Volume 318, number 1, 65–70 FEBS 12132 February 1993


# Distribution of β2-adrenergic receptor mRNA expression along the hamster nephron segments

Shigeo Taniguchi, Tsuyoshi Watanabe, Akihide Nakao, George Seki, Shu Uwatoko, Keiji Suzuki and Kiyoshi Kurokawa

*First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan*

Received 7 January 1993

Distribution of β2-adrenergic receptor mRNA expression along the microdissected hamster nephron segments was examined by the reverse transcription-polymerase chain reaction (RT-PCR) technique. Conventional RT-PCR using a set of primers on separate exons could not be applied for the detection of β2-adrenergic receptor mRNA because of its intronless nature. We used the 'rapid amplification of cDNA ends' protocol [(1985) Proc. Natl. Acad. Sci. USA 85, 8998–9002] as a maneuver for RT-PCR of an intronless gene. Using this method, we successfully located hamster β2-adrenergic receptor mRNA only in glomeruli and early proximal convoluted tubule along the nephron segments tested.

Receptor; β-Adrenergic; Messenger RNA; Polymerase chain reaction; Nephron

## 1. INTRODUCTION

The β-adrenergic system is one of the main regulators of the extracellular fluid volume, and its effect on the kidney has been studied extensively. However, there are discrepancies in reported data concerning the precise localization of its receptor and the subtype of the receptor involved (see section 4).

Polymerase chain reaction (PCR), combined with reverse transcription (RT), has realized the detection of mRNA from minimal tissue sample. This RT-PCR technique has been applied to microdissected nephron segments, and distribution of mRNA expression of several genes in the kidney has been reported [1,2]. Another advantage of this approach is that it allows the discrimination of similar proteins, such as hormone receptor subtypes, at the gene level. Application of this method for the β2-adrenergic receptor gene, however, has been hampered by the fact that it contains no introns [3], because PCR of reverse-transcribed cDNA is generally performed using a set of primers on exons separated by introns in order to avoid erroneous detection of amplified genomic DNA.

In the present study, we examined the distribution of β2-adrenergic receptor mRNA expression along the microdissected nephron segments, by modifying the RT-PCR protocol for the detection of mRNA of intronless genes from small quantities of tissue preparations. Following this approach, we have successfully located mRNA of the β2-adrenergic receptor in both glomerulus and early proximal convoluted tube.

*Correspondence address:* S. Taniguchi, First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Tokyo 113, Japan. Fax: (81) (3) 3812-5063.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Collagenase (type I) is the product of Sigma (St. Louis, MO, USA). AMV reverse transcriptase, RNase inhibitor, DNA tailing kit, digoxigenin (DIG)-dUTP, positively charged nylon membrane, chemiluminescent DIG detection kit, and DIG-labeled DNA weight marker (pBR 322 *BqlI* + pBR 328 *HinfI*) were purchased from Boehringer-Mannheim (Mannheim, Germany). *Taq* polymerase was purchased from Takara Shuzo Co. (Kyoto, Japan). Oligonucleotides used for RT and PCR primers or the hybridization probe were synthesized using DNA synthesizer (391, PCR-MATE; Applied Biosystems, Foster City, CA, USA), and purified with Sephadex G-25 quick spin columns (Boehringer-Mannheim, Mannheim, Germany). *AccI* restriction enzyme and its reaction buffer were purchased from Wako Chemicals (Osaka, Japan).

### 2.2. *Preparation of tissue samples*

Golden hamsters of both sexes weighing between 50 and 100 g were used. Nephron segments were microdissected as previously described [4] with minor modifications. In brief, left kidney was perfused from abdominal aorta with 'microdissection solution' (NaCl 137 mM, KCl 4 mM, $CaCl_2$ 1 mM, $KH_2PO_4$ 1 mM, $MgSO_4$ 1 mM, glucose 5 mM, lactate 5 mM, HEPES 10 mM, 0.1% of bovine serum albumin (BSA), and pH adjusted to 7.4 with NaOH), containing 0.1% collagenase, and sliced with a razor blade. Tissue slices were then incubated with 0.1% collagenase for 30 min at 37°C. Microdissection was performed at 4°C in microdissection solution. Microdissected nephron segments (10 glomeruli or 2 mm of each tubular segment) were transferred into fresh solution in another dish and rinsed carefully so that no cell debris or interstitial tissues were attached. The prepared segment was then transferred into a 500 μl Eppendorf tube with minimal solution (about 1 μl), and mRNA was extracted as follows.

### 2.3. *Extraction of mRNA*

Extraction of mRNA was performed by applying the acid guanidin-

ium-phenol-chloroform (AGPC) method reported by Chomczynski and Sacchi [5] with slight modifications. 100 μl of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol) was added to the tubes containing the microdissected nephron segments. Then 100 μl of phenol saturated with water (pH 4), 20 μl of chloroform/isoamyl alcohol (24:1), and 10 μl of 3 M sodium acetate (pH 5.3) were added, and the tubes were left on ice for 15 min. Samples were centrifuged at 10,000 × g for 10 min at 4°C, the aqueous phase was extracted again with chloroform/isoamyl alcohol (24:1), and was transferred to a fresh tube. Then 5 μg of tRNA was added as carrier of mRNA, and the solution was mixed with 200 μl of ethanol and stored at −20°C for precipitation. The tubes were then centrifuged, the pellet was washed with 70% ethanol, and centrifuged again. The pellet of RNA was used in the following steps.

### 2.4. *Reverse transcription and polymerase chain reaction*

Reverse transcription (RT) followed by selective amplification of specific cDNA was done following the 'rapid amplification of cDNA ends' (RACE) protocol reported by Frohman et al. [6]. The main strategy of this method is depicted in Fig. 1. In short, RT was performed using an oligo(dT) primer attached with an artificial adapter sequence, so that every antisense cDNA synthesized in this reaction carried the adapter sequence at the 5′ end. This adapter sequence primer was paired by a specific sense primer in the upstream region of the cDNA and used for subsequent PCR, where only the target cDNA was amplified.

The RT reaction was performed as follows. The pellet in the ethanol precipitation tube was dissolved in 17 μl of RT solution and divided into two fresh tubes for the RT-PCR reaction (thus, the amount of the sample in each tube was either 5 glomeruli or 1 mm of tubular segment). In one tube, 0.5 μl of RNase inhibitor and 1 μl of AMV reverse transcriptase were added. In the other tube, 1.5 μl of enzyme storage buffer was added for use as a control without reverse transcriptase. The final amount of RT reaction solution was 10 μl and its composition was: Tris-HCl (pH 8.3) 10 mM, KCl 50 mM, $MgCl_2$ 50 mM, dithiothreitol (DTT) 1 mM, primer (oligo(dT) + adapter) 1 μM, RNase inhibitor 5–25 U/10 μl, and AMV reverse transcriptase 10 U/10 μl. RT tubes were incubated at 42°C for 60 min, and the reaction was terminated by heating the tubes at 95°C for 5 min.

PCR was performed using a sense-specific primer (5′-CAGGCACGGAAAGCTTTGTG-3′ which corresponds to bases 2,371–2,390 of the reported cDNA sequence of the hamster β2-adrenergic receptor [7]) and adapter oligonucleotide (Fig. 1) as the antisense primer. PCR solution was added to the RT tube to make a final volume of 50 μl. The composition of the reaction solution was: Tris-HCl (pH 8.3 at room temperature) 10 mM, KCl 50 mM, $MgCl_2$ 2 mM, gelatin 0.01%, specific sense primer 200 nM, tail adapter primer 200 nM, dNTPs 0.2 mM each, and *Taq* polymerase 1.25 U/50 μl. Each cycle of PCR consisted of 93°C for 1 min of denaturating, 60°C for 1.5 min of annealing, and 72°C for 2 min of extension, and was repeated up to 40 cycles.

In most of the experiments, second step or nested PCR was performed. In these experiments, the first step PCR product was used after 10 times of dilution. PCR buffer composition and the reaction condition of each cycle were identical with the first step PCR. The primers used were 5′-GTACAAATGACTCACCGCTG-3′, which corresponds to bases 2,446–2,465 of the hamster β2-adrenergic receptor cDNA as sense primer, and 5′-TCACAGCAGAAAGGTCCAAG-3′, corresponding to bases 2,863–2,882 as antisense primer. The expected product size is 437 bp. The number of cycles ranged from 5 to 20, depending on the objective of the study.

### 2.5. *Restriction enzyme treatment of the PCR product*

In some experiments, the RT-PCR product was treated with the restriction enzyme *Acc*I, as discussed in section 3. The first step PCR product was extracted once with phenol/chloroform and precipitated with ethanol. The pellet was diluted with 20 μl of *Acc*I reaction buffer, and divided into 2 fresh tubes, one for the reaction and another to act as a control without enzyme. 1 μl of *Acc*I enzyme (9 U/μl) was added to the reaction tube and both tubes were incubated at 37°C overnight. The end product was analyzed by Southern hybridization.

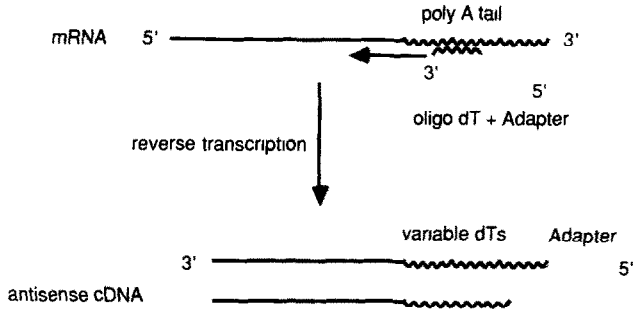


Fig. 1. The strategy of reverse transcription (RT) of the RACE protocol. RT was performed following the RACE (rapid amplification of cDNA ends) protocol reported by Frohman et al. [6] with slight modification. The primer used was a 37mer, which was composed of 17(dT) nucleotides at the 3′ end and an artificial adapter sequence at the 5′ end (3′-TTTTTTTTTTTTTTTTTAGGGCTACAGCTGAGCTCAG-5′). The oligo(dT) sequence of the primer matches arbitrarily with any part of the poly(A) tail of mRNA and the RT reaction ensues. Note that every synthesized antisense cDNA has the adapter sequence at the 5′ end, and that the (dT)*n* region before the adapter is of variable length, depending on which part of the poly(A) site of mRNA the primer matched for RT. The adapter sequence primer (3′-GGGCTACAGCTGAGCTCAG-5′) was paired by an upstream specific sense primer and used for subsequent PCR.

### 2.6. *Detection of the PCR product*

The RT-PCR product was detected by agarose gel electrophoresis and either ethidium bromide staining or Southern hybridization. The probe used for Southern hybridization was a sense 35mer oligonucleotide corresponding to bases 2,771–2,805 of hamster β2-adrenergic receptor cDNA (5′-CTCACTCGTCAAGTGTTAGGGGATACGCTGCTAGT-3′) labeled with DIG-dUTP using a DNA tailing kit. Hybridization was performed using positively charged nylon membrane. The hybridized samples were detected by using a chemiluminescent detection kit following the instructions of the manufacturer.

## 3. RESULTS

### 3.1. *RT-PCR of intronless genes*

We selected glomeruli β2-adrenergic receptor mRNA as a model to ascertain the relevance of our new approach of RT-PCR for intronless genes, since the presence of the β2-adrenergic receptor in glomeruli has already been demonstrated using autoradiography [8]. As the cDNA synthesized by the above-mentioned RT reaction has a variable length of dT's just before the adapter (Fig. 1), the PCR product was also not constant in its length and could not be detected as a band by normal gel electrophoresis. We used two distinct maneuvers to impose uniformity on the length of the product so that it could be detected by gel electrophoresis. One of them was to cut off the variable 3′ tail of amplified double-stranded cDNA using a restriction enzyme. Hamster β2-adrenergic receptor cDNA has a restriction site for *Acc*I (-GTAGAC-) at base 2,929, and it is the

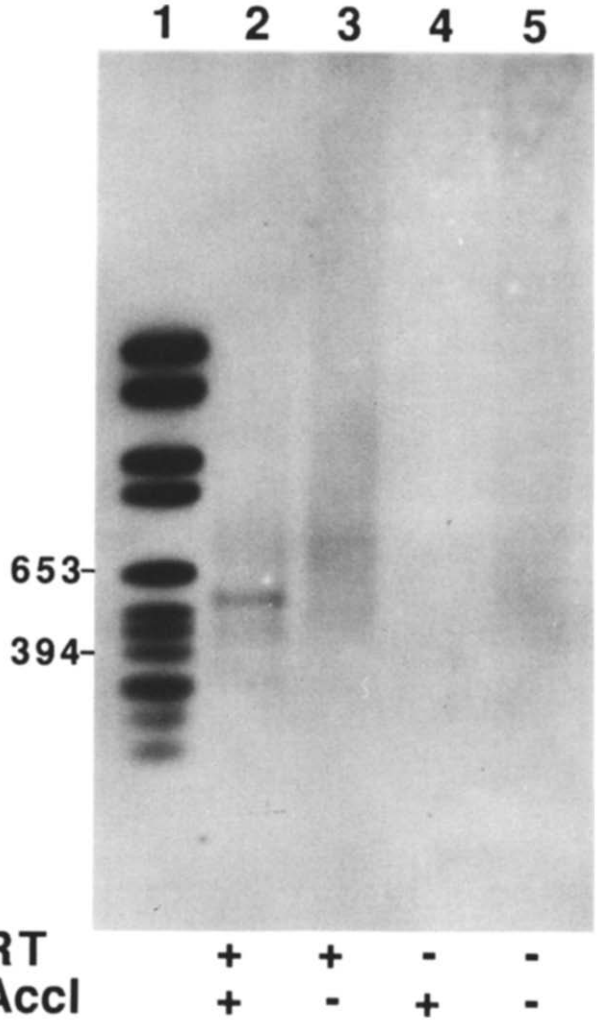


Fig. 2. The effect of *Acc*I treatment of the β2-adrenergic receptor mRNA RT-PCR product. Lane 1, DIG-labeled DNA weight marker. Lanes 2 and 3, results of the RT-PCR product with or without *Acc*I digestion, respectively. Lanes 4 and 5, results of the RT-negative control with and without restriction enzyme digestion.

only cutting site along the sequence which is amplified during PCR. The expected length of DNA after the enzyme treatment is 558 bp. Fig. 2 depicts the result of *Acc*I treatment of RT-PCR product from 5 glomeruli. The RT-PCR product without enzyme treatment shows only a vague broad signal, reflecting its variable length (lane 3). By digesting with *Acc*I, a single band of expected size appeared (lane 2). In the RT-negative control, a corresponding band cannot be seen even after restriction enzyme treatment (lane 4). The result implies that, even though the PCR product could not be detected as a band by electrophoresis, it had been sufficiently amplified during the reaction, and that the product has the restriction site at the expected position.

Another method we employed for detecting the RT-PCR product was to amplify a part of the product using a set of specific primers (nested PCR). The final product corresponds to a sequence where both ends of the first step PCR product have been truncated, and is of constant length. By ethidium bromide staining of agarose gel, we detected a single band at about 10 cycles of second step PCR amplification. Southern hybridization revealed the signal at 5 cycles of second step PCR (Fig. 3). This two-step PCR is sensitive, easy to perform and has a high degree of specificity. We used this method for screening the nephron distribution of mRNA.

### 3.2. *Nephron distribution of β2-adrenergic receptor mRNA*

The nephron segments tested were glomerulus, early proximal convoluted tubule (PCT), proximal straight tubule (PST), descending thin limb of Henle (DTL), medullary thick ascending limb (mTAL), cortical thick ascending limb (cTAL), distal convoluted tubule (DCT), cortical collecting duct (CCD), outer medullary collecting duct from inner stripe (OMCD), and terminal portion of inner medullary collecting duct (IMCD). For these 10 segments, we performed the two-step PCR. For each segment, experiments were repeated at least 5 times with essentially the same results. In each experiment 3–6 different nephron segments were studied, where glomeruli were always included as one of the segments and served as a positive control. For each segment, a sample without reverse transcriptase was always used as a negative control. In order to attain maximal sensitivity, second step PCR was continued up to 20 cycles. β2-Adrenergic receptor mRNA was detected only in glomerulus and early PCT. Fig. 4 depicts the results of two step PCR product agarose gel electrophoresis and Southern hybridization of 10 nephron segments.

Since we detected β-adrenergic receptor mRNA only in 2 of 10 nephron segments tested, we also examined α-tubulin gene (an example of a housekeeping gene) mRNA expression using the same protocol as reported here to ascertain the applicability of our method to negative segments. As expected, we detected its mRNA in all the nephron segments (data not shown).

## 4. DISCUSSION

RT-PCR of intronless genes has been reported by Grillo and Margolis [9]. They used DNase digestion of the sample prior to RT to minimize contamination of genomic DNA. However, the authors report faint signals in RT-negative controls in some experiments, the result indicating that minor contamination of genomic DNA is inevitable even after DNase treatment. We tried this method for microdissected nephron segments, and PCR products of the expected length were also seen in RT-negative controls only a few cycles later than in RT-positive samples (data not shown). The results may imply that, in our case, the quantity of sample mRNA was very small, and even after DNase treatment, the absolute difference between mRNA and the remaining DNA might have been insignificant; thus after PCR

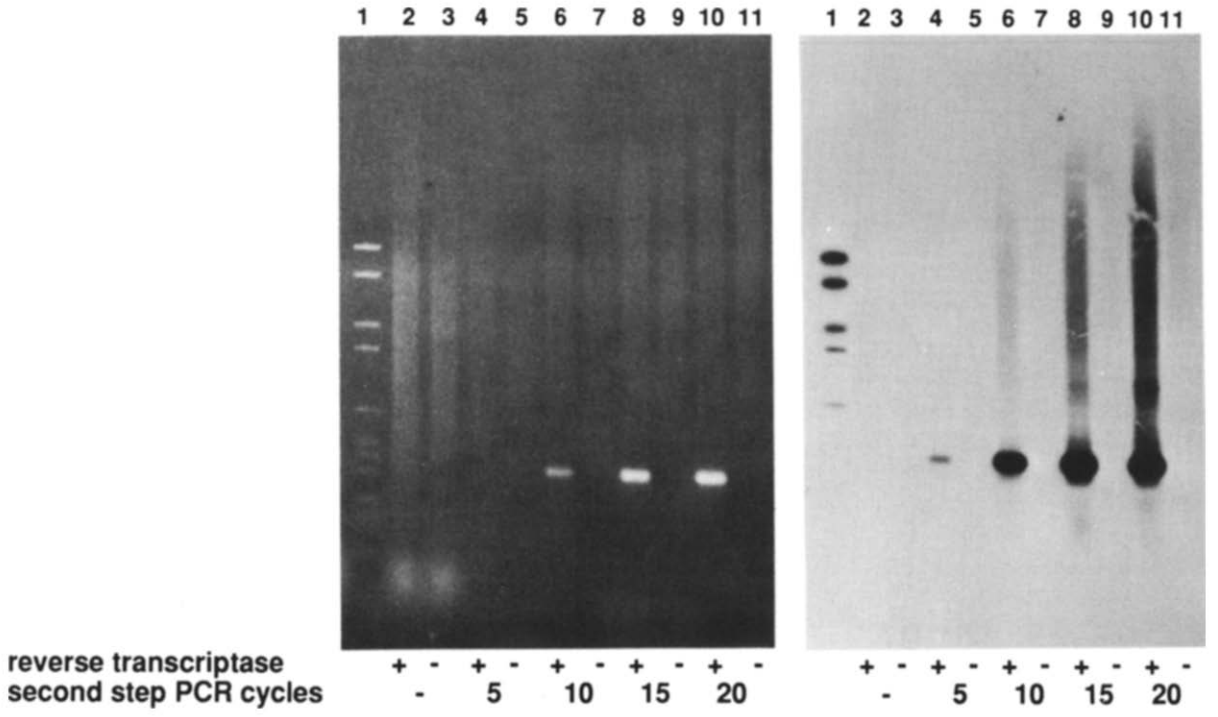


Fig. 3. Two-step PCR of hamster glomerular $\beta$2-adrenergic receptor mRNA. The figure demonstrates the results of different cycles (5, 10, 15, and 20 cycles) of second step PCR amplification. The product of each experiment was paired by a RT-negative control for detection. The left panel shows the result of agarose gel electrophoresis stained with ethidium bromide. The right panel depicts the result of Southern hybridization. In each panel, DIG-labeled DNA weight markers are shown in lane 1, and the first step PCR products are shown in lanes 2 and 3.

amplification, the origin of the final product became ambiguous. The procedure for RT-PCR of intronless genes by Grillo et al. [9] may not be adequate for the detection of mRNA expression in minute samples such as microdissected nephron segments.

For intronless genes, the cDNA sequence is essentially identical to that of the genomic DNA, so that conventional PCR cannot distinguish between the two. The only possible difference is elaborated by applying primers matching the poly(A) tail of mRNA at the step of RT. Efficient amplification of thus synthesized cDNA is reported by Frohman et al. as the RACE protocol [6]. In the present study, we have successfully applied this approach for RT-PCR of intronless genes.

Using the method reported here, we have located the $\beta$2-adrenergic receptor in glomeruli and early PCT microdissected from hamster kidney. The renal $\beta$-adrenergic system has been analyzed by many authors in various ways. Earlier clearance studies have demonstrated the direct effect of the $\beta$-adrenergic system on renal function [10]. Morphological investigations have shown adrenergic innervation in the kidney [11]. As for the precise nephron distribution of these receptors and the subtype involved, there are some discrepancies in the reported data. Cultured mesangial cells have been shown to respond to isoproterenol by increasing cAMP production [12]. As for tubular segments, some authors have shown distal distribution of $\beta$-receptors using biochemical [13–15] and physiological techniques [16,17], whereas others have reported that proximal tubular segments respond to $\beta$-adrenergic stimuli [10,18–21]. Most of those reports used isoproterenol as the agonist and/or propranolol as the antagonist (with the exception of Murayama et al. [20] who demonstrated by using specific antagonists that adrenergic receptors in microdissected PST are of the $\beta$2 subtype, and Teitelbaum et al. [15] who showed by using atenolol that $\beta$-adrenergic receptors of subtype other than $\beta$1 are present in cultured IMCD cells [15]), and from those studies it is not clear which subtype of receptors is involved in most of the nephron segments. Using subtype-specific agents, two groups have reported the distribution of $\beta$1 and $\beta$2 subtypes of adrenergic receptors in the kidney by autoradiography [8,22], but their results are not totally in agreement with each other. Summers et al. demonstrated that those receptors distributing in the distal nephron are mainly of the $\beta$1 subtype, while both $\beta$1 and $\beta$2 subtypes of receptors are present in glomeruli [8], data largely in agreement with our present results except for the lack of proximal $\beta$2 receptors. In contrast Healy et al. have reported that those receptors distributing in glomeruli are mainly of the $\beta$1 subtype whilst the $\beta$2 subtype of receptors are distributed in medullary tubules [22]. Although the reasons for the conflicting data reported in the literature, as well as some disagreements of our present results with others, may not be identified unequivocally, the problem of cross-reactivity of pharmacological agents should always be considered. We

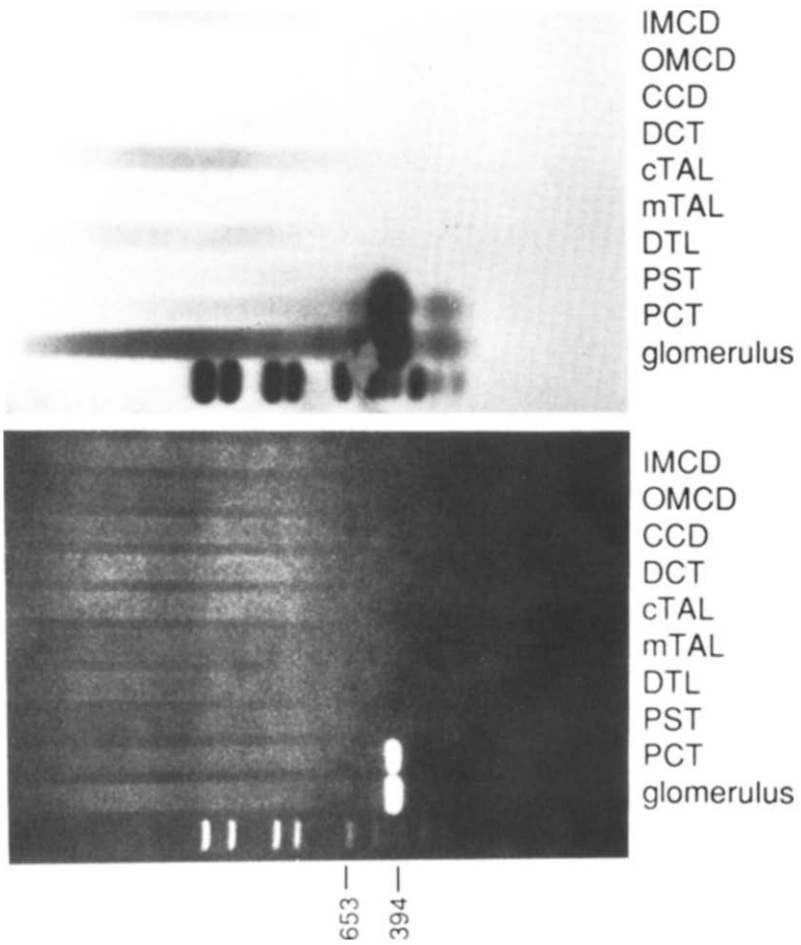


Fig. 4. Nephron distribution of hamster $\beta$2-adrenergic receptor mRNA. Distribution of $\beta$2-adrenergic receptor mRNA along the hamster nephron was examined using a two-step PCR method. The PCR products were detected by agarose gel electrophoresis and ethidium bromide staining (left panel) or Southern hybridization (right panel).

believe that the only way of definitely discriminating receptor subtypes is at the gene level, and RT-PCR should play a major role in this field. Finally, species difference remains as one of the candidates for some of the discrepancies of our data with others.

In conclusion, we have succeeded in detecting mRNA of the $\beta$2-adrenergic receptor gene, an example of an intronless gene, from small quantities of tissue preparation using the RACE protocol reported by Frohman, with slight modifications. By applying this approach we have successfully located its expression in microdissected hamster glomeruli and early PCT, but not in the other nephron segments studied.

*Acknowledgements:* We are grateful to Dr. Kazuhiko Koike for discussion and useful suggestions, and to Miss Hiromi Imaki for technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research, 02404036 and 03354019, from the Ministry of Education, Science, and Culture of Japan, and grants from the Mochida Memorial Foundation for Medical and Pharmaceutical Research, the Kato Memorial Trust for Nambyo Research, and the Uehara Memorial Foundation.

## REFERENCES

[1] Moriyama, T., Murphy, H.R., Martin, B.M. and Garcia-Perez, A. (1990) Am. J. Physiol. 258, F1470–F1474.
[2] Terada, Y., Moriyama, T., Martin, B.M., Knepper, M.A. and Garcia-Perez, A. (1991) Am. J. Physiol. 261, F1080–F1087.
[3] Kobilka, B.K., Frielle, T., Dohlman, H.G., Bolanowski, M.A., Dixon, R.A.F., Keller, P., Caron, M.G. and Lefkowitz, R.J. (1987) J. Biol. Chem. 262, 7321–7327.
[4] Morel, F., Chabardès, D. and Imbert, M., in: Methods of Pharmacology, Vol. 4B (M. Martinez-Maldono, Ed.), Plenum Press, New York, 1978, pp. 297–334.
[5] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[6] Frohman, M.A., Dush, M.K. and Martin, G.R. (1988) Proc. Natl. Acad. Sci. USA 85, 8998–9002.
[7] Dixon, R.A.F., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., Frielle, T., Bolanowski, M.A., Bennett, C.D., Rands, E., Diehl, R.E., Mumford, R.A., Slater, E.E., Sigal, I.S., Caron, M.G., Lefkowitz, R.J. and Strader, C.D. (1986) Nature 321, 75–79.
[8] Summers, R.J., Stephenson, J.A. and Kuhar, M.J. (1985) J. Pharmacol. Exp. Ther. 232, 561–569.
[9] Grillo, M. and Margolis, F.L. (1990) Biotechniques 9, 262–268.
[10] Gill Jr., J.R. and Casper, A.G.T. (1971) J. Clin. Invest. 50, 112–118.

[11] Barajas, L., Powers, K. and Wang, P. (1984) Am. J. Physiol. 247, F50–F60.
[12] Bonventre, J.V., Skorecki, K.L., Kreisberg, J.I. and Cheung, J.Y. (1986) Am. J. Physiol. 251, F94–F102.
[13] Chabardès, D., Imbert-Teboul, M., Montégut, M., Clique, A. and Morel, F. (1975) Pflügers Arch. 361, 9–15.
[14] Morel, F. (1981) Am. J. Physiol. 240, F159–F164.
[15] Teitelbaum, I., Strasheim, A. and Berl, T. (1989) Kidney Int. 35, 647–653.
[16] Iino, Y., Troy, J.L. and Brenner, B.M. (1981) J. Membrane Biol. 61, 67–73.
[17] Hayashi, M., Yamaji, Y., Iyori, M., Kitajima, W. and Saruta, T (1991) J. Clin. Invest. 87, 1153–1157.
[18] Jacobs, W.R. and Chan, Y.L. (1986) Biochem. Biophys Res Commun. 141, 334–339.
[19] Kudo, K., Kondo, Y., Abe, K., Igarashi, Y., Tada, K. and Yoshinaga, K. (1991) Am. J. Physiol. 261, F393–F399.
[20] Murayama, N., Ruggles, B.T., Gapstur, S.M., Werness, J.L. and Dousa, T.P. (1985) J. Clin. Invest. 76, 474–481.
[21] Weinman, E.J., Sansom, S.C., Knight, T.F. and Senekjian, H.O. (1982) J. Membrane Biol. 69, 107–111.
[22] Healy, D.P., Münzel, P.A. and Insel, P.A. (1985) Circ. Res. 57, 278–284.