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Biochemical and Biophysical Research Communications 319 (2004) 247–255
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AU-rich elements in the mRNA 3′-untranslated region of the rat receptor for advanced glycation end products and their relevance to mRNA stability☆

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Received 26 April 2004
Available online 14 May 2004

Abstract

Several putative polyadenylation sequences and an adenylate plus timidylate rich element (ARE) are present at the 3′ end of the rat advanced glycation end products receptor (RAGE) gene. Two transcripts are generated by the use of alternative polyadenylation sequences, one containing the ARE sequence in its 3′-untranslated region (3′-UTR). Transfections of CHO-k1 or NRK cells with constructs expressing the 3′-UTRs of the transcripts fused to a green fluorescence protein mRNA show that the ARE sequence has a negative effect on protein expression correlating with a decrease in the amount of mRNA, as shown in CHO-k1 transfected cells. When transfected cells were incubated in the presence of Actinomycin D the amount of fluorescence decreased in cells transfected with the ARE sequence, indicating that this sequence induces lower mRNA stability. Thus, alternative polyadenylation signals and an ARE sequence provide a novel mechanism for the regulation of the rat RAGE gene expression.

Keywords: 3′-untranslated region; Post-transcriptional regulation; Receptor for advanced glycation end products; Adenylate plus uridylate rich element; mRNA stability; Rat

The receptor for advanced glycation end products (RAGE) belongs to the immunoglobulin superfamily of cell surface molecules [1–3] and is able to bind different ligands such as advanced glycation end products (AGEs), several members of the S100/calgranulin family, β-sheet fibrillar structures, and amphoterin [4]. Accumulation of AGEs [5] and the intracellular responses triggered by their binding to RAGE (i.e., an increase in the oxidant stress and the liberation of cytokines and growth factors) [6] induce some of the long term complications of diabetes [7–9].

Human promoter has been characterized [10] and it has been suggested that RAGE expression in tissues is regulated at multiple levels. Regulation mechanisms involving alternative splicing of the receptor mRNA have been proposed in humans [11] and rats [12]. In this study, we describe the characterization of the 3′-flanking region of the rat gene. In this region, we have found the presence of several putative polyadenylation sequences and an adenylate plus timidylate rich element. We have demonstrated the existence of two transcripts generated by the use of alternative polyadenylation sequences that differ in their 3′-untranslated region (3′-UTR). One of the transcripts contains the adenylate plus uridylate rich element (ARE).

To further understand the molecular mechanisms that control the expression of the RAGE protein we have examined the ability of the ARE-containing 3′-UTR of the RAGE message to mediate post-transcriptional regulation of gene expression. To achieve this

☆ *Abbreviations:* ActD, actinomycin D; AGEs, advanced glycation end products; ARE, adenylate plus uridylate rich element; bp, base pairs; CMV, cytomegalovirus; GFP, green fluorescent protein; RACE, rapid amplification cDNA ends; RAGE, receptor for advanced glycation end products; SSC, sodium chloride/sodium citrate buffer; 3′-UTR, 3′-untranslated region.

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doi:10.1016/j.bbrc.2004.04.178

aim, we have studied the expression of the green fluorescent protein (GFP) derived from mRNA chimaeras containing the 3′-UTRs of these transcripts. The results presented here demonstrate that the ARE sequence of the 3′-UTR of RAGE can influence mRNA stability.

Experimental

Isolation and sequencing of the 3′-flanking region of a rat RAGE genomic clone. A Charon 4A rat genomic library [13] was screened by hybridization with a PCR product probe corresponding to a fragment of the rat RAGE gene [12]. Lifts were prepared and hybridized in Northern Max Prehyb/Hyb solution (Ambion, Austin, TX) with [α-^{32}P]dCTP random primer labelled probe (1×10^6 cpm/ml) according to standard techniques [14]. Positive plaques were selected and purified. DNA from positive plaques was digested with *Bam*HI and subcloned into plasmid pUC18 for DNA sequencing. A plasmid containing a 1.7-kb *Bam*HI insert corresponding to the 3′-flanking region of the rat RAGE gene was sequenced using an ABI 373 sequencer. Sequencing reactions were carried out using AmpliTaq FS polymerase and fluorescent-labelled chain terminators (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit) (Perkin–Elmer), (Servicio de Secuenciación, Instituto de Biomedicina y Parasitología López Neira, CSIC, Granada, Spain).

mRNA analysis. Total RNA was isolated using a guanidinium isothiocyanate extraction method [15]. First strand cDNA synthesis was performed on 5 μg total RNA in a final volume of 15 μl using a *Not*I-oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase as described by the manufacturer (Pharmacia First Strand Synthesis kit). PCR amplifications were accomplished using a set of oligonucleotide primers as indicated (Table 1). Reactions containing no reverse transcripted samples were run to demonstrate absence of genomic DNA contamination (data not shown). Reactions were performed in a DNA thermal cycler (PE 9700) in a 50 μl final volume of reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris–HCl, pH 9.0) containing 2.5 μl of reverse transcripted RNA, 50 pmol of each primer, and 0.2 mM dNTPs. Cycling conditions were: an initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 91 °C (1 min), annealing at 55 °C (1 min), and extension at 72 °C (2 min). PCR products were electrophoresed on agarose, isolated, and cloned into pST1-Blue vector (Novagen). To ensure that no errors were introduced by PCR, three independent clones were sequenced using M13 universal and reverse sequencing primers.

For Northern blot analysis, 10 μg of RNA was electrophoresed on a 1.0% agarose–5% formaldehyde gel and transferred using a 10× sodium chloride/sodium citrate buffer (SSC) solution to BrightStar-Plus positively charged nylon membranes (Ambion). After ultraviolet crosslinking, the filters were initially prehybridized for 3 h and then hybridized in Northern Max Prehyb/Hyb buffer for 18 h at 42 °C with a random primer labelled cDNA probe for GFP. Finally, the blots were washed once in 2× SSC and 0.1% SDS at room temperature and three times in decreasing concentrations (1×–0.1×) of SSC and 0.1% SDS at 65 °C. Blots were exposed to Kodak Biomax film and intensifying screen at −80 °C. The relative amount of mRNA in each sample was quantified by densitometric analysis using the NIH Image Software [16] and the data were normalized to those of 28S rRNA.

Design of expression constructs. The cDNAs corresponding to the 3′-UTR of the rat RAGE gene were amplified by PCR from a positive genomic DNA clone using *Taq* DNA polymerase and a set of primers (described in Table 1) forward and reverse with introduced *Bgl*II and *Mlu*I restriction endonuclease sites, respectively, under the following conditions: 94 °C (5 min) followed by 30 cycles of 91 °C (1 min), 55 °C (1 min), and 72 °C (1 min). After purification, the resulting fragments were digested by *Bgl*II and *Mlu*I, purified on agarose, and sub-cloned downstream from the GFP coding region into *Bgl*II–*Mlu*I digested pEGFP-C1 (Clontech, Palo Alto, CA, USA) in order to remove the 3′-UTR and polyadenylation signal of simian virus 40 from the original pEGFP-C1 plasmid and to replace it with those 3′-UTRs of the RAGE gene. The sequence of all constructs was verified by automated DNA sequencing.

Cell cultures and DNA transfection assays. Wild type Chinese hamster ovary (CHO-k1) and NRK (normal rat kidney) cells were grown in Dulbecco's modified Eagle's medium (Gifco Life Technologies, Barcelona, Spain) supplemented with 10% (v/v) fetal bovine serum (5% v/v for NRK cells), 2 mM glutamine plus 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma, St. Louis, MO, USA) in an atmosphere of air/CO_2 (95:5). NRK cells were also supplemented with non-essential aminoacids. For transfection experiments cells were used at 80–90% confluence. Transfection was performed using 4 μl Lipofectamine 2000 (Gifco Life Technologies) and 1.6 μg DNA/1×10^5 cells in 1 ml of culture medium, according to the manufacturer's instructions. RNA stability experiments were initiated by adding actinomycin D (1–5 μg/ml) to the growth medium.

Fluorescence microscopy. After transfection, cells were analyzed by fluorescence microscopy using an Olympus IX70 microscope equipped with a WB excitation filter. Digital image acquisition and processing were performed with an Olympus DP10 camera and the NIH image software package, respectively. At least 10 different cellular fields were examined in each plate at 100× or 200× magnification. Image acquisition within the same cell type studied was performed under constant conditions in every field examined and for each of the transfected plasmids. Fluorescence images were quantified by measurement of the relative fluorescence intensity/cell.

Alternatively, fluorescence intensity was assayed using a Shimadzu RF-5301PC spectrofluorophotometer. Transfected cells were scraped and the total amount of fluorescence in the cell extracts was measured at λ_{EX} 488 and λ_{EM} 507. Results were normalized using the total amount of protein of the cell extracts [17].

Table 1
Oligonucleotides used in this study

Oligonucleotide	Orientation	Sequence
*Not*I-oligo(dT)	Antisense	5′-AACTGGAAGAATTCGCGGCCGCAGGAAT$_{18}$-3′
RAGE-REF	Sense	5′-GGGGCCATCCTGTGGCG-3′
RAGE-COOH	Antisense	5′-AAGGTCCCCCTGCACCATTC-3′
Rage-31F	Sense	5′-AGA TCT *TAA* GAG CGC CCA GG-3′
Rage-32R	Antisense	5′-ACG CGT CAG ACA ACA AGC CGG-3′
Rage-33F	Sense	5′-AGA TCT *TAA* ACA CCT GAC ACA TGT TG-3′
Rage-34R	Antisense	5′-ACG CGT AAG ATT TCT TGA TTT GGT G-3′
GFP-C	Sense	5′-CATGGTCCTGCTGGAGTTCGTG-3′
RAGE-R1	Antisense	5′-AGCATGGATCATGTGGGCTCTG-3′
RAGE-R2	Antisense	5′-TCTTTCCACCCCTCACTAC-3′

Introduced *Bgl*II and *Mlu*I restriction endonuclease sites are underlined. Stop signals are in italics.

Results

Cloning and sequencing of the 3′-flanking region of the rat RAGE gene

A rat genomic library was screened by hybridization with a probe corresponding to a fragment of the rat RAGE gene [12]. DNA inserts from positive bacteriophages were subcloned into plasmid pUC18. A positive clone containing a 1.7 kb fragment of the rat RAGE gene corresponding to the 3′-flanking region was identified by sequencing. The DNA sequence corresponding to the 3′ end of the gene, including the translation stop codon, is shown in Fig. 1A.

Since a cDNA sequence of the rat RAGE has been previously described (GenBank Accession No. L33413) further analyses were allowed. The comparison with this sequence permitted the identification of a polyadenylation sequence, AAACA, which would match the location of the poly-A tail in the previously described sequence. Additionally, we have found two alternative canonical polyadenylation sequences, AATAAA and ATTAAA, which could produce alternative transcripts of the gene. A computer analysis [18] of the 550 bp complete 3′ end sequence also predicts the existence of a 49 bp ARE sequence. This region contains a single ATTTA motif in an adenine and timine rich environment, characteristic of class I ARE sequences [19], although this sequence would only be present in transcripts generated by the use of the putative new polyadenylation sequences.

Heterogeneity in the 3′ ends of rat RAGE cDNAs

To address the use of the new polyadenylation signals in the processing of the rat RAGE mRNA, a RACE reaction was carried out (Fig. 1B). For this purpose, total lung and kidney RNA were obtained and

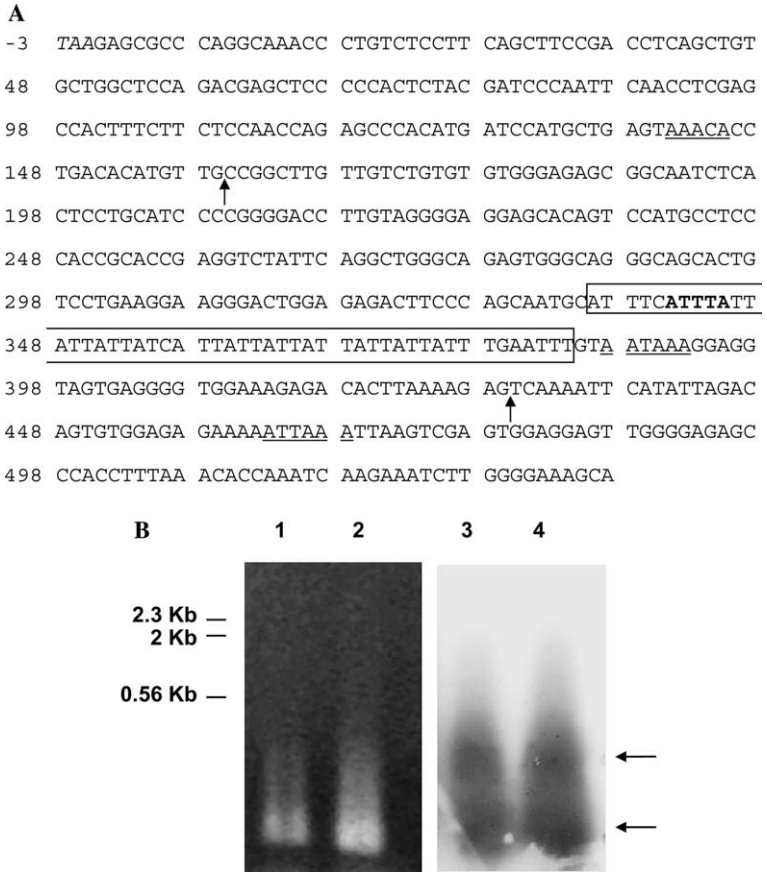

Fig. 1. Sequence of the rat RAGE gene 3′ end. (A) The sequence corresponding to the 3′ end of the gene is depicted. The initial TAA nucleotide sequence (−3 to −1) corresponds to the translation termination codon. Putative polyadenylation sequences are underlined. The positions of the polyadenylation tails are indicated by arrows. The ARE sequence is boxed and a sequence corresponding to an ATTTA pentamer is in bold. (B) Identification of transcripts of the rat RAGE gene by RACE. Total kidney and lung RNA were obtained and retro-transcribed to cDNA using a *Not*I-oligo(dT) primer as described in Experimental. PCR amplification reactions were carried out using the same *Not*I-oligo(dT) primer and a Rage-REF primer. The amplified products were electrophoresed on agarose (lanes 1 and 2) and the amplification products corresponding to the rat RAGE cDNAs were identified by Southern blot (lanes 3 and 4) using a digoxigenin-labelled oligonucleotide RAGE-COOH corresponding to the last 20 nucleotides of the rat coding sequence of the gene. Lanes 1 and 3, kidney samples; lanes 2 and 4 lung samples. Arrows mark bands that were isolated, cloned, and sequenced.

retro-transcribed to cDNA using a *Not*I-oligo(dT) primer as described in the Experimental. PCR amplifications were carried out using the same *Not*I-oligo(dT) primer and a Rage-REF primer that hybridizes 138 bases before the stop codon in the cDNA. The amplified products were electrophoresed on agarose and those corresponding to the rat RAGE cDNA were identified by Southern blot using a digoxigenin-labelled oligonucleotide probe corresponding to the last 20 nucleotides of the rat RAGE coding sequence. Using this technique, two amplification products were clearly identified, eluted, cloned, and sequenced. Similar results were obtained in brain (results not shown).

The analysis of the transcripts allowed us to clearly establish the use of two polyadenylation signals on the rat: One corresponding to the previously described transcript end and a second one generated by the use of the AATAAA polyadenylation signal. The use of the second polyadenylation signal produced a transcript containing the ARE sequence. The relative position of the poli-A tails identified by RACE is marked in Fig. 1A.

Reporter gene expression of RAGE 3′-UTRs in cell cultures

To study the effect of the rat RAGE 3′-UTRs on gene expression, constructs were designed (Fig. 2A), in which a reporter gene (GFP) under transcriptional control of the immediate early promoter of human cytomegalovirus (CMV) was followed by the RAGE 3′-UTRs. We have designed the GFP constructs in such a way that all the plasmids code for the same GFP and the polyadenylation signals for the processing of the transcript are provided only by the cloned 3′-UTR fragments. Construct GFP + 3′-UTR expressed the complete 3′-UTR of the rat gene including the two polyadenylation sequences described in this work. Construct GFPΔARE corresponded to the transcript generated by the use of the first polyadenylation sequence. Consequently, the ARE sequence was not included. Finally, construct GFP + ARE lacked the first polyadenylation signal and only the fragment containing the ARE sequence and the second polyadenylation signal of the 3′ end of the gene was expressed.

To confirm the use of the AATAAA polyadenylation sequence, cultures of the CHO-k1 cell line were transiently transfected with the constructs and the generated transcripts were analyzed by RT-PCR. Five micrograms of total RNA was retro-transcribed using a *Not*I-oligo(dT) primer and amplified by PCR using a GFP specific primer corresponding to the 3′ end of the GFP coding sequence, and two primers that hybridize in the RAGE 3′-UTR. Amplified products were analyzed by agarose electrophoresis. As shown in Fig. 2B, construct GFPΔARE produced a unique amplification product corresponding with a transcript generated by the use of the first polyadenylation signal. Obviously, no amplification was detected using the set of primers designed to amplify the transcripts generated by the second polyadenylation signal. On the other hand, construct GFP + ARE generated an amplification product of the expected size using the oligonucleotide that hybridizes close to the second polyadenylation sequence and no

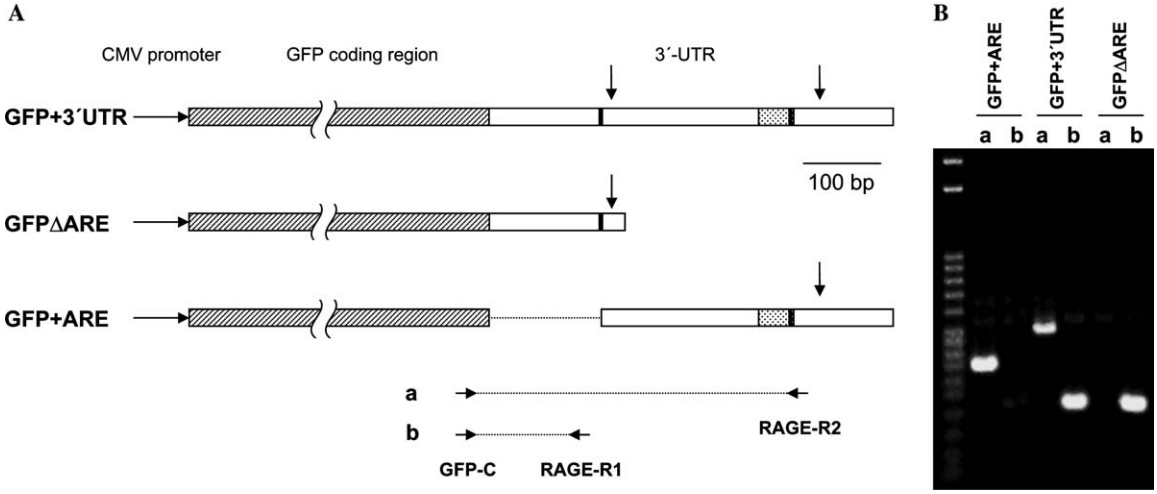

Fig. 2. Plasmids used in transfection experiments. (A) Schematic representation of the plasmids. The CMV promoter (CMV), GFP-coding region, and the different 3′-UTRs are indicated. The polyadenylation signals are in black boxes and the ARE sequence is indicated by a dotted box. The arrows mark the position of the poly-A tails. (B) Ethidium bromide-stained agarose gel of RT-PCR products corresponding to GFP mRNAs obtained from CHO-k1 transfected cells with the GFP constructs. Total RNA from transfected cells was retro-transcripted to cDNA using a *Not*I-oligo(dT) primer. The relative position of the oligonucleotides used in the PCRs is depicted in (A). The combination of oligonucleotides GFP-C and RAGE-R2 is named a; the combination of oligonucleotides GFP-C and RAGE-R1 is named b. In the agarose gel, the left lane is a 100-bp molecular mass marker.

amplification was detected when the first set of oligonucleotides was used as primers. Finally, in the construct GFP + 3′-UTR, amplifications were achieved using either set of primers.

Role of the 3′-UTRs of rat RAGE mRNA on gene expression

To address the influence of the different 3′-UTRs of rat RAGE mRNA on gene expression, we studied GFP protein expression in CHO-k1 and NRK cells. After transfection, stably transfected cells were selected in normal growth medium containing 500 μg/ml geneticin. Resistant colonies were isolated and subsequently used. In the isolated cells, the intensity of fluorescence due to the expression of GFP was assayed by fluorescence microscopy as described in Experimental. As shown in Fig. 3, the use of constructs containing the different 3′-UTRs of the rat RAGE cDNAs has a strong influence on the gene expression. In CHO-k1 cells, construct GFPΔ ARE, which lacks the ARE sequence, has the higher expression levels, while the construct GFP + ARE has an expression approximately 5-fold lower. As expected, construct GFP + 3′-UTR, which generates the two kinds of transcripts, has an intermediate expression level compared to those of the other two constructs. Similar results were obtained when transient transfections of CHO-k1 were analyzed (data not shown).

The effects of the different constructions on GFP expression were also analyzed in NRK cells. In this cell line, transient transfections showed similar behavior when compared to the CHO-k1 cells. GFPΔARE construct produces the highest relative expression levels (100 ± 3.7) compared to the GFP + ARE construct (13.1 ± 3.1, $P < 0.001$ vs GFPΔARE), while the GFP + 3′-UTR construct has an intermediate level of expression (36.6 ± 5.2, $P < 0.001$ vs GFPΔARE and $P < 0.005$ vs GFP + ARE).

Effects of the 3′-UTRs of rat RAGE cDNA on mRNA levels

Since the 3′-UTRs of the rat RAGE mRNA have a significant effect on gene expression, measured as GFP levels, we have studied the influence of these 3′-UTRs on the amount of mRNA corresponding to each construction in CHO-k1 isolated clones. To do this, we have carried out a Northern blot using a labelled fragment of the coding sequence of the GFP as a probe.

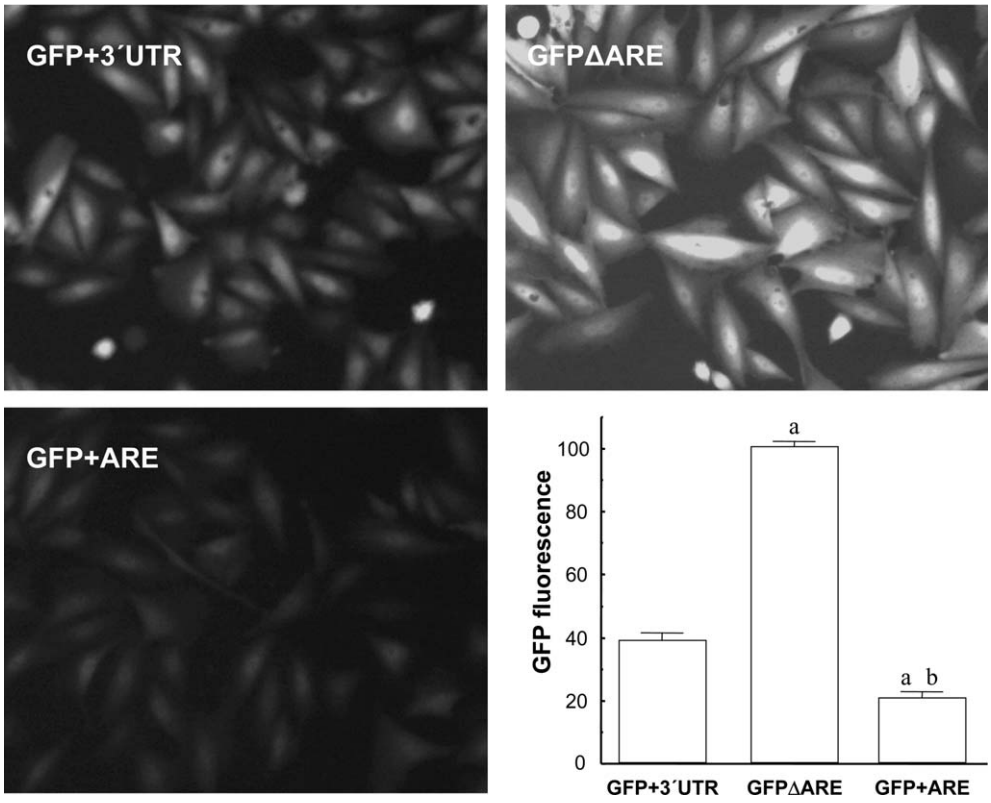

Fig. 3. GFP expression of the different 3′-UTR constructs in CHO-K1 cells. After transfection, stably transfected cells were selected in normal growth medium containing 500 μg/ml geneticin. Resistant colonies were isolated and subsequently used. In the isolated cells, the intensity of fluorescence due to the expression of GFP was assayed by fluorescence microscopy. At least 10 different fields were photographed at 20× magnification with the same exposure. Fields from representative experiments are shown. A histogram corresponding to the relative fluorescence intensity/cell of each construct is shown. Results are means ± SEM from at least 10 different fields for each construct. [a] $P < 0.001$ vs GFP+3′-UTR; [b] $P < 0.001$ vs GFPΔARE.

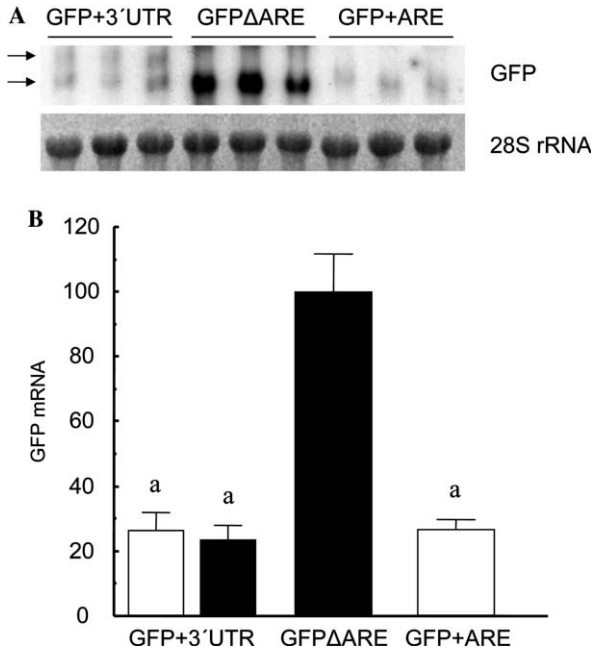

Fig. 4. Amount of GFP mRNA in CHO-K1 transfected cells. Ten micrograms of total RNA samples were analyzed by Northern blot as described in Experimental using a GFP probe. (A) Representative Northern blot showing GFP mRNAs in GFP + ARE, GFPΔARE, and GFP + 3′-UTR isolated clones. (B) Densitometric quantification of GFP mRNA abundance in the isolated clones. GFP signal density from GFPΔARE clones was assigned a value of 100%. Results were normalized to the 28S rRNA signal. Values are means ± SEM ($n = 5$). [a] $P < 0.005$ compared to GFPΔARE.

The processing of the RNA in the transfected cells is shown in Fig. 4. Cells transfected with GFPΔARE and GFP + ARE generated unique transcripts of the expected size, while cells transfected with the GFP + 3′-UTR construct expressed two transcripts due to the use of the two polyadenylation signals present in the 3′-cloned region. The intensity of the transcripts in the transfected cells varied considerably. The highest mRNA levels correspond to the GFPΔARE while the presence of the ARE sequence in GFP + ARE produced at least a 5-fold decrease in the amount of mRNA. Cells transfected with the GFP + 3′-UTR construct expressed two transcripts at intermediate levels compared to the other two constructs.

Effect of the ARE sequence of rat RAGE 3′-UTR on mRNA stability

To study the influence of the ARE sequence on mRNA stability we have used the GFP + ARE and GFPΔARE constructs. In CHO-k1 cells, the effects of these constructs on fluorescence intensity were assayed upon the addition of actinomycin D (ActD). For this purpose, cells were incubated with ActD, scraped and the intensity of fluorescence was measured with a fluorimeter. Results were normalized using the total amount of protein of the cell extracts. Previously, the effects of increasing amounts of ActD on fluorescence intensity were studied (data not shown) and the amount of 1 μg/ml of medium was selected since it was the lowest amount of ActD that has the highest effect on fluorescent intensity.

The time course of the effects of ActD on the fluorescence intensity of cells transfected with GFP + ARE and GFPΔARE constructs is shown in Fig. 5A. Compared to the GFPΔARE construct, the presence of the ARE sequence on the GFP+ARE has a significant effect on the fluorescence intensity of the cells treated with ActD. This result has been confirmed by Western blot, using an anti-GFP antibody. As shown in Fig. 5B, samples corresponding to CHO-k1 transfected with GFP + ARE construct have lower GFP protein levels,

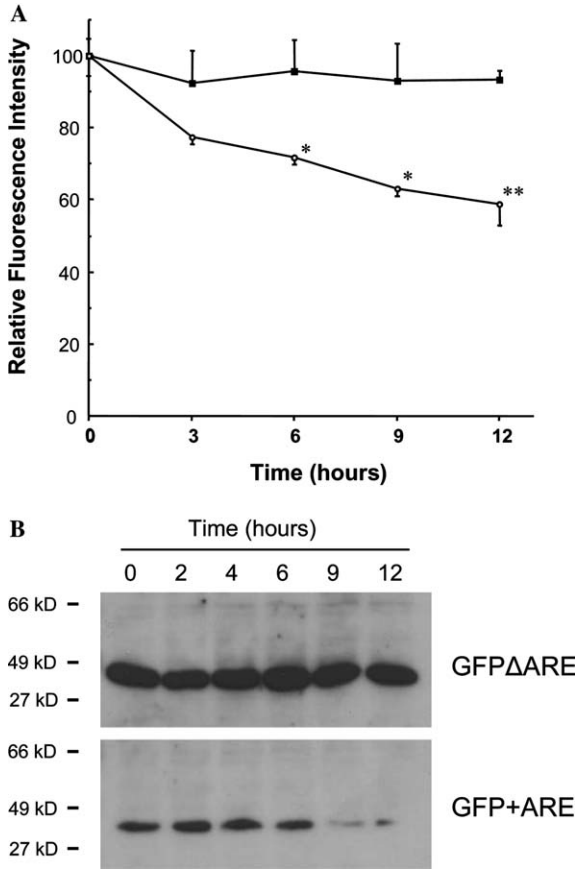

Fig. 5. The RAGE 3′-UTR sequence regulates the stability of GFP transcripts. GFP reporter constructs were transfected into CHO-k1 cells. The stability of GFP mRNA was determined by exposing cultures to actinomycin D (1 μg/ml). Samples were collected at various time points and fluorescence and GFP protein were measured. Results were normalized with the amount of proteins of each sample. (A) Fluorescence intensity of cells transfected with construct GFPΔARE (closed squares) and GFP + ARE (open circles). Results are normalized to the time 0 value for each construct. Values are means ± SEM ($n = 4$). *$P < 0.05$ and **$P < 0.005$ compared to GFPΔARE. (B) Western blot of extracts of CHO-k1 transfected cells using a monoclonal anti-GFP antibody (Sigma).

which rapidly decrease with time, while cells transfected with the GFPΔARE plasmid maintain constant expression levels at the assayed times.

Discussion

The RAGE is an integral protein, which together with a lactoferrin-like polypeptide, forms a membrane complex able to recognize AGEs [20,21]. Binding of ligands to RAGE does not accelerate their clearance or degradation, but triggers a cellular activation mediated by receptor-dependent signalling [4].

Since RAGE is involved in several pathologies, such as diabetes mellitus, great attention has been paid to the study of the regulation of the RAGE expression. High AGEs levels induce receptor overexpression through an NF-κB and SP1 stimulation of the human RAGE promoter [10,22]. Variability of the mRNA generated by alternative splicing has also been proposed as a mechanism to regulate RAGE expression in humans [11] and rats [12].

Here, we have studied a new mechanism of regulation of the rat RAGE expression. We show that the 3′-region of the rat RAGE gene plays an important role in the regulation of the receptor expression. In this region, several polyadenylation signals, as well as an adenylate and uridylate-rich (AU-rich) element (ARE), are present.

We demonstrate two functional polyadenylation signals in the rat gene. In the RACE experiments we have obtained two amplification products in kidney, lung, and brain. These transcripts are generated by the use of proximal and distal polyadenylation sequences. The position of the first polyA tail points to the use of an AAACA sequence. This is an uncommon polyadenylation sequence. However, the transcript that we identify matches that previously described for the rat mRNA and no other possible polyadenylation sequences are located nearby. The second functional polyadenylation signal, AATAAA, is located downstream and has not been described previously. This is probably due to the presence of a strong secondary structure in this transcript, making RACE amplifications difficult. Therefore, the alternate use of this second polyadenylation sequence will generate in the rat tissues mRNA isoforms that include an ARE sequence in their 3′-UTR.

The turnover of mRNA is a major control point in gene expression. The degradation of mRNA is a tightly regulated process, dependent on specific *cis*-acting sequences and *trans*-acting factors. A specific *cis*-acting element controlling the half-life of mRNA is ARE, found in the 3′-UTRs of many unstable mammalian mRNAs. ARE-containing mRNAs encode a broad group of functionally diverse proteins [23] and are the most widespread determinants of RNA instability. Moreover, ARE-directed mRNA degradation is influenced by many exogenous factors, including phorbol esters, calcium ionophores, cytokines, and transcription inhibitors [19].

AREs were first found in the 3′-UTR of several cytokine and oncoprotein genes [24] and are composed by a variable number of copies of the AUUUA pentamer or UUAUUUAUU nonamer. Based on the number and distribution of the AUUUA pentamer they contain, AREs have been classified into three categories [19]. Class I AREs are characterized by the presence of one to three pentamers, distributed within a large part of the 3′-UTR coupled with a nearby U-rich region. All three classes confer mRNA instability in cultured cells through different mechanisms, all of which imply mRNA deadenylation [25]. However, other functions have been adscribed to AREs, such as mRNA stabilization or regulation of mRNA translation efficiency [19,26].

To dilucidate the molecular mechanisms that regulate the expression of RAGE protein, we studied how the ARE-containing 3′-UTR of the RAGE message mediates post-transcriptional control of gene expression. Consequently, we have studied the expression of the green fluorescent protein (GFP) derived from mRNA chimaeras containing the 3′-UTRs of the above-described transcripts.

To carry out this study we have used two cell lines: CHO-k1, an epithelial cell line widely used for the expression of eukaryotic proteins, and (normal rat kidney) (NRK) which constitutively expresses RAGE and has been used as a model for the study of the long term complications of diabetes as a result of protein glycation in kidney [27,28]. Fluorescence intensity measurements in transfected cells indicate that rat RAGE ARE has a strong effect on protein expression since there is a 5-fold difference in the fluorescence of the CHO-k1 and NRK cells transfected with construct containing or lacking the ARE sequence.

Since AREs could regulate gene expression by mRNA instabilization or translational control, we have measured the amount of mRNA for the GFP in the CHO-k1 transfected cells. A Northern blot of total RNA from transfected cells shows that the ARE sequence also has a strong effect on mRNA relative amounts. The construct lacking the ARE has nearly 5-fold higher mRNA levels compared to the GFP + ARE. Results from construct GFP + 3′-UTR are interesting, since it is possible to distinguish in the Northern blot the generation of two transcripts confirming the use of the two polyadenylation sequences in the CHO-k1 cell line. The ratio fluorescence/mRNA is fairly constant for all the constructs transfected, indicating that the rat RAGE ARE exerts its effects through mRNA instabilization rather than through modulation of the translation efficiency.

To confirm the effect of the ARE on mRNA stability, we have determined fluorescence and amount of GFP protein in transfected cells treated with Act D. Since GFP has been clearly established as a useful reporter gene [29], GFP levels should reflect relative amount of mRNA. We have assayed two constructs, GFPΔARE and GFP + ARE, which differ in the presence of the ARE sequence. On blocking mRNA synthesis with Act D, GFPΔARE produces higher and more stable expression levels of GFP while GFP + ARE transfected cells have lower and faster decreasing levels of the protein. Thus, it is highly possible that the rat RAGE ARE has an mRNA destabilization function.

In conclusion, the results presented here demonstrate that the 3′-UTR of RAGE could influence mRNA stability. Analysis of the sequence within the 3′-UTR of the rat RAGE gene identified a 49 bp nucleotide AU-rich element probably responsible for rapid mRNA turnover. The use of alternative polyadenylation sequences that generate transcripts which differ in the presence or absence of this AU-rich sequence is a major regulation mechanism of the RAGE gene in rats.

Acknowledgments

The Charon 4A rat genomic library was kindly provided by Professor Richard C. Scarpulla, Northwestern University Medical School (Chicago, IL). This work was supported by grant PB1998-1292 from Ministerio de Ciencia y Tecnología (MCYT), Spain. J.J. Caballero was supported by a predoctoral fellowship from the MCYT, Spain. N. Sevillano is the recipient of a predoctoral fellowship from the Fundación Ramón Areces, Spain. We thank Robert Abrahams, BSc, for checking the manuscript.

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