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Molecular Cloning of a Bovine Renal G-Protein Coupled Receptor Gene (bRGR): Regulation of bRGR mRNA Levels by Amino Acid Availability

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A cDNA of 3.2kb, encoding a putative G protein-coupled receptor and hence called bRGR1, has been isolated from a cDNA library generated from the bovine renal epithelial cell line NBL-1. This cDNA consisted of 41 base pairs of 5′-untranslated sequence, an open reading frame of 1083 base pairs, and a 2.07kb fragment of 3′-untranslated sequence that includes a poly(dA) tail. The coding sequence predicts a protein of 361 residues. The ligand of the bRGR1 protein may be of low molecular weight, as deduced from the analysis of the predicted primary structure of the receptor protein and the comparison with other subtypes of the G protein-coupled receptor family. The amounts of bRGR1 mRNA significantly increase when NBL-1 cells are cultured in an amino acid-depleted medium. This effect can not be caused by a decrease in protein synthesis because cycloheximide did not mimic the increase in bRGR1 mRNA levels triggered by amino acid starvation. These data suggest that bRGR1 may be an amino acid-regulated gene.

Control of gene expression by nutrients is a common regulatory process in prokaryotes and lower eukaryotes. In contrast, mammalian cells are considered poorly responsive to nutritional stress. Nevertheless, increasing evidence supports the view that some genes may be modulated by the availability of metabolites (e.g. glucose, amino acids, fatty acids, cholesterol) (1-4). Several amino acid-regulated genes have been identified so far in mammalian cells (for review see 4,5). They include ribosomal proteins (6,7), transcription factors (8,9), enzymes (10), and plasma membrane proteins (11). Regulation of gene expression by amino acid availability may be understood as a key regulatory step in the nutritional control of cell physiology (4).

We have been using the bovine renal epithelial cell line NBL-1 as a model system to analyze the role of amino acid availability and anisotony on the activity and expression of selected plasma membrane proteins, such as the amino acid transport systems A (12,13) and Xag- (14) and the ion translocators Na+,K+,Cl− cotransporter (15) and Na+,K+-ATPase (16,17). Changes in substrate availability and medium osmolarity may have physiological relevance in absorptive epithelia. These four transport systems are modulated by medium tonicity (12-15). Systems A, Xag- and the sodium pump also seem to respond to amino acid starvation in this and other cell types (17-19). Nevertheless, the Na+,K+-ATPase α1 subunit gene is regulated by amino acid availability but only at the mRNA level, because the increase in α1 subunit mRNA following amino acid starvation does not increase the activity of the sodium pump (19). In an attempt to determine whether this type of "two-step" regulatory process occurs in the Na+,K+,Cl− cotransporter, we tried to isolate, by PCR-homology cloning, the cDNA encoding the bovine Na+,K+,Cl− cotransporter. We isolated a cDNA that hybridized at high stringency with a single mRNA species that appeared to be regulated by amino acid availability. Nevertheless, although it contained the sequence of the two primers used for the PCR reaction, this fragment showed negligible homology with the cDNAs that presumably encode the different isoforms of Na+,K+,Cl− cotransporters (NKCC1 and NKCC2). Indeed, we isolated a cDNA bearing high homology with members of the G protein-coupled receptors (GPR) (20), and, in particular, with an ovarian cancer G protein-coupled receptor (OGR1) whose ligand is unknown (21).

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The G protein-coupled receptor superfamily includes a large number of plasma membrane proteins that accept as ligands low molecular-weight peptides, neurotransmitters and other regulatory factors or sensory stimuli (see 20 for review). They all share some structural features. Analysis of the primary structure of GPRs predicts seven transmembrane spanning domains and, in most cases, shows an amino-terminal extracellular domain that may be glycosylated. The carboxyl-terminal cytoplasmic tail, along with other cytoplasmic loops, appears to be involved in coupling to G proteins. In general, ligand binding specificity is determined differentially depending on the GPR subtype.

This study reports the cloning of a putative renal bovine GPR family member that appears to be regulated by amino acid availability.

MATERIALS AND METHODS

Cell line and culture conditions. Bovine epithelial cells NBL-1 (MDBK) were originally obtained from Flow Laboratories (McLean, VA, USA) and routinely cultured as previously described (22). Cells used for Northern blot analysis were grown in 100 mm-diameter Petri dishes and fed every 2 days until they reached confluence, when they were used for experiments. To study the effect of amino acid starvation, cells were incubated for 3, 6, 9 and 12 hours in a medium known to stimulate amino acid-regulated membrane transporters in this cell line, like system A (12,21). The medium consisted of the inorganic salts of Ham's F-12 at pH 7.4 (0.3 mM $CaCl_2$, 10 nM $CuSO_4$, 3 μM $FeSO_4$, 3 mM KCl, 0.6 mM $MgCl_2$, 130 mM NaCl, 14 mM $NaHCO_3$, 1 mM Na_2HPO_4 and 3 μM $ZnSO_4$) supplemented with 10 mM glucose, 0.1% w/v BSA, 0.5 % Phenol Red and antibiotics as in the control. To determine whether the putative effects of amino acid starvation on bRGR1 mRNA levels were due to partial inhibition of protein synthesis, NBL-1 cells were also cultured for 12h in the presence of increasing concentrations of cycloheximide.

Total RNA extraction, polyA+ RNA purification and cDNA synthesis. Total RNA was extracted either from tissues or from confluent cell cultures using the guanidium thiocyanate method (23). PolyA+ RNA from NBL-1 cells was purified from total RNA with the PolyA-tract mRNA Isolation System III (Promega, Madison, WI) and cDNA was synthesized from it by the Reverse Transcription System using an oligo-dT primer (Promega, Madison, WI). The cDNA/RNA was treated with RNase H (Boehringer, Mannheim, Germany), purified with Clean-Up columns (Promega, Madison, WI) and used for PCR experiments.

PCR conditions, cDNA library screening and sequencing. Primers for the PCR were 5′-CTGGGGAGTCATGCTCTT-3′ (NKCC2F1) and 5′-AGTGGCTGCTGGGAAGAA-3′ (NKCC2R1) and were originally designed to be specific for the Na+,K+,Cl− cotransporter NKCC2 (24). The 50 μl PCR reaction mixture consisted of 5 μl of NBL-1 cDNA, 400 nM of each primer, 1.5 mM Mg_2Cl, 200 μM each of dATP, dCTP, dGTP and dTTP, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100 and 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI). The reaction was started by incubating the solution at 94°C for 5 min before the addition of the *Taq* DNA polymerase, cooling it to 80°C for 3 minutes and adding the enzyme. Afterwards PCR conditions were: 5 cycles of 1 min at 94°C, 1 min at 42°C and 3 min at 72°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C and 3 min at 72°C. Finally, an incubation of 10 min at 72°C was performed, followed by a treatment with Klenow fragment at 37°C for 1 h in order to blunt end the DNA products. The mixture was run in a 1% agarose gel and a single positive clear band of approximately 650 bp was purified using Gene-Clean (Bio 101 Inc, Vista, CA). The cDNA was subcloned into the EcoRV site of a Bluescript-KS vector and sequenced using the Auto Read Sequencing Kit and an A.L.F. DNA Sequencer (Pharmacia, Uppsala, Sweden).

A NBL-1 cDNA library was purchased from Clontech (Palo Alto, CA). It had been constructed using random and oligo-dT primers and cloning the cDNAs into the EcoRI site of the λgt10 vector. To screen the library up to 10^6 primary plaques grown in Petri dishes were transferred into nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), submerged for 30 seconds in 500 mM NaOH and 1.5 M NaCl, neutralized for 5 min in 1 M Tris, pH 8 and 1.5 M NaCl and fixed by UV radiation. Filters were prehybridized for 6 h at 42°C in 50% formamide, 2.5 × Denhart's solution, 4 × SSPE, 0.1 mg/ml sonicated salmon sperm DNA, 0.2% SDS, and 50 mM Na_2HPO_4 (pH 7.5). Hybridization was performed for 24 hours at 42°C in the same solution with 10^5 cpm/ml of the corresponding ^{32}P-labelled cDNA probe, synthesized by random priming of either the original PCR fragment (first round of screening) or the cDNA isolated from the first positive clone (second round of screening). Filters were washed once for 30 min at 65°C in 1 × SSC and 0.1% SDS, and once more in 0.2 × SSC and 0.1% SDS before autoradiography. Positive clones were isolated, amplified, subcloned into the EcoRI site of Bluescript-KS and sequenced as previously stated.

Northern blot analysis. Total RNA was separated on a 1% agarose gel in denaturing conditions. The gel was submerged for 5 min in 50 mM NaOH and 1.5 M NaCl and neutralized for 30 min in 1 M Tris, pH 6.8 and 1.5 M NaCl before an overnight transfer to nylon membranes (Hybond N, Amersham, U.K.) in 20×SSC (3M NaCl, 300 mM sodium citrate, pH 7.0). RNA was cross-linked to the filter under UV exposure. The blot was prehybridized for 6 h at 65°C in 20% formamide, 10% dextran sulfate, 2.5 × Denhart's solution (1g/l bovine serum albumin, 1g/l polyvinylpyrrolidone 40, 1g/l Ficoll), 4 × SSPE (600 mM NaCl, 40 mM NaH_2PO_4, 4 mM EDTA, pH 7.4), 0.1 mg/ml sonicated salmon sperm DNA, 0.2 mg/ml yeast RNA and 5% SDS. Blots were hybridized for 24 hours at 65°C in the same solution with 10^6 cpm/ml of a random primer ^{32}P-labelled cDNA probe. The bRGR1 cDNA used as a probe corresponds to the coding region of the nucleotide sequence between BamHI sites. The ribosomal protein L32 probe (25) was used to normalize the data, as previously reported (26). Filters were washed once for 30 min at 65°C in 1 × SSC and 1% SDS, and once more in 0.2 × SSC and 1% SDS before autoradiography. Films were scanned and the densitometric analyses were performed by means of the Phoretix software (Phoretix International Ltd, UK).

RESULTS

Cloning of the bovine renal G protein-coupled receptor, or bRGR1. The screening of a cDNA library generated from the bovine renal epithelial cell line NBL-1, using the PCR fragment obtained as indicated above, resulted in the first round in the isolation of a single positive clone (121) (Figure 1), which was thereafter used as a probe for a second round of screening. This time three positive cDNA clones were isolated. The four clones were sequenced and the longest (207), 3.2 kb, contained the totality of the coding sequence (Figure 1). The complete sequence of this clone (Figure 2) consists of 41 base pairs of 5′-untranslated sequence, an open reading frame of 1083 base pairs, and a 2.07 kb fragment of 3′-untranslated sequence that includes a polyadenylation signal and a poly(dA) tail. bRGR1 encodes a predicted protein of 361 residues (Figure 2)

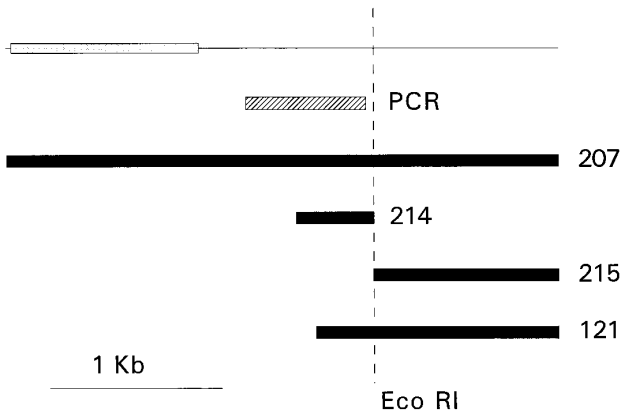

FIG. 1. cDNAs encoding the bRGR1 protein. The longest clone isolated from the NBL-1 cDNA library (207) consisted of an open reading frame, represented as a spotted bar, and a short 5′ and a long 3′ untranslated regions, shown by flanking lines on top of the figure. The original PCR product (see results) as well as other independent clones isolated from the cDNA library (214, 215 and 121) are also shown (black bars).

that shows an amino acid sequence identity of 89% with the OGR1 protein product. Indeed, the predicted amino acid sequence of the cytosolic loops of both protein products is almost identical. Identity is lower (65%) at the carboxy-terminal tail (from amino acids 321 to 361), where the sequence GAQG present in the predicted OGR1 gene product is lacking in the deduced protein encoded by bRGR1. Moreover, only three instead of four putative PKC phosphorylation sites are present in the bRGR1 carboxy-terminal tail. Other potential PKC phosphorylation sites are present in the second and third intracellular loops. bRGR1 shows several classical features of the G protein-coupled receptors: seven putative transmembrane spanning domains, as deduced from the hydrophobicity plots (Kyte-Doolittle), an amino-terminal extracellular fragment containing two N-linked glycosylation sites and a DRY motif followed by five amino acid residues and a proline in the second intracellular loop. Other GPR family members showed significantly less homology to bRGR1 than OGR1 (50% for the human orphan receptor GPR4 and 20% for the bovine type I angiotensin II receptor AT1). The 3′-untranslated fragment of 2.07 kb did not show significant homology with any of the sequences at the GenBank. Some sequence variability was found in the 3′-untranslated region between nucleotides 1937 and 1978. The PCR and clone 214 sequences showed a three-base-pair substitution and a deletion of a four-base-pair fragment when compared to clones 121 and 207 (Figure 3).

Expression of the bRGR1 gene. In Northern blot analysis of NBL-1 cells, the bRGR1 cDNA probe hybridized with a single mRNA species of approximately 3.5kb. Analysis of a set of rat tissue samples in which hybridization with the probe was at low stringency (65°C, 5% formamide in the hybridization buffer) gave a band of similar molecular weight in spleen, brain and kidney (not shown). In an attempt to determine whether the bRGR1 gene is under the control of stimuli that may have physiological relevance, we monitored the changes in bRGR1 mRNA in NBL-1 cells in different culture conditions, essentially anisotonic (hypo-osmotic and hyperosmotic) and amino acid depleted media. Neither anisotonic condition triggered any significant change in the mRNA amounts of the bRGR1 gene. Nevertheless, incubation of NBL-1 cells in an amino acid-free medium resulted in an increase in the bRGR1 mRNA, which was significant after 9 h of culture (Figure 4). Amino acid starvation did not modify the L32 mRNA levels. To rule out the possibility that the increase in bRGR1 mRNA levels is the consequence of protein synthesis inhibition, we monitored the effects of increasing concentrations of cycloheximide on the amounts of bRGR1 mRNA (Figure 5). In this case the protein synthesis inhibitor cycloheximide induced a dose-dependent decrease in bRGR1 mRNA levels.

DISCUSSION

This contribution describes the cloning of a cDNA that presumably encodes a membrane protein that belongs to the expanding family of G-protein-coupled receptors (GPR). This conclusion is based upon the close homology found between the cloned cDNA, called bRGR1, and the OGR1 gene described recently by others (21). This gene is characterized by its lack of introns. As indicated above, both genes may encode proteins that bear most of the characteristics of the GPR family (20). As for the OGR1 gene product, the bRGR1 protein is an orphan receptor for which the ligand is not known. OGR1 may encode a GPR with a low-molecular-weight ligand, given the alignment of the putative ligand binding regions comprised between the predicted transmembrane spanning regions II (TMII) and VII (TMVII). These regions matched the primary structure of receptors for angiotensin, bradykinin, opioids, purines, adenosine, endothelin and others (21). The identity of TMII to TMVII between OGR1 and bRGR1 is extremely high, which supports the view that the bRGR1 protein is also a low-molecular-weight-ligand receptor and may indeed be the bovine homologue of the human OGR1. Moreover, both the OGR1 and bRGR1 proteins lack the very large amino-terminal extracellular domain (often in excess of 300 amino acid residues) which is characteristic of glycoprotein hormone receptors (20).

As previously indicated, the long 3′ untranslated region of the bRGR1 mRNA does not match any other sequence in the data bank and shows some sequence variability. Although this feature is emphasized here,

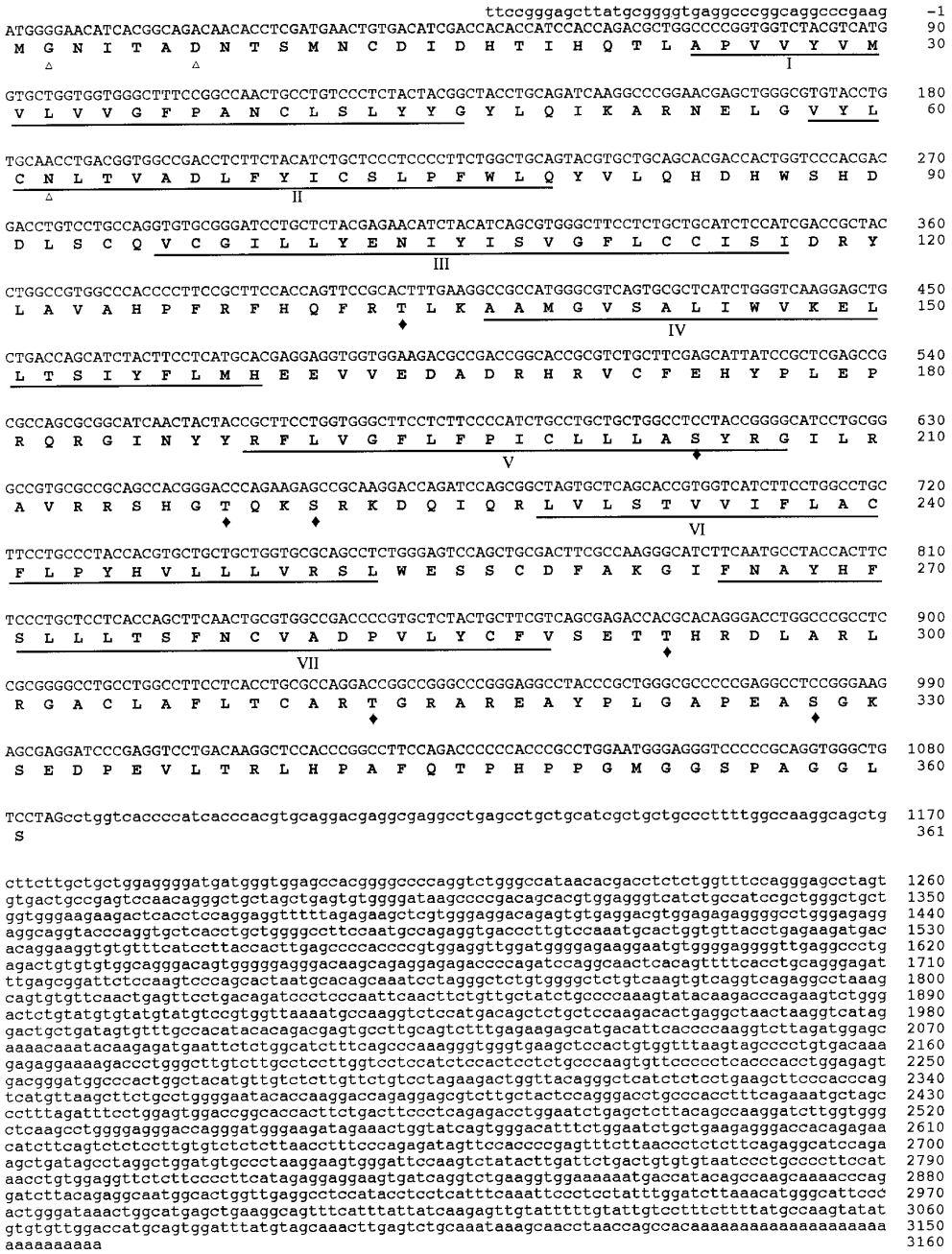

FIG. 2. Sequence of the bRGR1 cDNA. The complete nucleotide sequence of the bRGR1 cDNA is shown. Numbers indicate the nucleotide position. The predicted amino acid sequence of the bRGR1 protein is also shown below the cDNA sequence. Putative transmembrane spanning domains (I-VII) are underlined. Potential N-linked glycosilation sites on the amino acid sequence are indicated (△) as are possible PKC phosphorylation sites (♦).

we do not have an explanation. There may be polymorphisms in this cell line, either as a result of a multiclone origin of the NBL-1 cells or as a result of genetic variability associated with the number of passages.

An interesting finding of this study is that the bRGR1 mRNA amounts are modulated in NBL-1 cells by amino acid availability. The effect is clear and repetitive and results in about a two-fold increase in the mRNA levels. This may not be the result of a transcriptional activation of the bRGR1 gene since a change in mRNA stability cannot be ruled out. Moreover, the increase in bRGR1 mRNA amounts is not the result of the likely inhibition of protein synthesis triggered by amino acid starvation because cycloheximide did not

A

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AST:  EASGKS----EdPEvLTrLHPAFQTPhpPGmGGsPaGgLs   361
OGR1: EASGKSgaqgEePElLTkLHPAFQTPnsPGsGGfPtGrLa   365
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B

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121/207:  TGCCAaGGTcTCCaTGACAGCTCTGCTCCAAGACACTGAGGCTAActaaGGT   2016
214/PCR:  TGCCAgGGTtTCCgTGACAGCTCTGCTCCAAGACACTGAGGCTAA----GGT   2022
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FIG. 3. A) Comparison of the predicted sequence of the C-terminal tail of the bRGR1 and OGR1 proteins. B) Sequence variability in the 3′-untranslated region of the bRGR1 cDNA. Nucleotide and amino acid changes in the sequence are shown in bold lowercase letters. Deletions of nucleotides and amino acids are shown in dashes.

mimic this effect. Thus, bRGR1 may be considered to be an amino acid-regulated gene. Genes that are transcriptionally activated by amino acid starvation do not conform an homogeneous gene family because the specificity of the response seems to be different, as is the time-course of the effects triggered by amino acid availability changes (5,8-10). While most of these genes seem to be early-response genes characterized by rapid increases in their mRNA levels immediately after amino acid depletion, as reported for the ribosomal L17 and S25 mRNAs (6,7), the oncogenes c-jun and c-myc (8), the transcription factor CHOPS (9) and asparagine synthetase (10), the bRGR1 gene should be considered as a late onset gene since mRNA amounts were significantly increased only 9 hours after cells were depleted of amino acids. The physiological relevance of this finding remains to be elucidated as the ligand for the bRGR1 protein is also unknown.

In summary, this study reports the cloning of a bovine renal cDNA that is presumed to encode a member

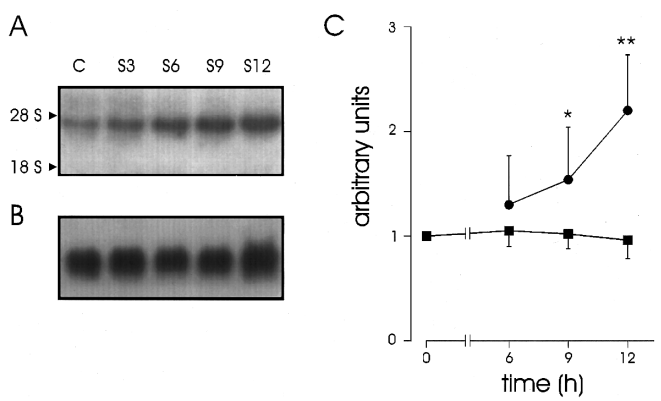

FIG. 4. Changes in the bRGR1 mRNA amounts in amino acid-deprived NBL-1 cells. A) Representative Northern blot analysis of the bRGR1 mRNA after starving NBL-1 cells for different time periods (3, 6, 9 and 12 hours). B) L32 mRNA, here used as a loading control, in the same cells and culture conditions. C) Densitometric measurements of Northern blots from five independent experiments showing the time-course of the increase in bRGR1 mRNA amounts after amino acid starvation of NBL-1 cells.

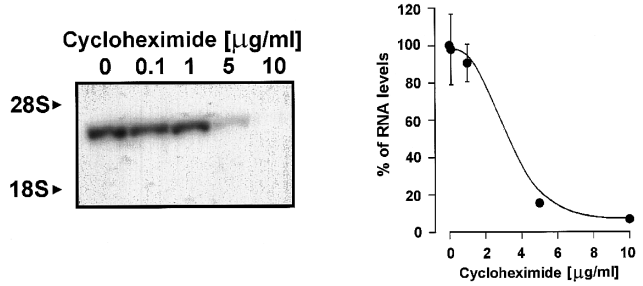

FIG. 5. Effects of cycloheximide on bRGR1 mRNA amounts in NBL-1 cells. NBL-1 cells were incubated for 12h in the presence of increasing concentrations of cycloheximide. Then, total RNA was extracted and used for Northern blot analysis. Left panel: a representative Northern blot. Right panel: mean±S.E.M. of the densytometric analysis of three independent experiments.

of a subtype of G protein-coupled receptors. The bRGR1 mRNA amounts seem to be modulated by amino acid availability. This finding opens the possibility that the expanding family of amino acid-regulated genes incorporates a new type of protein, a plasma membrane receptor.

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