents such as antagonists or agonists.

EXPERIMENTAL PROCEDURES

DNA extraction. Genomic DNA was prepared from human placenta which was mechanically pulverized in liquid nitrogen by a proteinase K/ NaCl protein precipitation procedure (19).

RNA extraction. Human lung and heart tissues were frozen in liquid nitrogen (Department of Pathology, University of Giessen). The RNA was extracted by homogenization of the respective tissue in a guanidine-thiocyanate-phenol solution using RNAzol according to the protocoll of the manufacturer (WAK Chemie, Medical GmbH, Bad Homburg, F.R.G.).

Polymerase chain reaction (PCR). Initially, PCR was performed from human genomic DNA using primers (MWG Biotech, Ebersberg, F.R.G.) corresponding to the second and fifth transmembrane domain of the rat AM-receptor sequence (2): am-r2 (GGC ATC ATC CTG TCT CTG CCT GTT TGG AT) and am-r5 (GAG AGG AAA AGG CAG

0006-291X/97 $25.00

TAG GAA GCC CAG GAT). The programming of the thermocycler (GeneAmp PCR System 2400, ABI, Perkin Elmer, Weiterstadt, F.R.G.) was 1min at 94 °C followed by 40 cycles of 10 sec at 94 °C, 1min at 55°C and 1.5 min. at 72 °C. After purification of the PCR product (Geneclean II, Bio101 Inc., La Jolla, USA) and ligation into the EcoRV cut T-tailed pBLUESCRIPT vector (Stratagene, USA) by a PCR product cloning procedure (20) the product was sequenced by the terminator dye chemistry using an automated capillary sequencer. (ABI Prism 310, Perkin Elmer, Weiterstadt, F.R.G.). For isolation of the 5′ end and 3′ end a marathon cDNA end amlification system (Clontech, Palo Alto, USA) was used according to the protocol of the company. In brief human lung cDNA was ligated with adaptors and used as template for PCR with primers binding to the adaptor sequence or to the initially established partial human homologue AM-receptor sequence. The 5′ end was amplified by nested PCR reactions using AP1 and AP2 primers (Clontech, Palo Alto, USA) and the specific primers 3ex (AAA GGT GCC ATG AAG AGG CAC ATG GGC TC) and 3in (TCC ACC AGC TGG ATG TGG ACC ACC TCA GGC). In the first PCR the primer combination AP1 and 3ex was used and for the nested PCR the combination AP2 and 3in. The 3′ end was amplified analogues using the AP1 and AP2 primers in combination with the primer 5ex (TGG AGG TCA CGC TGG ACT ACA CCT GGC TC and 5in (GGG CAG CTT CTC CTG CCG CTT CAC TCA CTA), respectively. The programming of the thermocycler was (1 min at 94 °C, followed by 5 cycles of 5 sec at 94°C, and 4 min at 72 °C, followed by 5 cycles of 5 sec at 94°C, and 4 min at 70°C, followed by 30 cycles of 5 sec at 94°C, and 4 min at 68 °C). The resulting 5′ end and 3′ end cDNAs were ligated into pBluescript plasmid as described above. For construction of an undivided full length DNA probe used for Northern-blot and Southern-blot experiments primers derived from the established 5′ end and 3′ end named MetHindIII: (TTA AGC TTC CCA AGG GAA ACT CAG GCG TGT GCT) overlapping the start of translation and containing an internal HindIII site and the primer tgaXhoI: (GAC TCC ATG ATC TTA AGT CGC CGG CGG AGC TCT T) overlapping the stop codon of translation and containing a XhoI site were used for PCR with genomic DNA. After digestion with HindIII and XhoI the fullength DNA fragment was cloned into a HindIII and XhoI digested T7 and SP6 promoter containing pCDNA3 plasmid (Invitrogen, San Diego, USA), which was used for labeling of a digoxigenin cRNA probe for Northern-blot and digoxigenin DNA probe for Southern-blot analysis.

Nucleic acid and amino acid sequence software. The homology studies of nucleic acid and amino acid sequences were performed using PCGENE software (Release 6.85, IntelliGenetics, Oxford, U.K.) and the HUSAR program (German Cancer Research Center, Heidelberg, F.R.G.). The hydrophobicity profile of hrhAMR was determined according to (21) using PCGENE software.

Northern-blot. Some Northern-blot experiments were performed with membranes commercially available (Clontech, Palo Alto, USA). In the case of heart left and right ventricle, left atria, and papillary muscle, RNA was denatured at 50°C for 60 min in glyoxal dimethylsulfoxide and its concentration spectrophotometrically determined. RNA was loaded in glycerol/sodium phosphate buffer to a 1.2% agarose gel prepared in 10 mM sodiumphosphate pH 7. After electrophoresis RNA was transfered to nylon membranes (Nytran N13, Schleicher&Schuell, Dassel, F.R.G.). A digoxigenin labeled cRNA probe was used for hybridization (Boehringer, Mannheim, F.R.G.). Labeling was performed according to the manufacturers protocol by in vitro transcription of the hrhAMR-pcDNA3 plasmid linearized withXhoI using SP6- RNA polymerase. The membranes were prehybridzided for 2 hours at 68°C in hybridization solution consisting of 50% formamide, 0.75 M sodium citrate, 0.075 M sodium chloride, 2% blocking agent (Boehringer Mannheim, F.R.G.), 0.1% N-lauroylsarcosine and 0.02% sodiumdodecylsulfate. Hybridization was carried out overnight at 68°C by using 50 ng digoxigenin-labeled RNA probe per ml hybridization solution (see above). Membranes were washed in a buffer containing 0.3 M sodium citrate, 0.03 M sodium chloride and 0.1% sodium dodecylsulfate for 10 minutes at room temperature and a buffer containing 0.03 M sodium citrate, 0.003 M sodium chloride and 0.1% sodium dodecylsulfate for 30 min at 68°C.

To detect the digoxigenin-labeled probes, the membrane was incubated for 30 minutes at room temperature with a 2% solution of blocking agent (Boehringer Mannheim, F.R.G.) in 100 mmol/l Tris-HCl (pH7.4) and 150 mM NaCl and then for 30 min at room temperature in the same solution containing 1:10 000 dilution of a polyclonal anti-digoxigenin sheep antibody fab fragment conjugated to alkaline phosphatase (Boehringer Mannheim, F.R.G.). The membrane was washed twice with 100 mM Tris-HCl (pH7.4) and 150 mM NaCl and then incubated for 10 min in 1 × phosphate-buffered-saline containing 0.1% Tween 20 and 0.2% I-block (Tropix, Masachusetts, USA) reagent. After the membrane was washed with 100 mM Tris-HCl (pH7.4) and 150 mM NaCl, alkaline phosphatase activity was determined by addition of 50 μl CSPD (Tropix, Masachusetts, USA) in 5.0 ml of 0.1 mM diethanolamin and 1mM $MgCl_2$ and exposure of the membrane to x-ray film.

Southern-blot. 20 μg DNA was digested by ApaI, EcoRI and Hind III respectively and run on a 0.7% agarose gel in tris acetate EDTA buffer which was transferred to a neutral nylonmembrane (Schleicher Schüll, Nytran N13, Dassel, F.R.G.) by vacuum blotting in NaCl /NaOH with 0.1 bar for 1.5h. A digoxigenin-dUTP labeled cDNA probe was synthesized by PCR using the hrhAMR-pCDNA3 plasmid as template and the primers corresponding to the 5′ end and 3′ end of the AMR sequence, respectively. The probe was purified by agarose gel electrophoresis (Geneclean II, Bio101 Inc., La Jolla, USA). Hybridization was performed in a buffer containing 50% formamide at a temperature of 55 °C. Autoradiography by chemiluminescent reaction was performed as described in Northern-blot.

RESULTS AND DISCUSSION

Using primers derived from the second and fifth transmembrane region of a rat adrenomedullin receptor a partial 399 bases DNA fragment was synthesized by PCR from human genomic DNA. Sequencing revealed a homology of 87 % with the corresponding region of the rat AM receptor sequence. Using perfectly matched primers derived from the established partial human sequence the 5′ cDNA end and 3′ cDNA end were isolated by amplification of cDNA derived from human lung RNA which was ligated with adaptors serving as external anchors for corresponding primers used in combination with the internal sequence specific primers. The two resulting overlapping cDNA fragments were cloned into plasmid and analyzed by sequencing. The 5′ end containing the ATG start codon and the 3′ end containing a TGA stop codon had a common overlapping region of 224 bases and encompassed an open reading frame of 404 amino acids. On nucleic acic level the sequence named hrhAMR showed a homology of 83 % and on amino acid level a homology of 73 % with the rat AM receptor sequences (Fig. 1, 2). More distantly related receptor sequences include a human orphan receptor (RDC1, Swissprot Acc. No. P25106) with a homology of 34 % in a stretch of 148 amino acids and the human type 1a angiotensin receptor (Swissprot Acc.No. P30556) with a homology of 33 % in a stretch of 138 amino acids, respectively. The

```
CAGCCTCCTCACAGCTCCCCATAGCCTGGACCTGCCGGCCCTCCCTCCAGGACCGAGGGG      60
CTCCCAAGGGAAACTCAGGCGTGTGCTGGTCCCAATGTCAGTGAAACCCAGCTGGGGGCC     120
                                    M  S  V  K  P  S  W  G  P
TGGCCCCTCGGAGGGGGTCACCGCAGTGCCTACCAGTGACCTTGGAGAGATCCACAACTG     180
  G  P  S  E  G  V  T  A  V  P  T  S  D  L  G  E  I  H  N  W
GACCGAGCTGCTTGACCTCTTCAACCACACTTTGTCTGAGTGCCACGTGGAGCTCAGCCA     240
  T  E  L  L  D  L  F  N  H  T  L  S  E  C  H  V  E  L  S  Q
GAGCACCAAGCGCGTGGTCCTCTTTGCCCTCTACCTGGCCATGTTTGTGGTTGGGCTGGT     300
  S  T  K  R  V  V  L  F  A  L  Y  L  A  M  F  V  V  G  L  V
GGAGAACCTCCTGGTGATATGCGTCAACTGGCGCGGCTCAGGCCGGGCAGGGCTGATGAA     360
  E  N  L  L  V  I  C  V  N  W  R  G  S  G  R  A  G  L  M  N
CCTCTACATCCTCAACATGGCCATCGCGGACCTGGGCATTGTCCTGTCTCTGCCCGTGTG     420
  L  Y  I  L  N  M  A  I  A  D  L  G  I  V  L  S  L  P  V  W
GATGCTGGAGGTCACGCTGGACTACACCTGGCTCTGGGGCAGCTTCTCCTGCCGCTTCAC     480
  M  L  E  V  T  L  D  Y  T  W  L  W  G  S  F  S  C  R  F  T
TCACTACTTCTACTTTGTCAACATGTATAGCAGCATCTTCTTCCTGGTGTGCCTCAGTGT     540
  H  Y  F  Y  F  V  N  M  Y  S  S  I  F  F  L  V  C  L  S  V
CGACCGCTATGTCACCCTCACCAGCGCCTCCCCCTCCTGGCAGCGTTACCAGCACCGAGT     600
  D  R  Y  V  T  L  T  S  A  S  P  S  W  Q  R  Y  Q  H  R  V
GCGGCGGGCCATGTGTGCAGGCATCTGGGTCCTCTCGGCCATCATCCCGCTGCCTGAGGT     660
  R  R  A  M  C  A  G  I  W  V  L  S  A  I  I  P  L  P  E  V
GGTCCACATCCAGCTGGTGGAGGGCCCTGAGCCCATGTGCCTCTTCATGGCACCTTTTGA     720
  V  H  I  Q  L  V  E  G  P  E  P  M  C  L  F  M  A  P  F  E
AACGTACAGCACCTGGGCCCTGGCGGTGGCCCTGTCCACCACCATCCTGGGCTTCCTGCT     780
  T  Y  S  T  W  A  L  A  V  A  L  S  T  T  I  L  G  F  L  L
GCCCTTCCCTCTCATCACAGTCTTCAATGTGCTGACAGCCTGCCGGCTGCGGCAGCCAGG     840
  P  F  P  L  I  T  V  F  N  V  L  T  A  C  R  L  R  Q  P  G
ACAACCCAAGAGCCGGCGCCACTGCTTGCTGCTGTGCGCCTACGTGGCCGTCTTTGTCAT     900
  Q  P  K  S  R  R  H  C  L  L  L  C  A  Y  V  A  V  F  V  M
GTGCTGGCTGCCCTATCATGTGACCCTGCTGCTGCTCACACTGCATGGGACCCACATCTC     960
  C  W  L  P  Y  H  V  T  L  L  L  L  T  L  H  G  T  H  I  S
CCTCCACTGCCACCTGGTCCACCTGCTCTACTTCTTCTATGATGTCATTGACTGCTTCTC    1020
  L  H  C  H  L  V  H  L  L  Y  F  F  Y  D  V  I  D  C  F  S
CATGCTGCACTGTGTCATCAACCCCATCCTTTACAACTTTCTCAGCCCACACTTCCGGGG    1080
  M  L  H  C  V  I  N  P  I  L  Y  N  F  L  S  P  H  F  R  G
CCGGCTCCTGAATGCTGTAGTCCATTACCTTCCTAAGGACCAGACCAAGGCGGGCACATG    1140
  R  L  L  N  A  V  V  H  Y  L  P  K  D  Q  T  K  A  G  T  C
CGCCTCCTCTTCCTCCTGTTCCACCCAGCATTCCATCATCATCACCAAGGGTGATAGCCA    1200
  A  S  S  S  S  C  S  T  Q  H  S  I  I  I  T  K  G  D  S  Q
GCCTGCTGCAGCAGCCCCCCACCCTGAGCCAAGCCTGAGCTTTCAGGCACACCATTTGCT    1260
  P  A  A  A  A  P  H  P  E  P  S  L  S  F  Q  A  H  H  L  L
TCCAAATACTTCCCCCATCTCTCCCACTCAGCCTCTTACACCCAGCTGAGGTACTAGAAT    1320
  P  N  T  S  P  I  S  P  T  Q  P  L  T  P  S  -
TCAGCGGCCGCTGAATTCTAG
```

FIG. 1. The nucleic acid sequence of hrhAMR and its derived amino acid sequence using the one letter code is shown below (EMBL Database Acc. No.Y13583). Possible glycosylation sites are shown by bold letters, the seven transmembrane regions are underlined.

hydrophobicity profile of hrhAMR, being quite similar to that of the rat AM receptor fits into the seven transmembrane motif. Among further common structural features of G-protein coupled receptors are two asparagin residues at position 28 and 37 of the amino terminal extracellular region which serve as potential glycosylation sites according to the Asn-Xaa-Ser/Thr rule. A typical cystein residue in the extracellular loop between the 2. and 3. transmembrane region was found and several typical serin and threonin residues at the intracellular C-terminal end serving as substrates for phosphorylations are present.

Additionally to the overlapping separated 3′ and 5′ cDNA ends an undivided PCR product derived from genomic DNA using primers overlapping the start codon ATG and stop codon TGA of translation, respec-

```
hrhAMR    - MSVKPSWGPGPSEGVTAVPTSDLGEIHNWTELLDLFNHTLSECHVELSQS -50
ratAMR    - MSVIPSSRPVS----TLAPDNDFREIHNWTELLHLFNQTFSDCRMELNEN -46

hrhAMR    - TKRVVLFALYLAMFVVGLVENLLVICVNWRGSGRAGLMNLYILNMAIADL -100
ratAMR    - TKQVVLFVFYLAIFVVGLVENVLVICVNCRRSGRVGMLNLYILNMAVADL -96

hrhAMR    - GIVLSLPVWMLEVTLDYTWLWGSFSCRFTHYFYFVNMYSSIFFLVCLSVD -150
ratAMR    - GIILSLPVWMLEVMLEYTWLWGSFSCRFIHYFYLANMYSSIFFLTCLSID -146

hrhAMR    - RYVTLTSASPSWQRYQHRVRRAMCAGIWVLSAIIPLPEVVHIQLVEGPEP -200
ratAMR    - RYVTLTNTSPSWQRHQHRIRRAVCAGVWVLSAIIPLPEVVHIQLLDGSEP -196

hrhAMR    - MCLFMAPFETYSTWALAVALSTTILGFLLPFPLITVFNVLTACRLRQPGQ -250
ratAMR    - MCLFLAPFETYSAWALAVALSATILGFLLPFPLIAVFNILSACRLRRQGQ -246

hrhAMR    - PKSRRHCLLLCAYVAVFVMCWLPYHVTLLLLTLHGTHISLHCHLVHLLYF -300
ratAMR    - TESRRHCLLMWAYIVVFAICWLPYHVTMLLLTLHTTHIFLHCNLVNFLYF -296

hrhAMR    - FYDVIDCFSMLHCVINPILYNFLSPHFRGRLLNAVVHYLPKDQTKAGTCA -350
ratAMR    - FYEITDCFSMLHCVANPILYNFLSPSFRGRLLSLVVRYLPKEQARAAGGR -346

hrhAMR    - SSSSCSTQHSIIITKGDSQPAAAAPHPEPSLSFQAHHLLPNTSPISPTQP -400
ratAMR    - ASSSSSTQHSIIITKEGSLLAAADLHTHAIRNVQASSLPPNTSP-TLCNS -395

hrhAMR    - LTPS -404
ratAMR    - IA-S -398
```

FIG. 2. The homolgy between the amino acid sequences of hrhAMR and the rat AM receptor (EMBL Database Acc. No.209249) is shown. Amino acid identity is shown by a line.

tively according to the established cDNA sequence was synthesized. After cloning into a plasmid sequencing revealed the identical sequence as predicted from the separated 5′ and 3′ ends with the exception of one c insted of t at position 694 which did not result in a change of the genetic codon and may be a variant or polymorphism. Furthermore the intronless of the gene at least in the open reading frame was proofed. This full length cDNA probe was used for genomic characterization by Southern-blot analysis (Fig. 3). In the case of EcoRI and Hind III digestion two bands of about 5 kb and in the case of ApaI restriction two smaller bands of a length of about 3 kb and 2 kb were autoradiographically visualized. The finding of two bands in the case of ApaI restriction is conform with two in close distance located internal ApaI sites at position 686 and 739. The Southern-blot analysis confirms the existence of a single hrhAMR gene. Performing Northern-blot analysis with a full length hrhAMR cRNA probe under stringent conditions, a major common band of a length of about 1.8 kb was observed in RNA extracts of human tissues (Fig. 4). High expression was found in heart and skeletal muscle, liver, pancreas, and stomach, spleen, lymph node and bone marrow, adrenal gland and tyroid. Lower expression was found in brain, lung, placenta, small intestine, thymus and leukocytes as well as in fetal brain, lung, liver and kidney. The high expression found in heart was analyzed in more detail showing a similar expression strength in left, and right ventricle and atria. This together with the high expression found in skeletal muscle suggests that hrhAMR is expressed in myocytes. Interestingly, binding experiments have shown AM binding in a rat muscle cell line (22) a finding which let the authors speculate that AM may be involved in the regulation of glucose metabolism in myocytes. The high expression of hrhAMR in parts of the immune sytem suggests its potential immunological function. AM has been shown to be upregulated in smooth muscle cells by lipopolysaccharide, interleukin 1 and tumor-necrosis-factor-alpha (7). Furthermore, circulating AM was shown to be increased in sepsis (23).

Additionally to the major 1.8 kb, which probably corresponds to the polyadenylated hrhAMR mRNA, several higher molecular weight bands in the range between 4 and 7 kb were observed, especially in the case

of adrenal, spleen, lymph node and liver. Pancreas and liver showed an additional pronounced band of less than 1 kb. The origin of these bands is not clear. The expression pattern in human differs considerably compared to that of rat tissues when the homologue rat adrenomedulin receptor probe was used (2). In rat the highest expression was found in lung while a much lower expression was found in heart. Comparable signal strengths were found in adrenal gland of rat and human.

The important question of functional characterization of the receptor sequence by expression studies and identification of the corresponding receptor ligand as well as the elucidation of the second messenger pathways has not been adressed in this study and will be subject of further studies. Because of the 73 % homology on the amino acid level with rat AM receptor, we speculate that potential ligands will be members of the CGRP/ Amylin/ Adrenomedullin peptide family.

Taken together we have isolated a new human seven transmembrane receptor by PCR cloning on RNA and partially on DNA level which is homologous to a rat adrenomedullin receptor and is highly expressed in the heart, skeletal muscle, the immune system and liver.

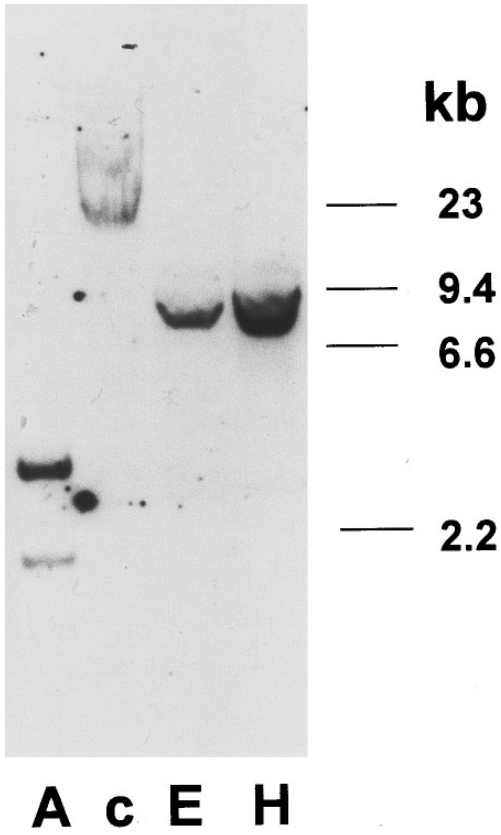

FIG. 3. Southern-blot analysis of hrhAMR: 20 μg of human genomic DNA digested with ApaI (A), EcoRI (E), HindIII (H), and undigested control (C), respectively.

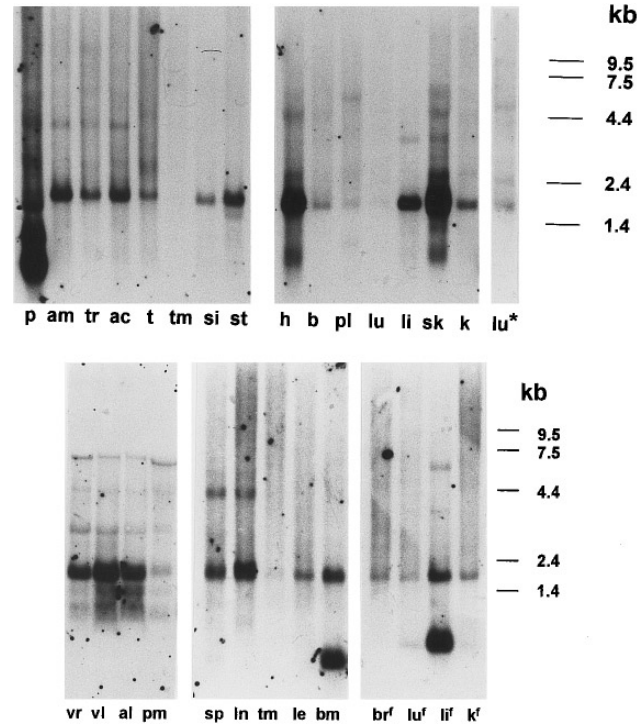

FIG. 4. Northern-blot analysis of hrhAMR transcripts in various human tissues. A common major band of 1.8 kb was observed beside several longer and shorter bands as outlined in the text. The tissues analyzed include 2μg of poly A^+ RNA from pancreas (p), adrenal medulla (am), thyroid (tr), adrenal cortex (ac), testis (t), thymus (tm), small intestine (si), stomach (st), heart (h), brain (b)m, placenta (pl), lung (lu), liver (li), skeletal muscle (sk), kidney (k), lung with 3 fold longer exposure time (lu*), spleen (sp), lymph node (ln), thymus (tm), leukocytes (le), bone marrow (bm), fetal brain (br^f), fetal lung (lu^f), fetal liver (li^f), fetal kidney (k^f), respectively, and 25 μg total RNA of right ventricle (vr), left ventricle (vl), left atria (al), respectively, and 8 μg total RNA of papillary muscle (pm).

ACKNOWLEDGMENTS

The study has been supported by a grant of the Deutsche Forschungsgemeinschaft (SFB 547). We wish to thank Prof. Schulz, Department of Pathology, University of Giessen for providing us with human tissues. The excellent technical assistence of Mrs. U. Jacobs and Mrs. C. Magel is gratefully acknowledged.

REFERENCES

1. Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M. J., Dumont, J. E., and Vassart, G. (1989) *Science* **244,** 569–572.
2. Kapas, S., Catt, K. J., and Clark, A. J. L. (1995) *J. Biol. Chem.* **270,** 25344–25347.
3. Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H., and Eto, T. (1993) *Biochem. Biophys. Res. Commun.* **192,** 553–560.
4. Morris, H. R., Panico, M., Etienne, T., Tippins, J., Girgis, S. I., and MacIntyre, I. (1984) *Nature* **308,** 746–748.

5. Cooper, G. J., Willis, A. C., Clark, A., Turner, R. C., Sim, R. B., and Reid, K. B. (1987) *Proc. Natl. Acad. Sci.* **84,** 8628–8632.
6. Sugo, S., Minamino, N., Kangawa, K., Miyamoto, K., Kitamura, K., Sakata, J., Eto, T., and Matsuo, H. (1994) *Biochem. Biophys. Res. Commun.* **201,** 1160–1166.
7. Sugo, S., Minamino, N., Shoji, H., Kangawa, K., Kitamura, K., Eto, T., and Matsuo, H. (1995) *Biochem. Biophys. Res. Commun.* **207,** 25–32.
8. Ueta, Y., Kitamura, K., Isse, T., Shibuya, I., Kabashima, N., Yamamoto, S., Kangawa, K., Matsuo, H., Eto, T., and Yamashita, H. (1995) *Neurosci. Lett.* **202,** 37–40.
9. Heaton, J., Lin, B., Chang, J. K., Steinberg, S., Hyman, A., and Lippton, H. (1995) *Am. J. Physiol.* **268,** H 2211–2215.
10. Haynes, J. M., and Cooper, M. E. (1995) *Eur. J. Pharmacol.* **280,** 91–94.
11. Kanazawa, H., Kurihara, N., Hirata, K., Kudoh, S., Kawaguchi, T., and Takeda, T. (1994) *Biochem. Biophys. Res. Commun.* **205,** 251–254.
12. Murphy, T. C., and Samson, W. K. (1995) *Endocrinology* **136,** 2459–2463.
13. Mazzocchi, G., Rebuffat, P., Gottardo, G., and Nussdorfer, G. G. (1996) *Life-Sciences* **58,** 839–844.
14. Sato, A., Canny, B. J., and Autelitano, D. J. (1997) *Biochem. Biophys. Res. Commun.* **230,** 311–314.
15. Jougasaki, M., Wie, C. M., Aarhus, L. L., Heublein, D. M., Sandberg, S. M., and Burnett, J. C. (1995) *Am. J. Physiol.* **268,** F657–663.
16. Entzeroth, M., Doods, H. N., Wieland, H. A., and Wienen, W. (1994) *Life Sci.* **56,** PL19–25.
17. Zimmermann, U., Fischer, J. A., and Muff, R. (1995) *Peptides* **16,** 421–424.
18. Shimekake, Y., Nagata, K., Ohta, S., Kambayashi, Y., Teraoka, H., Kitamura, K., Eto, T., Kangawa, K., and Matsuo, H. (1995) *J. Biol. Chem.* **270,** 4412–4417.
19. Miller, S. A., Dykes, D. D., and Polesky, H. F. (1988) *Nucleic Acids Res.* **16,** 1215.
20. Clark, J. M. (1988) *Nucleic Acids Res.* **16,** 9677–9686.
21. Novotny, J., and Auffray, C. (1984) *Nucleic Acids Res.* **12,** 243–255.
22. Coppock, H. A., Owji, A. A., Bloom S. R., and Smith, D. M. (1996) *Biochem. J.* **318,** 241–245.
23. Hirata, Y., Mitaka, C., Sato, K., Nagura, T., Tsunoda, Y., Amaha, K., and Marumo, F. (1996) *J. Clin. Endocrin. Metabol.* **81,** 1449–1453.