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Cloning and sequencing of rat liver cDNAs encoding the regulatory protein of glucokinase*

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cDNAs encoding the rat liver regulatory protein of glucokinase were cloned and sequenced. The deduced protein contains 568 amino acids for a molecular mass of 62,867 Da. Northern blot analysis showed the presence of a major RNA species of 2.35 kb in rat liver. No signal was observed with muscle, brain, heart, testis, intestine or spleen RNA. Recombinant regulatory protein expressed in *Escherichia coli* was insoluble and inactive, and was presumably contained in inclusion bodies. Western blot analysis showed that the recombinant protein was recognized by antibodies raised against regulatory protein purified from rat liver.

Glucokinase; Regulatory protein; Glycolysis

1. INTRODUCTION

Rat liver contains a regulatory protein, which inhibits glucokinase competitively with respect to glucose [1]. The effect of this protein is greatly reinforced by fructose 6-phosphate and antagonized by fructose 1-phosphate. The regulatory protein has been purified to near-homogeneity and identified as a 62 kDa polypeptide [2]. It inhibits glucokinase by forming an inactive complex with this enzyme (reviewed in [3]). The regulatory protein is present in the livers of species that have glucokinase, although not in others (Vandercammen and Van Schaftingen, unpublished results). There are also indications that regulatory protein is present in β-cells of Langerhans islets [4], where glucokinase plays the role of a glucose-sensor [5]. Here, we report the cloning of cDNAs encoding rat liver regulatory protein.

2. MATERIALS AND METHODS

Radiolabelled compounds, nitrocellulose filters (Hybond C-extra), nylon filters (Hybond-N) and Megaprime labelling kit were from Amersham, restriction and modifying enzymes from Boehringer, GeneAmp DNA Amplification Kit from Perkin Elmer Cetus, and T7 Sequencing kit from Pharmacia.

2.1. *Protein sequencing*

Rat liver regulatory protein purified to near-homogeneity [2] was submitted to two-dimensional gel electrophoresis [6]. The proteins were then electroblotted onto Immobilon Membranes (Millipore Corp.), which were submitted to in situ digestion with trypsin [7]. The generated peptides were purified by HPLC as in [7] on a 0.46 × 25 cm C_{18} reversed phase column. Peptides were detected by absorbance at 214 nm and manually collected. Sequence analysis was carried out with a 477A Applied Biosystems pulsed liquid phase sequenator equipped with an on-line phenylthiohydantoin amino acid analyser (model 120A). For the sequencing of the amino-terminal end, regulatory protein was purified as in [8] and used without being submitted to electrophoresis.

2.2. *Obtention of a DNA probe*

We used the protocols described in [9] for all standard molecular biology techniques. Rat liver cDNA was prepared by reverse transcription of total rat liver RNA (see section 2.4) with an oligo-dT primer. A PCR with 1 ng of cDNA and 25 pmol of two degenerate primers [5'-CGAAGCTTTACCAGCATGTGAT(TCA)GA(AG)A-C(TGCA)CC-3' and 5'-CGGGATCCTTCTCTGTGATIGG-(TGCA)AC(TGCA)GC-3'] was performed as described in [10] with an annealing temperature of 37°C during 3 cycles and of 52°C during 30 cycles. The PCR product was purified by electrophoresis in a low-melting agarose gel, digested overnight with *Hin*dIII and *Bam*HI, cloned in pBluescript KS(+) (Stratagene) and sequenced.

2.3. *Isolation and sequencing of cDNA clones*

About 100,000 clones of a rat liver cDNA library (Clontech, no. RL1001b) constructed in λgt11 were plated with *E. coli* Y1088 as host, and grown overnight. The DNA from the plaques was transferred to nitrocellulose filters, which were subsequently prehybridized in a solution containing 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, 0.1 mg/ml denatured herring sperm DNA, 1 mM EDTA and 10 mM sodium phosphate buffer, pH 7.1. Hybridization was performed overnight at 65°C with 200,000 dpm/ml of the cloned probe (specific activity: 700,000 dpm/ng DNA), labelled by random priming. The filters were rinsed 3 × 5 min in 2 × SSC, 0.1% SDS at room temperature, washed 30 min with 0.2 × SSC, 0.1% SDS at 65°C and autoradiographed. Positive plaques were picked up and submitted to a secondary screening. Their phage DNA was purified and the *Eco*RI inserts were subcloned in pBlueScript KS(+).

About 100,000 phages of a second library constructed in λZAPII (Stratagene, no. 936513) were plated with *E. coli* XL1Blue as host and

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*The nucleotide sequence of the cDNA described in this article has been deposited in the GenBank/EMBL data base under accession number X68497.

screened as above, with a probe corresponding to nucleotides 1–745 of pBS-LPR17, a clone obtained in the first screening. The pBlueScript II KS(+) phagemids containing the cDNA were excised in vivo with ExAssist Phage Helper as described by the manufacturer.

Plasmid DNA was purified by the boiling lysis method [11] and sequenced on both strands after alkaline denaturation [12].

2.4. *RNA preparation and Northern blot analysis*

Total RNA was prepared from various tissues by the guanidinium isothiocyanate/CsCl procedure, electrophoresed in 1% agarose/formaldehyde gels and transferred to nylon filters by capillarity. The filters were prehybridized in 50% formamide, 5 × SSPE (1 × SSPE is 180 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.5), 0.5% SDS, 5 × Denhardt's solution and 0.5 mg/ml denatured herring sperm DNA and hybridized at 42°C with a probe corresponding to nucleotides 1–1,800 of pBS-LPR17 labelled by random priming (1.5×10^6 cpm/ml). The filters were washed 3 × 30 min at 50°C in 1 × SSC, 0.1% SDS and autoradiographed.

2.5. *Expression of a recombinant protein*

The protein was expressed in the expression system of Studier and Moffatt [13]. A *Nde*I restriction site was introduced at the initial ATG in the coding sequence of pBS-LPR17 by PCR mutagenesis [14] with a primer containing *Eco*RI and *Nde*I sites followed by the first 18 nucleotides of the coding sequence and a reverse primer corresponding to nucleotides 1,135–1,118 of pBS-LPR17. An *Eco*RI–*Pvu*II fragment containing the first 368 nucleotides of the coding sequence was excised from the amplified DNA and ligated to a fragment derived from pBS-ZPR1, which contained the rest of the coding sequence. The resulting construct was inserted in pBlueScript KS(+), checked by sequencing and inserted in pET3a expression plasmid. After cloning and amplification in XL1-Blue, the resulting plasmid, termed pET-PR, was used to transform competent *E. coli* BL21 (DE3)pLysS. The cells were grown in M9 medium containing 1% glucose, 25 μg/ml ampicillin and 30 μg/ml chloramphenicol until A_{600} reached 0.5. The inducer isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to final concentrations ranging from 0.01 to 0.4 mM and the cultures were incubated overnight at 22°C or 3 h at 37°C. The cells were pelleted by centrifugation, washed in cold buffer B (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol) and centrifuged again. They were resuspended in cold buffer B containing 1 mM phenylmethanesulfonyl fluoride, 2.5 μg/ml leupeptin, 2.5 μg/ml antipain, 2.5 μg/ml aprotinin, 2 mM $ZnCl_2$ and 2.5 μg/ml pepstatin, and disrupted in a French Press (Aminco) at 96 MPa. Western blots were performed as in [15], using a rabbit antibody directed against rat liver regulatory protein, and revealed as in [16].

3. RESULTS AND DISCUSSION

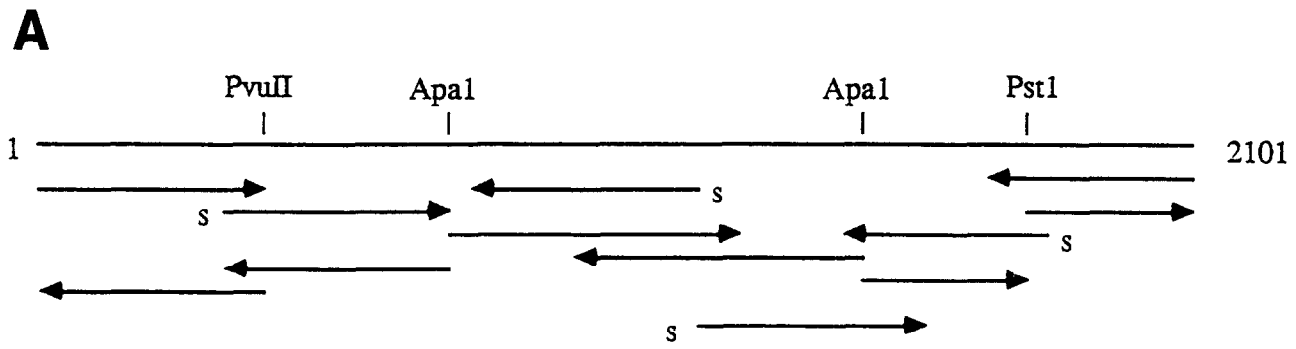
3.1. *Isolation and characterization of regulatory protein clones*

As shown in Fig. 1B the longest of the 4 tryptic peptides that were sequenced contained 27 amino acids. The extremities of this peptide were used to design PCR primers, which were degenerate at their 3′ end. A PCR reaction was performed with rat liver cDNA and a single 90 bp product was cloned and sequenced. The sequences of three clones that were determined matched that of the 27 amino acid peptide.

Using the cloned PCR product as a probe, we screened about 100,000 plaques of a rat liver cDNA library from Clontech, and obtained ten positive clones. The longest of them (pBS-LPR17) was completely sequenced (Fig. 1A) and found to contain two partially overlapping open reading frames, one from nucleotide 22 to 1,425, and a second from nucleotide 1,370 to 1,729. The first one contained a potential start codon at nucleotide 22 and coded for a protein of 51.5 kDa, 10 kDa smaller than rat liver regulatory protein. Partial sequencing of another long clone indicated that it corresponded to nucleotides 51 to 2,081 of PBS-LPR17. This clone contained however a stretch of 4 thymidines instead of 5 as found at position 1,411–1,415 of PBS-LPR17. This change extended the longest open reading frame by an additional 300 nucleotides, bringing the predicted molecular mass of the encoded polypeptide to a value of approximately 63 kDa.

Two positive clones, termed pBS-ZPR1 and 2, were obtained from the Stratagene library. Both clones contained only 4 T at position 1,411–1,414 of pBS-LPR17, confirming that the fifth T was the result of a mutation as can be found at sites of reiterated bases [17]. pBS-ZPR1, which was completely sequenced, corresponded to nucleotides 45–2,101 of PBS-LPR17, plus 9 nucleotides and a poly A-tail of 45 residues at its 3′ end. In addition, this clone contained a G-to-A transition at position 924, which did not affect the deduced amino acid sequence. PBS-ZPR2, which was only partially sequenced, corresponded to nucleotides 55–2,101 of PBS-LPR17, plus an additional 15 nucleotides, but without poly-A tail.

Fig. 1B shows the sequence of the cDNA that could be reconstructed from the various clones, as well as the deduced protein sequence. The initial ATG codon is surrounded by a sequence similar to the Kozak consensus sequence (GCC(A/G)CCATGG). Identification of this ATG as the start codon was confirmed by sequencing of the NH_2-terminus of rat liver regulatory protein. The obtained sequence (XGTKRYQXVIETPEPGE) corresponded to amino acids 2–18 of the deduced sequence, indicating that the initiator methionine of the regulatory protein was excised in vivo, as is often observed [18]. The open reading frame encoded a protein of 568 amino acids (excluding the initiator methionine), in which the sequence of the four peptides can be identified. The predicted molecular mass is 62,867 Da, in good agreement with the mass of regulatory protein purified from rat liver. Interestingly, the C-terminus of the protein is a basic tetrapeptide, Arg-Arg-Lys-Arg. The same sequence is found internal in retroviral envelope proteins, where it serves as a cleavage signal for proteolytic processing [19]. A similar sequence (Arg-Lys-Arg-Arg) serves a similar role in the insulin receptor precursor protein [20]. Due to its position, the basic tetrapeptide cannot play this role in liver regulatory protein. It could, however, participate in the interaction of this protein with glucokinase, as this interaction was shown to be partially ionic in nature [8]. Sequence comparisons did not reveal a significant degree of similarity with other DNA (GenBank release no. 73) or protein (Swissprot release no. 23) sequences.



B

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GAGGGTCCACAGTGTGGGACCATGCCAGGCACCAAACGATATCAGCATGTGATCGAGACCCCTGAGCCTGGTGAATGGGAGTTGTCAGGG   90
                  M  P  G  T  K  R  Y  Q  H  V  I  E  T  P  E  P  G  E  W  E  L  S  G
TATGAAGCGGCTGTGCCAATCACAGAGAAATCCAACCCACTGACCCGAAACCTGGACAAAGCAGATGCAGAGAAAATTGTCAAACTGCTG   180
 Y  E  A  A  V  P  I  T  E  K  S  N  P  L  T  R  N  L  D  K  A  D  A  E  K  I  V  K  L  L
GGGCAGTGTGATGCTGAGATATTCCAGGAGGAGGGGCAGATTGTGCCCACCTACCAGCGACTATACAGCGAATCAGTTCTGACCACCATG   270
 G  Q  C  D  A  E  I  F  Q  E  E  G  Q  I  V  P  T  Y  Q  R  L  Y  S  E  S  V  L  T  T  M
TTGCAAGTGGCTGGAAAAGTCCAGGAAGTTCTGAAGGAGCCAGATGGGGGTCTGGTAGTGCTGAGTGGAGGGGGAACCTCTGGTCGTATG   360
 L  Q  V  A  G  K  V  Q  E  V  L  K  E  P  D  G  G  L  V  V  L  S  G  G  G  T  S  G  R  M
GCATTTCTCATGTCTGTGTCTTTCAACCAGCTGATGAAAGGCCTGGGACAAAAGCCTCTTTACACCTACCTCATTGCAGGAGGTGACAGG   450
 A  F  L  M  S  V  S  F  N  Q  L  M  K  G  L  G  Q  K  P  L  Y  T  Y  L  I  A  G  G  D  R
TCTGTTGTGGCCTCTCGTGAACAGACAGAAGATAGCGCCCTACACGGGATCGAGGAGCTGAAGAAGGTGGCTGCTGGGAAGAAGAGAGTG   540
 S  V  V  A  S  R  E  Q  T  E  D  S  A  L  H  G  I  E  E  L  K  K  V  A  A  G  K  K  R  V
GTCGTCATAGGCATCTCTGTGGGACTCTCTGCGCCCTTTGTGGCAGGTCAGATGGACTACTGCATGGATAACACAGCCGTCTTCTTGCCG   630
 V  V  I  G  I  S  V  G  L  S  A  P  F  V  A  G  Q  M  D  Y  C  M  D  N  T  A  V  F  L  P
GTTCTGGTTGGCTTCAATCCAGTGAGCATGGCCAGAAATGACCCCATTGAAGACTGGAGATCAACATTCCGGCAAGTGGCAGAGCGGATG   720
 V  L  V  G  F  N  P  V  S  M  A  R  N  D  P  I  E  D  W  R  S  T  F  R  Q  V  A  E  R  M
CAAAAGATGCAGGAGAAACAGGAAGCTTTTGTGTCTCAATCCTGCCATCGGGCCCGAGGGGCTCAGCGGCTCTTCCCGAATGAAAGGTGGA   810
 Q  K  M  Q  E  K  Q  E  A  F  V  L  N  P  A  I  G  P  E  G  L  S  G  S  S  R  M  K  G  G
GGTGCCACCAAGATTCTACTGGAAACCCTGCTACTAGCAGCCCATAAGACTGTGGACCAGGGTGTTGTGTCCTCTCAAAGATGCCTTCTG   900
 G  A  T  K  I  L  L  E  T  L  L  L  A  A  H  K  T  V  D  Q  G  V  V  S  S  Q  R  C  L  L
GAAATCCTGAGGACATTTGAGCGGGCTCATCAGGTGACCTACAGTCAAAGTTCCAAAATTGCCACGCTGATGAAACAAGTCGGCATCAGC   990
 E  I  L  R  T  F  E  R  A  H  Q  V  T  Y  S  Q  S  S  K  I  A  T  L  M  K  Q  V  G  I  S
CTGGAGAAGAAAGGCCGAGTGCACTTGGTTGGCTGGCAGACTCTCGGCATCATTGCCATTATGGACGGAGTAGAGTGCATCCACACTTTT  1080
 L  E  K  K  G  R  V  H  L  V  G  W  Q  T  L  G  I  I  A  I  M  D  G  V  E  C  I  H  T  F
GGTGCTGATTTCCAAGATATCCGTGGCTTTCTTATTGGTGACCACAGTGACATGTTTAACCAGAAGGATGAACTCACCAACCAGGGTCCC  1170
 G  A  D  F  Q  D  I  R  G  F  L  I  G  D  H  S  D  M  F  N  Q  K  D  E  L  T  N  Q  G  P
CAGTTCACCTTCTCCCAGGATGACTTCCTGACTTCCATCCTGCCATCCCTCACGGAGACTGACACCGTGGTCTTCATTTTTACCCTGGAT  1260
 Q  F  T  F  S  Q  D  D  F  L  T  S  I  L  P  S  L  T  E  T  D  T  V  V  F  I  F  T  L  D
GATAACCTCACAGAAGTACAGGCCCTGGCAGAAAGAGTGAGAGAGAAGTGCCAGAACATCCAGGCCCTGGTGCACAGCACTGTGGGGCAG  1350
 D  N  L  T  E  V  Q  A  L  A  E  R  V  R  E  Q  N  I  Q  A  L  *  V  H  S  T  V  G  Q  S  L
TCCTTGCCGGCCCCTCTAAAGAAACTCTTTCCCTCACTCATCAGTATCACGTGGCCACTTCTTTTCTTCGATTATGAAGGGACCTATGTT  1440
 P  A  P  L  K  C  K  K  L  F  P  S  L  I  S  I  T  W  P  L  L  F  F  D  Y  E  G  T  Y  V
CAGAAGTTCCAGCGTGAGTTAAGCACCAAGTGGGTGTTGAATACAGTGAGTACTGGGGCCCATGTACTGCTGGGGAAGATCCTACAGAAC  1530
 Q  K  F  Q  R  E  L  S  T  K  W  V  L  N  T  V  S  T  G  A  H  V  L  L  G  K  I  L  Q  N
CACATGCTGGACCTCCGCATCGCCAACTCCAAGCTCTTCTGGAGGGCGCTGGCCATGTTGCAGAGGTTCTCTGGACAGTCCAAGGCTCGC  1620
 H  M  L  D  L  R  I  A  N  S  K  L  F  W  R  A  L  A  M  L  Q  R  F  S  G  Q  S  K  A  R
TGCATTGAGAGCCTCCTTCAAGCAATCCACTTTCCTCAACCACTGTCGGATGATGTCGCGCCGCTCCCATCTCCTGCCACGTCCAGGTTG  1710
 C  I  E  S  L  L  Q  A  I  H  F  P  Q  P  L  S  D  D  V  A  P  L  P  S  P  A  T  S  R  L
CCCACGAGAAGGAAAAGGTGATCCCCACAGCCTTGCTGAGCCTCCTACTCCGGTGCTCCATCTCTGAGGCTAAGGCACGCCTGTCTGCAG  1800
 P  T  R  R  K  R
CTTCTTCAGTCTGTGAGGTTGTTAGGAGCGCCCTCTCTGGGCCGGGTCAGAAGCGCAGCACGCAAGCCCTTGAAGACCCTCCCGCCTGTG  1890
GGACCCTGAATTGATATTTCTAGAACCATGGAGGGGCAGAGTCTCCGTCCACTTCCAAGGGGACATGTGCCAGCAGTACACGCTGTGGGA  1980
AGAACTCAGTTTCGGGTGGGTGGGGCCTAACTGCCCAGAATTGGGGAAGAGCCCTGTTCTCAACCGGATTATTTCCATTTTTACTGGTGT  2070
CTTCTGAACTCAGAAATAAAACTAAATGTCTTGTTTTGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 1. Sequencing strategy and sequence of the cDNA encoding rat liver regulatory protein. (A) Sequencing was performed on subclones, or using specific primer(s). (B) Nucleotide and deduced amino acid sequence. The asterisk indicates the position where an additional T was found in pBSLPR17. The sequences of the tryptic peptides are underlined.

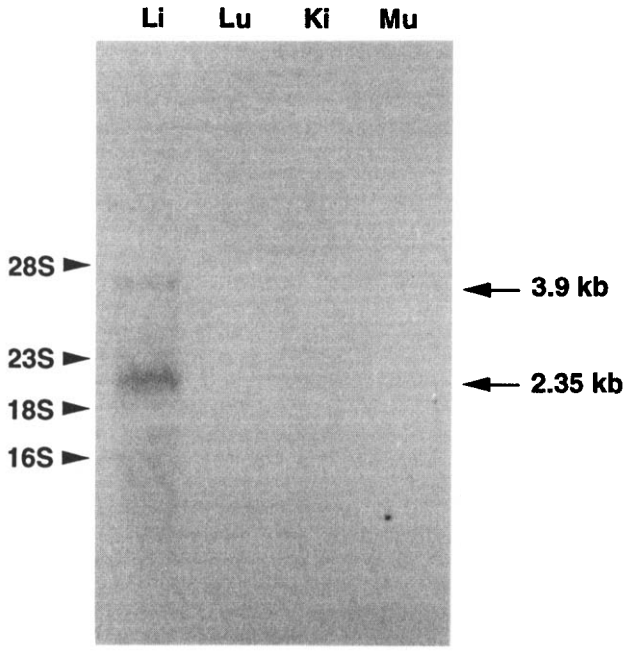

Fig. 2. Northern blot analysis of different tissues. Twenty μg of total RNA was loaded per lane. Li, liver; Lu, lung; Ki, kidney; Mu, muscle.

3.2. *Northern blot analysis*

As shown in Fig. 2, Northern blot analysis revealed the existence in rat liver of a major 2.35 kb transcript, and a minor 3.9 kb band, possibly an immature transcript. A faint signal with a size of 2.35 kb was also observed in some lung samples (± 3% of the rat liver signal). No positive signal was observed with RNA from skeletal muscle, brain, intestine, testis, spleen and heart. These results are consistent with the presence of regulatory protein in liver but not in other tissues.

3.3. *Expression of regulatory protein in bacteria*

The coding sequence of the regulatory protein was reconstructed from pBS-LPR17 and pBS-ZPR1 and inserted in the expression vector pET3a [13]. The resulting recombinant plasmid was introduced in *E. coli* BL21 (DE3)pLysS. Addition of isopropylthiogalactoside to the culture medium resulted in the appearance of a ca. 60 kDa polypeptide in the cells harboring the recombinant plasmid although not in cells containing the expression vector without the insert (not shown). Western blots indicated that this 60 kDa protein cross-hybridized with antibodies directed against rat liver regulatory protein (not shown). The majority of the recombinant protein present in cell-free extracts sedimented upon a 10 min centrifugation at 5,000 × g, indicating that it was present in inclusion bodies. Attempts at obtaining a soluble recombinant protein by lowering the temperature during induction (22°C) or by lowering the concentration of isopropylthiogalactoside failed.

Not surprisingly because of its insolubility, the recombinant protein present in extracts of cells did not inhibit glucokinase. Our attempts to renature the regulatory protein present in inclusion bodies by dilution from concentrated urea solutions [21] were so far unsuccessful.

To confirm that the product of the cloned gene corresponded to the regulatory protein of glucokinase, we electrophoresed the recombinant protein and transferred it onto a nitrocellulose membrane. Strips corresponding to the major band were used to immunopurify antibodies [9]. These purified antibodies were found to

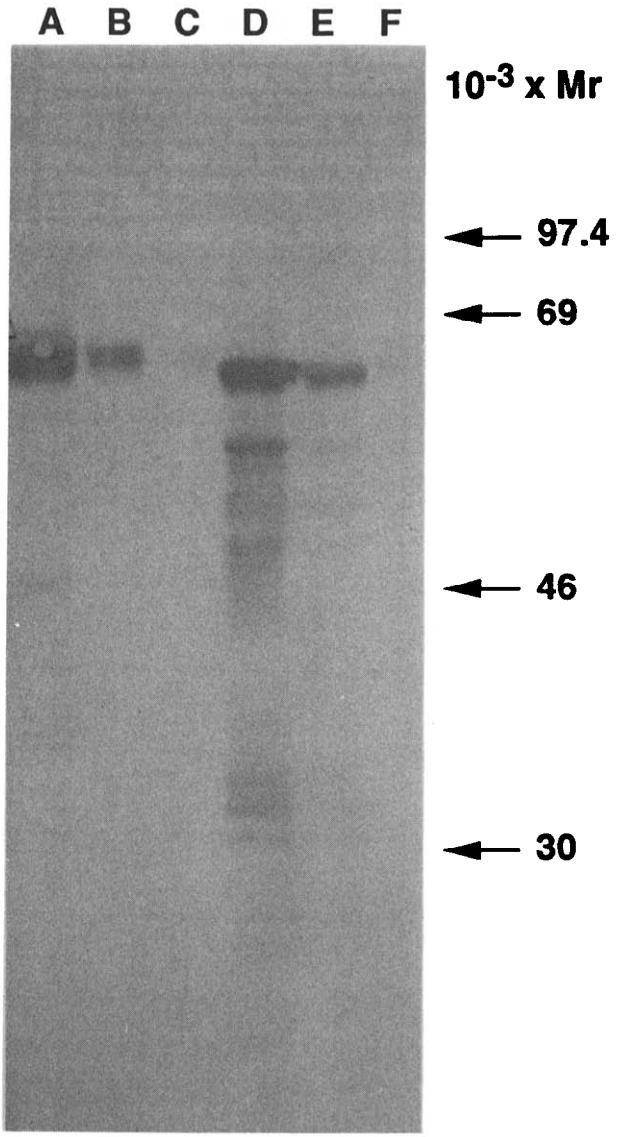

Fig. 3. Western blot analysis of rat liver regulatory protein and of recombinant regulatory protein. The following samples were electrophoresed: Lanes A–C: 5 (A), 1 (B) and 0.2 (C) U of regulatory protein purified from rat liver [2], corresponding to ca. 1, 0.2 or 0.04 μg pure protein. Lanes D–F: similar amounts of the recombinant 60 kDa protein as in lanes A–C, as judged from a Coomassie blue-stained gel. The blot was developed with antibodies immunopurified [9] on immobilized recombinant regulatory protein.

react with regulatory protein purified from rat liver and with recombinant regulatory protein with similar sensitivity. These results indicated that the cloned cDNA does not correpond to a protein that would still contaminate our preparation of apparently homogeneous regulatory protein. The molecular mass of the recombinant protein is slightly smaller than that of the protein purified from liver. This could be due to proteolysis of the first, or to post-translational modification of the latter.

As a conclusion, we have obtained the cDNA containing the entire coding sequence of rat liver regulatory protein. Our identification is based on: (1) the identity between partial peptide sequences obtained from homogeneous regulatory protein and the sequence deduced from the cDNA; (2) the good agreement between the mass of the regulatory protein purified from rat liver and the recombinant protein; (3) the recognition of the recombinant regulatory protein by antibodies directed against rat liver regulatory protein.

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