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Isolation and characterization of multiple isoforms of the rat urokinase receptor in osteoblasts

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Abstract

A rat urokinase receptor (uPAR) cDNA fragment was amplified by RT-PCR from RNA of the rat osteoblastic cell line CFK-1. Using this DNA species as a hybridization probe two rat uPAR cDNAs were isolated from a CFK-1 cDNA library. These two cDNAs encode an identical uPAR protein except for a single base mutation which results in the substitution of cysteine to serine at amino acid 71 in one variant. PCR analysis of rat genomic DNA revealed the presence of an additional uPAR arising from alternate splicing which is expressed in a variety of tissues. These studies provide the tools for examining uPAR function in fibrinolysis, tumor invasion and metastasis in the rat and for identifying the mechanism of species specificity in uPA actions.

Key words: Urokinase receptor; Plasminogen activator; Alternate splicing; PCR; Growth factor

1. Introduction

Urinary plasminogen activator or urokinase (uPA) belongs to the family of serine proteases which cause fibrinolysis by plasminogen activation and is also believed to play a major role in tumor invasion and metastasis [1–4]. It is produced by various tumors and tumor cell lines including prostate cancer and the prostate cancer cell PC-3 [5]. Binding of uPA to a cell surface receptor is essential for activation of plasminogen and for site directed extracellular proteolysis by tumors which produce this protease [6,7]. In previous studies we have also shown that uPA can act as a growth factor for the cells of the osteoblast phenotype [5] and have localized these effects of uPA within its receptor binding domain i.e. amino acids 4–43. Furthermore fucosylation of uPA at Thr^{18} was shown to be critical for these effects [8].

Recently cDNAs encoding human and mouse uPA receptors (uPARs) have been isolated [9,10]. These are cysteine rich highly glycosylated 50–60 kDa proteins attached to the cell membrane by a glycosyl-phosphotidylinositol (GPI) anchor. The mature uPAR consists of three domains of internal homology. In addition, the NH_2-terminal 87 residues are believed to constitute the uPA binding domain. In both the human and the mouse, variant uPARs have been cloned which are believed to be due to alternative splicing. These isoforms differ from the original uPARs in their carboxy-terminal regions. In both species however, the alternatively spliced forms retain the critical NH_2-terminal ligand binding domain [11]. By in situ hybridization the alternately spliced forms of uPARs have been reported to be differentially expressed in the mouse gastrointestinal tract [10].

In the present study we report the isolation and characterization of two uPAR isoforms from the rat osteoblastic cell line, CFK-1, which differ by a single amino acid within their critical ligand binding domain. Furthermore analysis of rat genomic DNA demonstrates the presence of an additional rat uPAR isoform resulting from alternate splicing in the second repeat of the uPAR. This alternately spliced receptor is expressed in multiple rat tissues.

2. Materials and methods

2.1. Materials

All PCR reactions were performed in a Gene amp PCR 9600 apparatus, Perkin-Elmer-Cetus Norwalk, CT. The rat osteoblastic cell line, CFK-1, which was employed has previously been described [12]. The CFK-1 cDNA library was a generous gift from Dr. John Wozney, Genetics Institute, Cambridge, MA.

2.2. PCR amplification of rat uPAR

Total cellular RNA was isolated from CFK-1 cells using 6M guanidium thiocyanate–phenol–chloroform extraction. 3 μg of total RNA was used for reverse transcription using random primer (Pharmacia) and 200 units of reverse transcriptase (BRL) in a final volume of 20 μl and incubating at 23°C for 10 min, at 42°C for 50 min and at 70°C for 15 min. Rat uPAR cDNA was amplified using two PCR primers 1 and

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2 corresponding to nucleotides 521–538 and 1034–1051, respectively, of the reported nucleotide sequence of human uPAR [9,13]. The PCR reaction included 30 cycles of denaturation (90°C, 20 s), annealing (50°C, 30 s), and extension (72°C, 60 s), followed by a final (10 min) extension step in a volume of 100 μl. One-tenth of the reaction mixture was analyzed by electrophoresis on a 1.1% DNA agarose gel and then visualized by ethidium bromide staining to identify the amplified DNA products.

To identify the alternately spliced rat uPAR, 1 μg of rat genomic DNA was isolated from CFK-1 cells and subjected to PCR using two flanking primers, A (5′-GAGCAAAGCCTGCAG-3′) and B (5′-TTGCTATGGAAACC-3′) which were synthesized based on the reported nucleotide sequence of the alternately spliced form of mouse uPAR [10]. To examine the tissue specific expression of this uPAR isoform 3 μg of total RNA was extracted from CFK-1 cells, kidney, liver and brain and analyzed by RT-PCR using primers A and B as described above. All PCR reactions were analyzed on 1.1% DNA agarose gel containing ethidium bromide.

2.3. cDNA cloning

The amplified rat specific uPAR DNA fragment was labeled with [^{32}P]dCTP by the random primer method [14]. 1×10^6 plaques from a CFK-1 cDNA library in lambda ZAP vector were screened on duplicate filters (Amersham) at 40°C in 50% formamide. After 24 h incubation, filters were washed four times for 20 min, each with 1 × SSC (10 × SSC = 1.5 M NaCl, 0.5 M sodium citrate, pH 7.0), 0.1% SDS at 40°C [15,16]. Autoradiography of filters was carried out at −70°C using XAR film (Kodak Co, Rochester, NY) with two intensifying screens.

3. Results

3.1. Amplification of rat uPAR DNA

Using RT-PCR a predicted 520-bp DNA fragment was amplified from the rat osteoblast cell line, CFK-1 (Fig. 1), the authenticity of this DNA as encoding a rat uPAR was confirmed by DNA sequence analysis.

3.2. Isolation and characterization of rat uPAR cDNA

Using the amplified rat specific uPAR DNA as a hybridization probe, ten positive clones were isolated from the CFK-1 cDNA library. These rat uPAR clones were further purified by secondary screening and subjected to nucleotide sequence analysis. By these methods two 1.3-kb cDNAs were identified each of which contained the full-length coding region of rat uPAR. These cDNAs started 11 bp upstream of the initiation codon, were followed by a 984 bp open reading frame and extended for 275 bp of untranslated sequence in the 3′ noncoding region. These two cDNAs (rat uPAR-1a and 1b) had identical restriction maps and each encoded a mature peptide of 304 amino acids (Fig. 2). Three silent base changes were found between ruPAR-1a and ruPAR-1b, however, a single base mutation in rat uPAR-1b results in a change from Cys to Ser in uPAR-1b. This amino acid change occurs in the critical ligand binding domain of the uPAR at amino acid 71 (Fig. 2).

A comparison of the nucleotide sequences of the isolated rat uPAR cDNAs showed 67% and 83% homology with human and mouse uPAR respectively (Fig. 3). Furthermore, rat uPAR is 59% and 87% homologous in its amino acid sequence to the human and mouse uPAR respectively in its ligand binding domain, amino acid (1–87).

3.3. Amplification of an alternatively spliced rat uPAR from rat genomic DNA

Two PCR primers of rat uPAR flanking the unique sequence of the alternatively spliced form of mouse uPAR were synthesized to attempt to isolate an alternatively spliced rat uPAR from rat genomic DNA. These PCR primers amplified a DNA band of 148 bp corresponding to our previously isolated receptor isoforms uPAR-1a and 1b. In addition, a 665 bp DNA fragment was also amplified from rat CFK-1 genomic DNA using these primers (Fig. 4).

This DNA fragment was subcloned into the pBluescript KS(+) vector and sequenced. The sequence analysis revealed an open reading frame of 258 bp followed by a stop codon. Although the sequence was homologous to the alternately spliced mouse uPAR-2, substantial differences were seen at both nucleotide and amino acid levels between these two species (Fig. 5). As in the mouse alternate splicing of rat uPAR (ruPAR-2) occurs in its second domain.

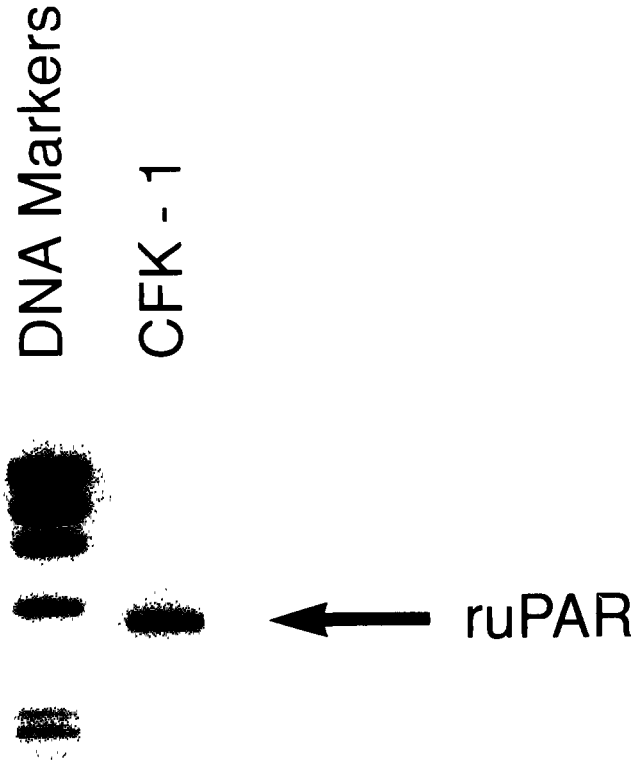

Fig. 1. Amplification of uPAR from rat osteoblasts cell line CFK-1 by PCR. 3 μg of total RNA from CFK-1 cells was reverse transcribed as described in section 2. Amplification of rat uPAR was done by uPAR primers 1 and 2 (described in section 2) and analyzed on a 1.1% DNA agarose gel.

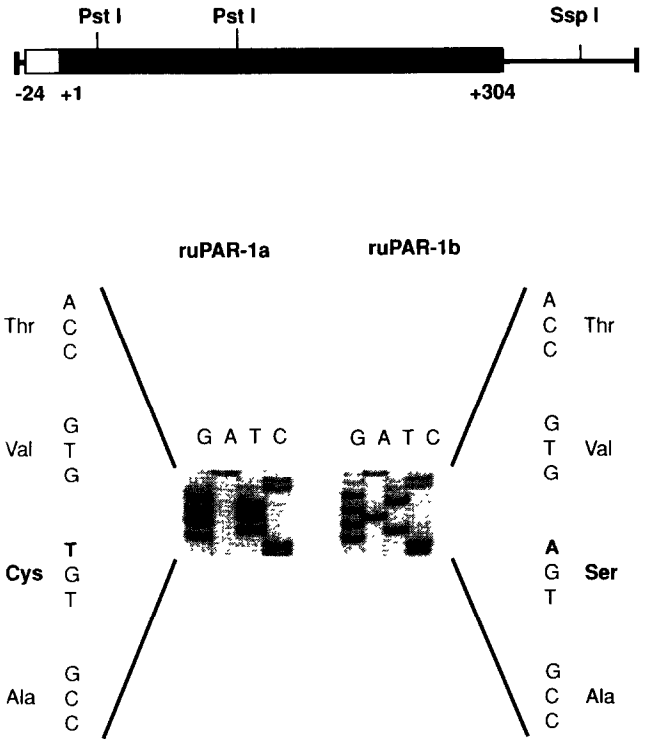

Fig. 2. Restriction map of rat uPAR. The entire sequence (Fig. 3) was obtained from the full length rat uPAR-1a and 1b. Pst1 and SSPI restriction sites and amino acids numbers of uPAR protein are indicated (upper panel). Nucleotide sequencing (lower panel) identified the substitution of A in uPAR-1b for T in uPAR-1a resulting in Ser instead of Cys.

3.4. Partial organization of the rat uPAR gene

Based on the nucleotide sequence of rat uPAR-1a and 1b, and the sequence of the additional uPAR product amplified from genomic DNA it is predicted that uPAR-2 arises by alternate splicing. The same exon appears to generate two species of mRNA by alternate splicing due to the presence of two donor sites. These mRNAs can result in the expression of mature proteins of 304 amino acids in the case of uPAR-1a and 1b (differing in one amino acid as a result of a single base mutation) and 196 amino acid in the case of rat uPAR-2 (Fig. 6).

3.5. Expression of alternately spliced ruPAR

Using flanking PCR primers, expression of alternately spliced form of ruPAR was examined in various tissues by RT-PCR. This amplification results in two DNA bands of 148 bp and 665 bp corresponding to ruPAR-1 and ruPAR-2, respectively, in rat CFK-1 cells, kidney, liver and brain RNA. The authenticity of these amplified DNA bands as ruPAR-1 and ruPAR-2 was further confirmed by DNA sequence analysis.

4. Discussion

In the present study we have isolated and characterized three isoforms of the rat uPA receptor. Rat uPAR-1a and 1b each encode a protein with a predicted 24 amino acid signal peptide followed by a mature protein of 304 amino acids. Both receptors have a very high cysteine content whose number and location appear to dictate the occurrence of three internal repeats as they do in the corresponding mouse and human uPARs. A num-

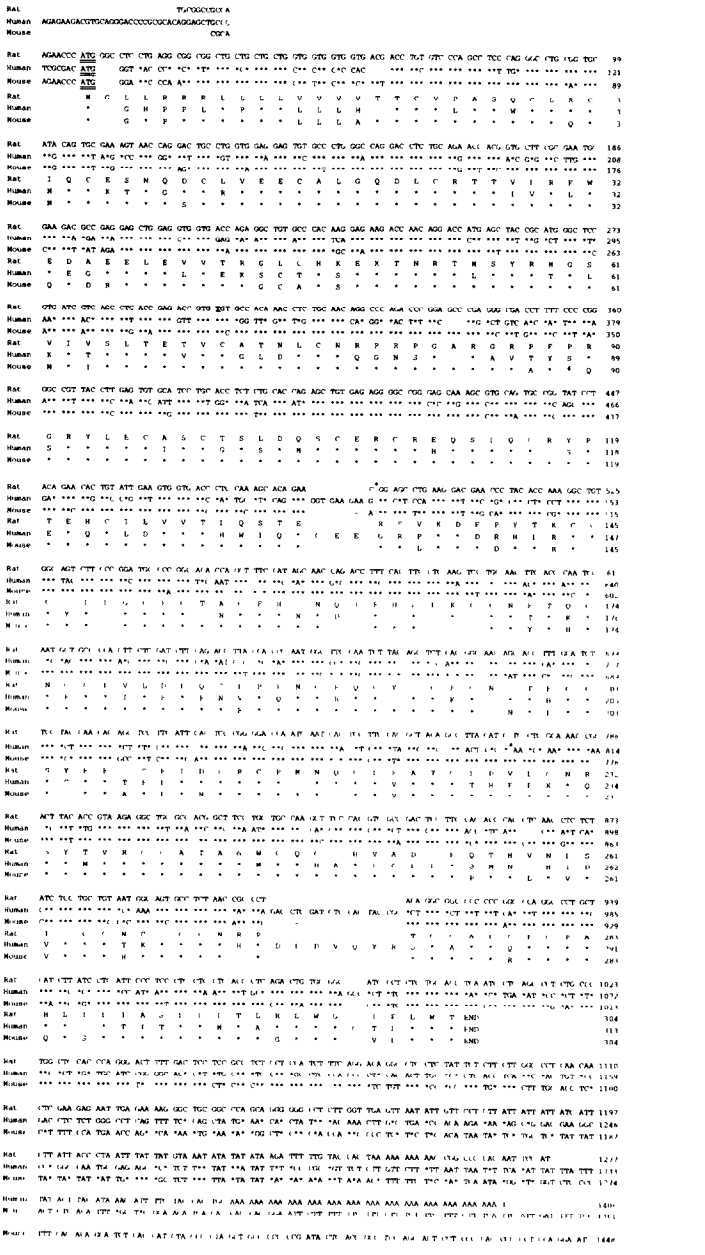

Fig. 3. Comparison of nucleotide and amino acid sequences of human, mouse and rat uPARs. The sequence of rat uPAR-1a is aligned with the human and mouse uPAR sequences for comparison. The areas of alternate splicing in human, mouse and rat uPARs are marked by +. Substitution of T to S in ruPAR-1b is underlined. * indicates identical nucleotides and amino acids in human and mouse uPAR. EMBL accession no.: X71898 and X71899.

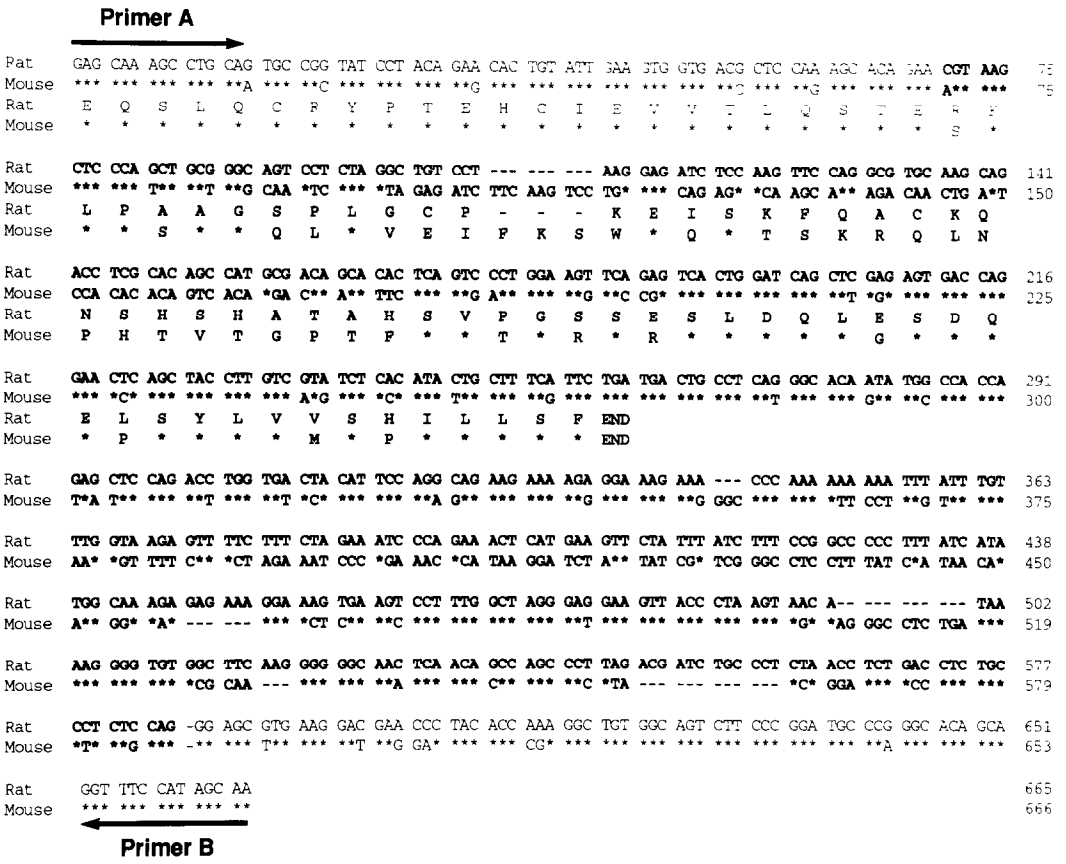

Fig. 4. Genomic DNA sequence of alternately spliced rat uPAR. The alternately spliced form of rat uPAR (ruPAR-2) was amplified from genomic DNA using PCR primers A and B as described in section 2. The data is aligned with mouse uPAR-2 for comparison. * denotes identical nucleotides and amino acids in rat and mouse uPAR-2. The sequence unique to ruPAR-2 (relative to ruPAR-1) is shown in bold. EMBL accession number: X-76129.

ber (43%) of amino acid differences between these rat forms and the corresponding human uPAR are found in the uPA binding domain (residues 1–87). Fewer differences (13%) occur in this region between the rat and corresponding mouse uPARs. These interspecies amino acid substitutions may underlie the apparent increased affinity of each receptor for its homologous ligand which has been reported at least for mouse and human uPA. Furthermore the substitution of Ser in rat uPAR-1b for Cys in rat uPAR-1a at position 71 may have important consequences for the ligand–receptor interaction in the rat.

Both mouse and human uPARs are believed to be anchored to the cell membrane by a glycosyl-phosphatidyl-inositol (GPI) linkage after removal of a carboxy-terminal hydrophobic peptide. The Gly conserved at position 275 in rat uPAR-1a and 1b and in muPAR-1 has been suggested to be the site of GPI attachment in these species whereas the Gly at position 283 in the huPAR1 may serve a similar function [10]. The rat uPARs are also highly glycosylated proteins as are the mouse and human uPARs. The rat uPARs have 5 putative N-glycosylation sites in the same position as in the mouse and human receptors and have 2 additional sites in common only with the mouse receptor.

Analysis of genomic DNA has revealed the presence of another uPAR isoform which corresponds to uPAR-2 in the human and mouse. The NH_2 terminal region of uPAR-2 is identical to that of uPAR-1a and 1b but includes a unique carboxyl-terminal peptide of 63 amino acids. This appears to be due to alternative splicing from the same gene. Thus excision of an intron occurs at different positions in uPAR-1a and 1b versus uPAR-2 due to two different upstream donor splice sites and the same downstream acceptor sites. Comparison of rat uPAR-2 with mouse uPAR-2 reveals strong homology in both coding and noncoding regions. Furthermore as in the mouse, alternative splicing occurs in the second internal repeat contrast to the splicing event in human uPAR-2 which occurs in the third repeat. Due to the presence of a stop codon at nucleotide 258 ruPAR-2 only encodes a mature protein of 196 amino acids. As with mouse and human uPAR-2, rat uPAR-2 retains the ligand binding site. The sequence unique for these isoforms in each case is hydrophillic and lacks a potential GPI anchoring site. Consequently as with the other spe-

cies of alternatively spliced receptors uPAR-2 may represent a water-soluble secreted protein [11].

We and others have observed the expression of uPAR isoforms in a variety of tissues, however the potential significance of these isoforms is uncertain. Several studies in heterologous systems have shown that uPA can promote tumor invasions however these studies were limited in their scope due to an interspecies blockade of uPA actions. In recent studies we have developed a homologous rat model to study the role of uPA in tumor metastasis associated with prostate cancer. Accumulating evidence has suggested an important role for uPAR in the process of tumor invasion in a variety of malignancies. Knowledge of the rat uPAR sequence and availability of a uPAR cDNA will now allow us to characterize the role played by these receptors in the process of tumor invasion and metastasis and to determine the potential therapeutic benefit of modulating uPAR levels using gene transfer techniques.

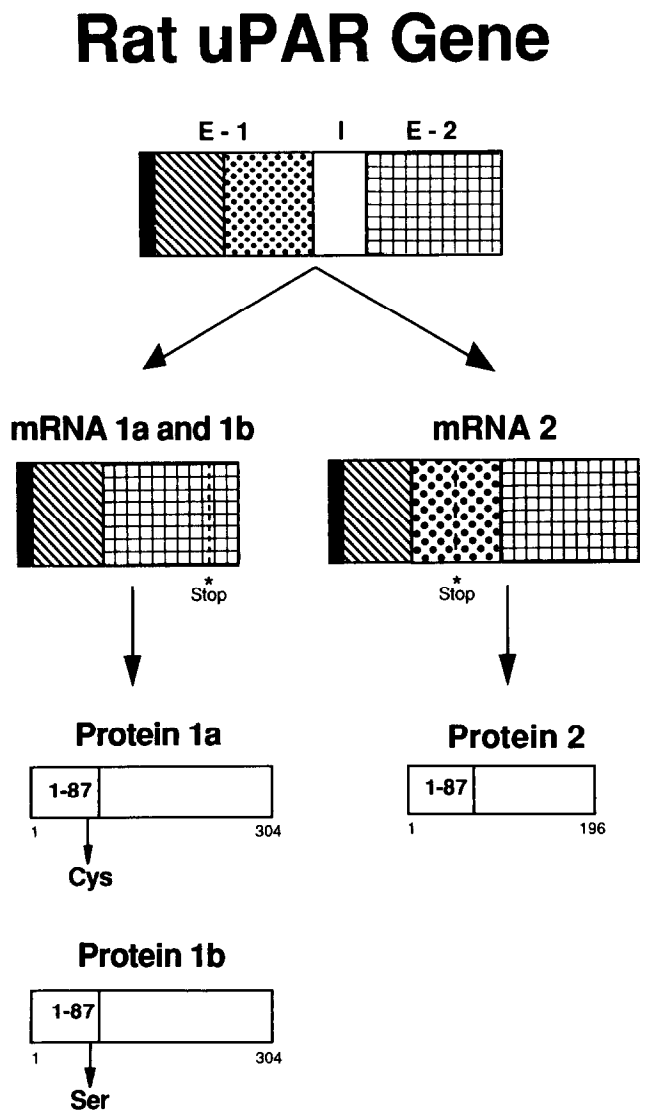

Fig. 5. Partial organization of the rat uPAR gene. Schematic representation of the rat uPAR gene and its mRNA and protein products. The coding region of the ruPAR gene consists of two exons E-1 and E-2. Two alternate donor sites in E-1 result in alternative splicing to E-2 and consequent variations in the size of the intervening sequence (I). This alternative splicing accounts for mRNA 1a and 1b or mRNA-2. A single base mutation in E-1 appears to account for mRNA 1a and 1b.

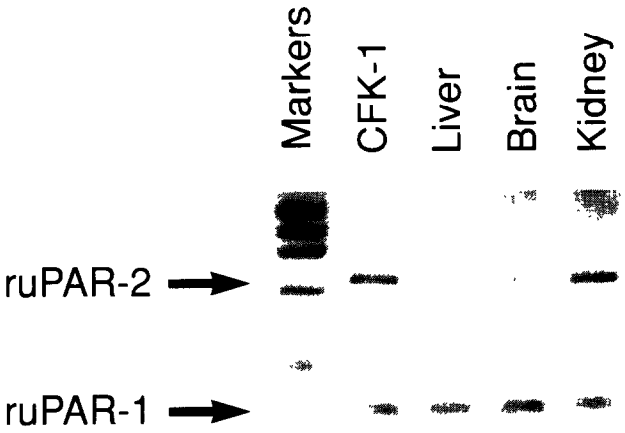

Fig. 6. Expression of alternately spliced ruPAR. 3 μg of total RNA from CFK-1 cells rat, kidney, liver and brain were reverse transcribed as described in section 2. Amplification of alternately spliced ruPARs was performed with flanking primers A and B as described in section 2. Amplified PCR products were analyzed on a 1.1% DNA agarose gel. Amplification of ruPAR-1 and ruPAR-2 are marked by arrows.

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