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# The amino acid sequence of a glutamic acid-rich protein from bovine retina as deduced from the cDNA sequence

(visual transduction/cGMP phosphodiesterase/neurofilaments/rod outer segments/repeated sequences)

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ABSTRACT **cDNA clones encoding a glutamic acid-rich protein were isolated from a bovine retina cDNA expression library. The cDNA sequence contained an open reading frame of 1770 base pairs encoding a protein of 590 amino acids (64,509 Da) and untranslated regions of 60 and 490 base pairs at the 5′ and 3′ ends, respectively. The cDNA hybridized to a 2.4-kilobase retinal mRNA. The amino acid sequence derived from the cDNA sequence contains a glutamic acid-rich domain in which 68 of 109 amino acids are glutamic acid. In addition, this domain contains four repeats of a peptide of 11 amino acids and two repeats of a peptide of 26 amino acids. A polyclonal antibody raised against a decapeptide corresponding to the undecapeptide repeat sequence reacted with a protein in an extract of bovine rod outer segments, whose molecular mass, 65 kDa, corresponded to that of the above glutamic acid-rich protein. The retinal glutamic acid-rich protein showed homology with glutamic acid-rich proteins from bovine brain and the C-terminal region of mammalian neurofilaments.**

Several proteins have been shown to be involved in visual transduction in the vertebrate rod cell. Rhodopsin is the photoreceptor; upon absorption of light, it undergoes a structural change that allows it to activate transducin, the GTP-binding protein. Transducin exchanges bound GDP for GTP, and the $\alpha$ subunit of transducin dissociates from rhodopsin and the $\beta$ and $\gamma$ subunits of transducin. The complex of the $\alpha$ subunit of transducin and GTP activates the rod outer segment (ROS) cGMP phosphodiesterase (PDE). The resulting decrease in cGMP levels leads to the closing of cGMP-gated channels in the ROS plasma membrane. Thus, the overall process culminates in hyperpolarization of the rod cell, which generates a neuronal signal. In addition to the proteins implicated in the above steps, rhodopsin kinase is known to be involved in phosphorylation of rhodopsin, and arrestin (a 48-kDa protein) appears to be involved in dark adaptation of rhodopsin. Considering the delicately regulated functions of the visual sensory system, it seems highly likely that additional proteins will be discovered in retina that play specific roles in visual transduction. Indeed, recently, a retina-specific cDNA that encodes a soluble 33-kDa protein has been isolated by means of differential colony hybridization to retina and brain (1). This protein has been shown to form a complex with the $\beta$ and $\gamma$ subunits of transducin (2), suggesting its possible role in visual transduction. These results support the possibility that more proteins that participate in visual transduction remain to be discovered.

During work on identification of the genes for PDE of bovine retinal ROS (3), we isolated a cDNA clone that encoded a protein different from any of the PDE subunits and from any of the other known proteins. Assuming that this clone specified an as yet unknown protein of interest in the retina, we pursued its characterization. We now report that this cDNA sequence‡ specifies a protein of 590 amino acids in which one domain is strikingly rich in glutamic acid: 68 out of 109 amino acids are glutamic acid. The glutamic acid-rich domain contains four repeats of an undecapeptide and two repeats of 26-amino-acid-long peptide sequence.

## METHODS

ROS membranes were isolated from bovine retina according to Papermaster and Dreyer (4). After extensive washing with isotonic buffer (100 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/1 mM dithiothreitol/0.1 mM EDTA/0.01 mM phenylmethylsulfonyl fluoride), the ROS membranes were extracted with hypotonic buffer (5 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/0.1 mM EDTA/0.01 mM phenylmethylsulfonyl fluoride) in the light. The extract was further fractionated by column chromatography on DE-52 cellulose and Sephacryl S-200 to obtain partially purified PDE.

Approximately 10 $\mu$g of the protein, with an equal volume of complete Freund's adjuvant, was injected into rabbits as described (5).

The methods for the preparation and screening of the $\lambda$gt11 cDNA library have been described (5, 6). Hybridization was carried out at 65°C for 15 hr as described (7).

The 5′ end of cDNA was amplified by the PCR technique (8). Retinal poly(A)$^+$ RNA was reverse transcribed with primer I (nucleotides 230–261). After tailing the $(dA)_n$ adapter to the first-stranded reaction product, PCR was accomplished with the $(dT)_{17}$ primer and primer II (nucleotides 186–206).

DNA sequences were determined by the dideoxynucleotide chain-termination method of Sanger *et al.* (9), using T7 DNA polymerase (Sequenase, United States Biochemical).

The decapeptide Glu-Gly-Arg-Glu-Lys-Glu-Glu-Glu-Glu-Gly was assembled stepwise by the Merrifield solid-phase method (10) and then purified by Sephadex G-15 column chromatography. The amino acid composition, determined by analytic HPLC, was consistent with that theoretically expected. The protocol used for immunization of rabbits was that of Goldsmith *et al.* (11).

A crude extract of bovine ROS was resolved by SDS/PAGE and then transferred to a nitrocellulose membrane (Schleicher & Schuell). The blots were incubated with antibodies directed against the PDE-rich extract or the repeated sequence peptide (serum dilution, 1:400). Antibody binding was then measured by incubating the blots with the $^{125}$I-labeled goat anti-rabbit $F(ab')_2$ fragment (NEN).

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Abbreviations: PDE, cGMP phosphodiesterase; ROS, rod outer segment(s).

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M61185).

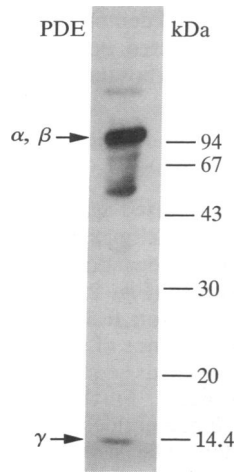


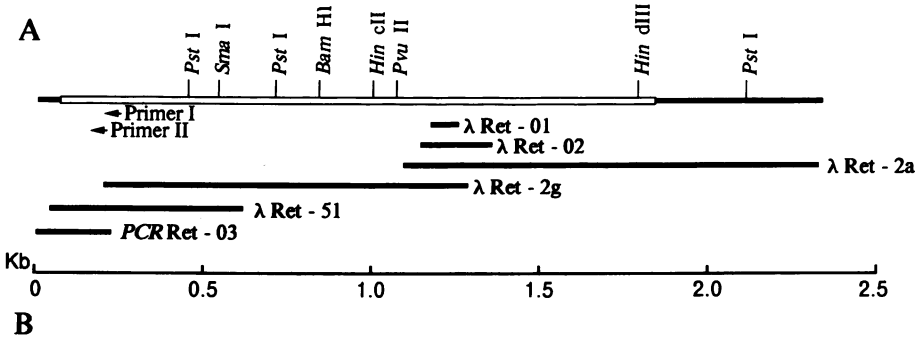

FIG. 1. Immunoblot analysis of the PDE-rich extract by using polyclonal antibody preparation raised against the extract. Fifty micrograms of the crude extract from ROS was separated by SDS/PAGE on a 10% gel. The positions of molecular mass standards and of the α, β, and γ subunits of PDE are shown. Immunopositive bands against the antibody preparation include the α and β subunits (94 kDa), unknown bands smaller than 67 kDa, and the γ subunit (14.4 kDa).

## RESULTS

**Polyclonal Antibodies Against a Retinal Extract Rich in cGMP PDE.** Antibodies prepared against a PDE-rich retinal extract were tested by immunoblotting as shown in Fig. 1. The antibody preparation reacted with each of the subunits (α, β, and γ) of PDE. It also gave a positive reaction against some additional proteins as shown in Fig. 1.

**Identification of a cDNA Clone with a 1770-Base-Pair (bp) Coding Region in the Retinal cDNA Library Corresponding to a Protein of 590 Amino Acids with a Glutamic Acid-Rich Domain.** On screening 80,000 recombinants of a retinal cDNA library with the above antibody preparation, six positive clones were obtained. Of these, two clones (λ Ret-01 and λ Ret-02) contained identical DNA sequences. Because the two clones contained only short DNA sequences, the λ Ret-02 clone was used as a plaque hybridization probe to isolate longer cDNA clones. Fig. 2*A* shows a restriction map of the cDNA clones thus obtained. The clones λ Ret-2a and λ Ret-2g had overlapping 5′ and 3′ ends, respectively. A fragment at the 5′ end of the *Sma* I digest of λ Ret-2g was used as a plaque hybridization probe, and the clone λ Ret-51 (Fig. 2*A*) was isolated. The three λ Ret clones provided the entire amino acid coding region of the protein. Another 20-bp extension (Fig. 2*A*) of the sequence at the 5′ end of the DNA was obtained by the primer extension method, as described in *Methods*.

Fig. 2*B* shows the total nucleotide and deduced amino acid sequences of the cDNA clones. The cDNA contains a coding region of 1770 bp, corresponding to 590 amino acid residues,




B

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                                                -60 GGAGAGAAGCAGGAAGAAGGCAAGTCCTGGCTGGTGGACGGCATCCGAGCAGGTGTCAGG   -1

ATGCTGGGCTGGGTCCAGAGGGTGCTGCCTCAGCCCCCAGGGACCCCTCAGAAGACCAAGCAAGAGGAGGAGGGGACAGAACCAGAGCCAGAGTTGGAGCCGAAG   105
 M  L  G  W  V  Q  R  V  L  P  Q  P  P  G  T  P  Q  K  T  K  Q  E  E  E  G  T  E  P  E  P  E  L  E  P  K    35

CCTGAAACAGCTCCCGAGGAGACTGAGCTAGAGGAAGTGTCCCTGCCACCAGAGGAGCCCTGCGTGGGGAAGGAAGTGGCTGCGGTTACTCTGGGTCCTCAGGGA   210
 P  E  T  A  P  E  E  T  E  L  E  E  V  S  L  P  P  E  E  P  C  V  G  K  E  V  A  A  V  T  L  G  P  Q  G    70

ACCCAGGAGACTGCTCTCACTCCGCCCACATCCCTCCAGGCTCAGGTCTCTGTGGCTCCCGAAGCTCACAGCAGTCCCAGAGGCTGGGTGCTGACCTGGCTCAGG   315
 T  Q  E  T  A  L  T  P  P  T  S  L  Q  A  Q  V  S  V  A  P  E  A  H  S  S  P  R  G  W  V  L  T  W  L  R   105

AAGGGTGTGGAGAAGGTTGTCCCGCAGCCCGCCCACAGCAGCAGGCCCTCCCAGAACATTGCTGCAGGCCTGGAGAGTCCAGATCAGCAGGCAGGAGCACAGATC   420
 K  G  V  E  K  V  V  P  Q  P  A  H  S  S  R  P  S  Q  N  I  A  A  G  L  E  S  P  D  Q  Q  A  G  A  Q  I   140

CTTGGGCAGTGCGGCACTGGGGGCTCAGATGAACCCAGTGAACCCTCCAGGGCCGAAGACCCCGGGCCTGGGCCCTGGCTCCTCCGGTGGTTCGAACAGAATCTG   525
 L  G  Q  C  G  T  G  G  S  D  E  P  S  E  P  S  R  A  E  D  P  G  P  G  P  W  L  L  R  W  F  E  Q  N  L   175

GAGAAAATGCTGCCTCAGCCCCCGAAAATCTCTGAGGGCTGGAGAGATGAGCCTACAGATGCTGCCTTGGGTCCAGAGCCCCCAGGACCCGCCTTGGAGATCAAG   630
 E  K  M  L  P  Q  P  P  K  I  S  E  G  W  R  D  E  P  T  D  A  A  L  G  P  E  P  P  G  P  A  L  E  I  K   210

CCCATGCTGCAGGCCCAGGAGAGCCCCTCCCTGCCAGCTCCTGGCCCCCCGGAGCCTGAGGAGGAACCGATCCCAGAGCCCCAGCCCACTATTCAAGCCTCCTCC   735
 P  M  L  Q  A  Q  E  S  P  S  L  P  A  P  G  P  P  E  P  E  E  E  P  I  P  E  P  Q  P  T  I  Q  A  S  S   245

CTGCCGCCCCCCGCAGGACTCTGCCAGGTTGATGGCATGGATCCTGCACAGGCTGGAGATGGCCCTGCCACAGCCAGTGATCCGTGGGAAGGGTGGGGAGCAGGAG   840
 L  P  P  P  Q  D  S  A  R  L  M  A  W  I  L  H  R  L  E  M  A  L  P  Q  P  V  I  R  G  K  G  G  E  Q  E   280

TCTGATGCCCCAGTGACATGTGACGTGCAGACCATCAGCATCCTCCCTGGAGAGCAAGAGGAGTCGCATCTCATCCTAGAAGAGGTTGACCCTCACTGGGAGGAG   945
 S  D  A  P  V  T  C  D  V  Q  T  I  S  I  L  P  G  E  Q  E  E  S  H  L  I  L  E  E  V  D  P  H  W  E  E   315

GATGAACACCAGGAGGGCAGCACCAGCACCAGCCCGCGGACCTCAGAGGCAGCTCCAGCTGATGAAGAGAAGGGCGAGGTGGTGGAGCAGACGCCCAGGGAGCTG  1050
 D  E  H  Q  E  G  S  T  S  T  S  P  R  T  S  E  A  A  P  A  D  E  E  K  G  E  V  V  E  Q  T  P  R  E  L   350

CCTCGGATTCAAGAGGAGAAAGAAGATGAGGAGGAGGAGAAGGAAGACGGAGAGGAGGAAGAAGAGGAAGGCAGAGAGAAAGAGGAGGAAGAAGGTGAAGAAAAA  1155
 P  R  I  Q  E  E  K  E  D  E  E  E  E  E  K  E  D  G  E  E  E  E  E  G  R  E  K  E  E  E  E  G  E  E  K   385

GAGGAGGAGGAAGGCAGAGAGAAAGAGGAGGAAGAAGGCGAAAAAAAAGAGGAAGAAGGCAGAGAGAAAGAGGAGGAAGAAGGTGGAGAGAAAGAGGAAGAAGAAGGCAGAGGGAAAGAGGAGGTGGAAGGCAGAGAG  1260
 E  E  E  E  G  R  E  K  E  E  E  E  G  E  K  K  E  E  E  G  R  E  K  E  E  E  E  G  G  E  K  E  D  E  E   420

GGCAGAGAGAAAGAGGAGGAAGAAGGCAGAGGGAAAGAGGAAGAAGAAGGTGGAGAGAAAGAGGAAGAAGAAGGCAGAGGGAAAGAGGAGGTGGAAGGCAGAGAG  1365
 G  R  E  K  E  E  E  E  G  R  G  K  E  E  E  E  E  G  E  K  E  E  E  E  G  R  G  K  E  E  V  E  G  R  E   455

GAGGAGGAAGACGAAGAGGAGGAGCAGGATCACAGTGTCCTGCTGGATAGTTATCTGGTGCCACAGAGCGAGGAGGACCAGAGTGAAGAAAGTGAAACCCAGGAC  1470
 E  E  E  D  E  E  E  E  Q  D  H  S  V  L  L  D  S  Y  L  V  P  Q  S  E  E  D  Q  S  E  E  S  E  T  Q  D   490

CAGAGTGAAGTGGGCGGAGCCCAGACCCAGGGCGAAGTGGGCGGAGCCCAGGCCCTGAGTGAAGAAAGTGAAACCCAGGACCAGAGTGAAGTGGGCGGAGCCCAG  1575
 Q  S  E  V  G  G  A  Q  T  Q  G  E  V  G  G  A  Q  A  L  S  E  E  S  E  T  Q  D  Q  S  E  V  G  G  A  Q   525

GACCAGAGTGAAGTGGGCGGAGCCCAGGCCCAGGGCGAAGTGGGTGGAGCCCAGGAGCAGGACGGAGTAGGCGGAGCCCAGGACCAGAGCACCTCACACCAGGAG  1680
 D  Q  S  E  V  G  G  A  Q  A  Q  G  E  V  G  G  A  Q  E  Q  D  G  V  G  G  A  Q  D  Q  S  T  S  H  Q  E   560

CTGCAAGAAGAGGCCTTGGCCGACAGCTCAGGAGGAAGCTTCCAAATGTCTCCATTCGAGGCCCTTCAAGAGTGTGAGGCCCTGAAGAGGTAAAATTGTCACTAC  1785
 L  Q  E  E  A  L  A  D  S  S  G  G  S  F  Q  M  S  P  F  E  A  L  Q  E  C  E  A  L  K  R  *              590

ATGCAGATGACATGATACTATACGTAGGAAACCCTAAAGACTCCACGCAAACACTGCTGCTGCTGCTGCTACGTCGCTTCAGTCGTGTCCAACTCTGCTCGGCCC  1890
CATAGACGGCAGCCCACCAGGCTCCCCCGTCCCTGGGATTCTCCAGGCAAGAACACTGGAGTGGGTTGCCATTTCCTTCTCCAATGCATGAAAGTGAAAGGTGAA  1995
AGGGAAGTCGCTCAGTCGTGTCTGACTCTTGGCGACCCCATGGACTGCAGCCTACCAGGCTCCTCCGTCCATGGGATTTTCCAGACAAGAGTACTGGAGTGGGGT  2100
GCCATTGCCTTCTAATAAATGAATTTAGCGAAGTAGCGGGATTCAAGATTAATATTAAAAAATGAGTTGCAACACTTTATAATAACAATGAAATATCAGAAAAGG  2205
AATGTAAAAAAAAGAAACCCTCTAAAAATAGCACCAAAAAAAAAAAAAAAAAAAA                                                    2260
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FIG. 2. (*A*) Restriction map of cDNAs encoding the glutamic acid-rich protein. The protein coding region is indicated by an open bar. Arrows indicate the primers used to amplify the 5′ end of the cDNA. (*B*) Nucleotide sequence of the cDNA encoding the glutamic acid-rich protein and its deduced amino acid sequence. Single and double underlines indicate repeated sequences. Two possible polyadenylylation signals are boxed. ATG begins with nucleotide 1, whereas termination is at TAA, shown by an asterisk (nucleotide 1771).

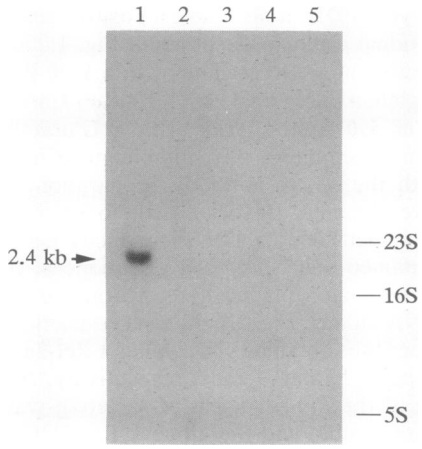


FIG. 3. Northern hybridization of total RNA from various bovine tissues. Total RNA (12 μg) from various tissues was separated by electrophoresis on a 1.0% agarose gel. The 629-bp fragment upstream of the *Bam*HI-digested λ Ret-2g insert was used as a probe. Lanes: 1, retina; 2, brain; 3, liver; 4, adrenal gland; 5, spleen. The size markers are 5S, 16S, and 23S rRNA.

and untranslated regions of 60 and 490 bp at the 5′ and 3′ ends, respectively. The 3′ untranslated region contains two putative polyadenylylation signals, AATAAA and ATTAAA, at positions 2114 and 2154, respectively.

The protein contains a 109-amino acid domain that is rich in glutamic acid residues (68 out of 109 amino acids are glutamic acid). This domain contains four repeats of a glutamic acid-rich 11-amino acid peptide (double underlining in Fig. 2*B*) and two repeats of a 26-amino acid peptide (single underlining in Fig. 2*B*).

**Retinal mRNA Corresponding to the cDNA Clones.** Northern blot analysis was carried out to investigate the transcript size of the derived protein as well as tissue specificity. As shown in Fig. 3, the cDNA probe recognized a 2.4-kilobase mRNA species in retina. Furthermore, hybridization was specific to the retinal mRNA; the probe did not hybridize to the mRNA from brain, liver, adrenal gland, or spleen.

**Identification of a 65-kDa Protein in Bovine Retinal ROS That Is Recognized by a Polyclonal Antibody Against a Glutamic Acid-Rich Polypeptide Fragment in the Protein Sequence.** We synthesized a decapeptide corresponding to the repeated glutamic acid-rich undecapeptide to raise an antibody against it, as described in *Methods*. Fig. 4 demonstrates that the anti-synthetic peptide antibody reacted positively with a 65-kDa protein in the crude extract from bovine retinal ROS.

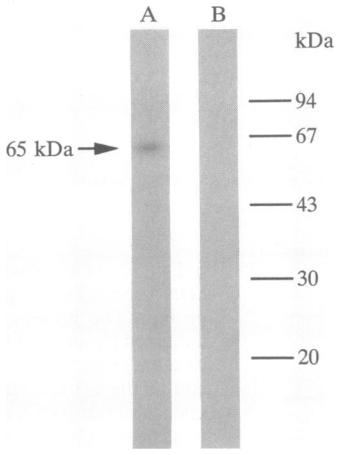


FIG. 4. Identification of the 65-kDa protein by immunoblotting. Fifty micrograms of the crude extract from bovine ROS was separated by SDS/PAGE. Immunoblotting was performed as described in *Methods*. (*A*) Immunoblot with antibody raised against the repeated peptide Glu-Gly-Arg-Glu-Lys-Glu-Glu-Glu-Glu-Gly. (*B*) Immunoblot with preimmune antibody.

## DISCUSSION

By using a polyclonal antibody preparation raised against an extract of ROS, we have isolated cDNA clones whose sequence analysis shows that the cDNA encodes a glutamic acid-rich protein of 590-amino acid residues (Fig. 2*B*). The ATG codon at nucleotide 1 in the translation frame was concluded to be the initiator codon. The presence of adenine at position −3 was consistent with the consensus sequence at initiation sites (12) in eukaryotic messengers. Further, extension of the 5′ end with a primer by 20 nucleotides gave a sequence that contained no ATG codon. The size of the mRNA (2.4 kilobases) identified was in good agreement with that of the cDNA sequence (2320 nucleotides) (Fig. 2*B*). The characteristic feature of the DNA sequence of this protein is its extremely high purine content in the nucleotide region between 1060 and 1390. The cDNA-derived amino acid sequence in the same region was also remarkably distinct. Thus, in a run of 109 amino acids (residues 355–463) in this region, there were 68 glutamic acid residues. Further, this domain contained four repeats of an undecapeptide sequence with high glutamic acid content (Glu-Glu-Gly-Arg-Glu-Lys-Glu-Glu-Glu-Glu-Gly) and an additional two repeats of 26 amino acids also rich in glutamic acid.

The expression of the above glutamic acid-rich protein in the retina was further supported by the results of an immunological experiment (Fig. 4). An antibody raised against the specific glutamic acid-rich peptide sequence in the protein specifically recognized a protein with a molecular mass of 65 kDa; this is in excellent agreement with the amino acid composition derived from the cDNA sequence (64.8 kDa).

Northern hybridization showed that the mRNA encoding the glutamic acid-rich protein was present only in retina and not in any of the other bovine tissues tested (Fig. 3). A search of the GenBank DNA sequence data base (release 65.0) and the National Biomedical Research Foundation protein data base (release 25.0) showed that this cDNA sequence has not been described previously and, further, that there is no significant homology with any of the known proteins, except

**A**

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     354
RGP QEEKEDEEEEKEDGEEE-EEEGREKEEEEGEEKEEEEGREKEEEEG
       :  :        : : :::: :::: : :   ::::   :::
NFL EAAKAEEAKDEPPSEGEAEEEGKEKEEAEAEAEAEEEGAQEEEEAA
     460
                                        428
RGP EKKEEEGREKEEEEGEEKEDEEGREKEEEE
    :: : :   :::: ::    ::  : ::::
NFL EKEESEE-AKEEEGGEGDQGEETKEAEEEE
                                        534
```

**B**

```
     362
RGP EEKEDGEEEEEEGREKEEEEGEEKEEEEGREKEEEEGEKKEEEGRE
:     :: :::: :::: : :   ::::   :: :  :   :
BGP EPPSEGEAEEEE-KEKEEAEAEAEAEEEGAQEEEAEAAKEDAEE-A
    3
                              428
RGP KEEEEGEEKEDEEGREKEEEE
    :::: ::   : ::  : ::::
BGP KEEEGGEGEEAEETKEAEEEE
                              67
```

FIG. 5. Homology of the retinal glutamic acid-rich protein with other tissue proteins. (*A*) Comparison of the bovine retinal glutamic acid-rich protein (RGP) with porcine neurofilament L (NFL). (*B*) Comparison of the bovine brain glutamic acid-rich protein (BGP) with the bovine retinal glutamic acid-rich protein. Identical amino acids between the sequences of two proteins are indicated by two dots. The amino acid numbers of each protein sequence are shown at both ends.

in the repeated glutamic acid-rich domains. The highest scores have been obtained for neurofilaments from the brains of various animals (13–15) and for glutamic acid-rich proteins from bovine brain (16) (Fig. 5). Although the function of the brain micro glutamic acid-rich protein is not clear, it was deduced to be the C-terminal endpiece of the neurofilament 68-kDa protein from its primary sequence (17). The N-terminal and C-terminal domains of the 68-kDa protein are believed to allow it to polymerize into the filament structure and to provide a scaffold for the interaction with other cellular components (17). We therefore consider the possibility that the retina-specific glutamic acid-rich protein that shows homology with the C-terminal region of neurofilaments may participate in the process of visual transduction by interacting with other proteins in bovine ROS.

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