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Biochemistry

AUH, a gene encoding an AU-specific RNA binding protein with intrinsic enoyl-CoA hydratase activity

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ABSTRACT **AU-rich elements within the 3′ untranslated region of transcripts of lymphokines and some protooncogenes serve as signal for rapid mRNA degradation. By using an AUUUA matrix, we have affinity-purified a 32-kDa protein, microsequenced it, and cloned the corresponding cDNA. *In vitro*, the recombinant protein bound specifically to AU-rich transcripts, including those for interleukin 3, granulocyte/macrophage colony-stimulating factor, c-*fos*, and c-*myc*. Sequence analysis revealed an unexpected homology to enoyl-CoA hydratase (EC 4.2.1.17), and the recombinant protein showed a low degree of the enzymatic activity. Thus, this gene, designated *AUH*, encodes an RNA binding protein with intrinsic enzymatic activity. Protein immobilized on an AUUUA matrix was enzymatically active, suggesting that hydratase and AU-binding functions are located on distinct domains within a single polypeptide.**

Control of gene expression occurs by transcriptional and posttranscriptional regulation. The expression of certain protooncogenes and lymphokines is regulated by specific and rapid decay of their transcripts. Control mechanisms involve cis-acting elements found in the 3′ untranslated region (UTR) and, in some examples, also in the coding region of mRNA (1). A common cis element found in the 3′ UTR of rapidly decaying mRNA is an AU-rich element (ARE), containing various numbers of reiterated AUUUA pentamers, at times associated with a general AU-richness with a surplus of uridylic residues (2, 3). In hybrid constructs, ARE is able to confer rapid degradability to otherwise stable reporter transcripts (2, 3).

Different transcripts with similar yet distinct AREs are regulated differentially, for example, granulocyte/macrophage colony-stimulating factor (GM-CSF) and c-myc mRNAs, arguing that different trans-acting proteins are involved (4). Furthermore, several groups have recently identified a number of mRNA-binding proteins with AU specificity (5–10), two of which have been cloned (11, 12).

Physiological calcium-dependent interleukin 3 (IL-3) production during IgE-triggered mast cell activation involves mRNA stabilization (13, 14), and tumors derived from v-H-*ras*-expressing mast cells were found to have a defect in IL-3 mRNA degradation (15, 16). Such IL-3 transcripts could be destabilized by cyclosporin A through a mechanism depending on an intact ARE (16). To approach ARE-dependent regulatory mechanisms, we purified an ARE-specific binding protein and cloned the corresponding cDNA.§ We report an unexpected bifunctionality of this RNA binding protein.

MATERIALS AND METHODS

Protein Purification. Human brain was homogenized in buffer A [10 mM Hepes, pH 7.9/40 mM KCl/5% (wt/vol) glycerol/1 mM dithiothreitol/0.3 mM phenylmethylsulfonyl fluoride/0.2% Nonidet P-40] supplemented with 0.5 M NaCl. After centrifugation at 40,000 rpm for 1 h in a Ti 45 rotor (Beckman), cleared supernatant was dialyzed and subjected to heparin-Sepharose chromatography (Pharmacia, type CL-6B). After washing with buffer A containing 0.15 M NaCl, bound material was eluted with 0.5 M NaCl and dialyzed against buffer A. Finally, the dialysate was subjected to affinity chromatography as described by Neupert *et al.* (17). The oligonucleotide 5′-ATTTATTTATGTATTTATGTATTTATTT-ATTTA-3′ (nt 2738–2770 of the IL-3 gene) (18) was subcloned into pGEM-3Z (Promega) between the T7 promoter and a $(A)_{18}$ tract. Polyadenylylated $(AUUUA)_6$ transcripts were bound to oligo(dT)-cellulose (Pharmacia, type 7). Proteins bound to this matrix in the presence of 0.25 M NaCl were eluted with buffer A supplemented with 10 mM EDTA, 1 M NaCl, and 10 mM $MgCl_2$, dialyzed, and concentrated.

Northwestern Blot Analysis. After SDS/PAGE and electroblotting, proteins on nitrocellulose were renatured in buffer A supplemented with 3 mM $MgCl_2$, 0.1 mM EDTA, and bovine serum albumin at 5 mg/ml. A radiolabeled transcript corresponding to the 3′ UTR of GM-CSF containing an ARE (6) (6×10^6 cpm) was added to the blot in RNA binding buffer (RB buffer = 10 mM Hepes, pH 7.9/150 mM KCl/5 mM $MgCl_2$/0.2 mM dithiothreitol/8% glycerol) supplemented with yeast tRNA (50 μg/ml) and incubated at 37°C for 20 min and at room temperature for 2 h. Unbound RNA was removed by RNase T1 (150 units/ml).

Western Blot Analysis. Anti-peptide antisera (1:300 dilution) and ^{125}I-labeled donkey anti-rabbit antibody (Amersham) in 3% (wt/vol) nonfat milk in PBS were used. Antibodies were raised against a synthetic peptide, EMKTEDEL-RVRHLEEENR, residues 70–87 of AUH. Monoclonal antibody 4E4 recognizing heterogeneous nuclear ribonucleoproteins (hnRNPs) was provided by J. Wilusz (19) and used with peroxidase-conjugated goat anti-mouse antibody (Cappel).

Peptide Sequencing. Tryptic peptides were obtained from protein blotted onto ProBlott membrane (Applied Biosystems) as described (20). Peptides were purified by reverse-phase HPLC and sequenced (21). The N-terminal sequence was determined as described (22).

PCR Cloning. Degenerate oligonucleotides were synthesized for reverse transcription–PCR. Sources of RNA were human brain and HeLa and IMR32 cells. To generate a PCR product corresponding to peptide N, the forward primer 1 (see below) and the reverse primer 2 were used. Based on the obtained internal sequence, a forward primer 3 was designed and used with the reverse oligo(dT) anchor primer M108 (23). The products were reamplified with an internal forward

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Abbreviations: UTR, untranslated region; ARE, AU-rich element; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL-3, interleukin 3; hnRNP, heterogeneous nuclear ribonucleoprotein; MBP, maltose binding protein; IRE-BP, iron-response-element binding protein.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. X79888).

primer 4 and the reverse anchor primer M45 (23). The 5′ end was further extended by using 5′-AmpliFinder Race kit (Clontech) (clone HB8). PCRs were performed for 30–40 cycles at 94°C for 1 min, 46–60°C for 2 min, and 72°C for 0.5–3 min. PCR products were subcloned and sequenced. The primer sequences were as follows: 1, 5′-GA(GA)ATGAA(GA)ACNGA(GA)GA-3′; 2, 5′-TT(TC)TC(TC)TC(TC)TCNA(AG)(AG)TG-3′; 3, 5′-ATGAA(GA)ACNGA(GA)GACGAGCT-3′; 4, 5′-GAGCTGCGGGTGCGGCA-3′.

cDNA Library Screening. Poly(A)$^+$ RNA prepared from IMR32 neuroblastoma cells was used to construct a library in the λ Uni-Zap vector (Stratagene). For screening, a 570-bp fragment spanning peptide N sequence and an internal peptide C sequence (see Fig. 2*A*) was produced by PCR using primers 5 (5′-AGGACGAGCTGCGGGTGCGGCACCTGG-3′) and 6 [5′-TANGCNGC(AG)TCNCC(TC)TC-3′] and was used to screen 500,000 primary λ phage plaques. Two clones, IMR1 and IMR7, were obtained and sequenced.

Expression and Purification of Recombinant Proteins. (*i*) AUHp32. A PCR fragment encoding the sequence between the codon for Ser^{68} and the stop codon of *AUH* (see Fig. 2*A*) was subcloned into the vector pQE-9 (Qiagen). (*ii*) AUHp40–MBP—i.e., AUHp40 fused to maltose-binding protein (MBP). Clone IMR1 (see Fig. 2*A*) was subcloned into the vector pMalc2 (New England Biolabs). Both recombinant proteins were expressed in *Escherichia coli* and purified on a nickel nitrilotriacetic acid agarose column (for AUHp32) or an amylose column (for AUHp40–MBP) under nondenaturing conditions. As a control, MBP was expressed from the empty pMalc2 vector and purified as was AUHp40–MBP.

UV Cross-Linking. Protein (0.5–1 μg) was incubated with radiolabeled RNAs (50,000 cpm, 7–45 fmol per reaction mixture) in RB buffer supplemented with tRNA (200 μg/ml) for 20 min at 30°C and then UV-cross-linked (Stratalinker, four cycles of 120 mJ/cm^2). The mixture was treated with 10 units of RNase T1 at 37°C for 15 min. Samples were heat-denatured in gel-loading buffer and subjected to SDS/PAGE. In the competition analyses, the amount of purified AUHp40–MBP protein was reduced to 15 ng (170 fmol) per reaction mixture to ensure sensitivity and incubated with 10,000 cpm (6 fmol) of radiolabeled $(AUUUA)_6$ probe in the presence of the indicated nonradioactive competitors. Thus, the molar ratio of protein to the labeled RNA probe was 1:28 in each reaction. RNA probes were labeled by using [α-^{32}P]UTP and purified from urea/polyacrylamide gels.

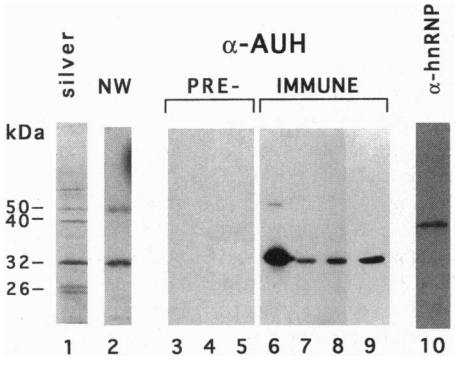

FIG. 1. Purification and analysis of AU-binding proteins from brain extract. Silver staining of affinity-purified preparation from human brain (lane 1, 20 ng of protein). Northwestern blot analysis (NW, lane 2) showing RNA-binding activity of proteins from lane 1. Western blots probed with preimmune (lanes 3–5), anti-AUH peptide (lanes 6–9), or anti-hnRNP sera (lane 10) are shown. Lanes: 3 and 6, recombinant AUHp32 (0.1 μg); 4 and 7, crude human brain extract a (male); 5 and 8, crude extract b (female) (100 μg); 9 and 10, affinity-purified preparation (20 ng).

Hydratase Measurement. A coupled assay of the hydration and subsequent dehydration reactions was performed as described (24). In brief, 1 mM 3-acetylpyridine adenine dinucleotide, 3.6 units (25 μg) of porcine heart L-hydroxyacyl-CoA dehydrogenase, and the indicated amounts of enoyl-CoA hydratase or recombinant proteins were mixed in 500 μl of 0.1 M Tris·HCl (pH 8.0) containing 0.1% Triton X-100. Reactions

A

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  -4  CAAC
   1  ATGGCGGCCGCGGTGGCGGCGGCACCTGGGGCCTTGGGATCCCTGCATGCTGGCGGCGCC   60
 (1)  M  A  A  A  V  A  A  A  P  G  A  L  G  S  L  H  A  G  G  A    (20)

  61  CGCCTGGTGGCCGCTTGCAGTGCGTGGCTCTGCCCGGGGTTGAGGCTGCCCGGCTCGTTG  120
(21)  R  L  V  A  A  C  S  A  W  L  C  P  G  L  R  L  P  G  S  L    (40)

 121  GCAGGCCGGCGAGCGGGCCCGGCGATCTGGGCCCAGGGCTGGGTACCTGCGGCCGGGGGT  180
(41)  A  G  R  R  A  G  P  A  I  W  A  Q  G  W  V  P  A  A  G  G    (60)

 181  CCCGCCCCGAAAAGGGGCTACAGCTCTGAGATGAAGACGGAGGACGAGCTGCGGGTGCGG  240
(61)  P  A  P  K  R  G  Y  (S)  S  E  M  K  T  E  D  E  L  R  V  R   (80)
                              X  X          peptide N
 241  CACCTGGAGGAGGAGAACCGAGGAATTGTGGTGCTTGGAATAAACAGAGCTTATGGCAAA  300
(81)  H  L  E  E  N  R  G  I  V  V  L  G  I  N  R  A  Y  G  K    (100)

 301  AATTCACTCAGTAAAAATCTTATAAAAATGCTATCAAAAGCTGTGGATGCTTTGAAATCT  360
(101) N  S  L  S  K  N  L  I  K  M  L  S  K  A  V  D  A  L  K  S   (120)

 361  GATAAGAAAGTACGGACCATAATAATCAGGAGTGAAGTCCCAGGGATATTCTGTGCTGGT  420
(121) D  K  K  V  R  T  I  I  I  R  S  E  V  P  G  I  F  C  A  G   (140)

 421  GCTGACCTTAAGGAAAGAGCCAAAATGAGTTCCAGTGAAGTTGGTCCTTTTGTCTCCAAA  480
(141) A  D  L  K  E  R  A  K  M  S  S  S  E  V  G  P  F  V  S  K   (160)

 481  ATAAGAGCAGTGATTAACGATATTGCTAATCTTCCAGTGCCAACAATTGCAGCAATAGAT  540
(161) I  R  A  V  I  N  D  I  A  N  L  P  V  P  T  I  A  A  I  D   (180)

 541  GGACTCGCTTTAGGTGGTGGTCTTGAACTGGCTTTAGCCTGTGATATACGAGTAGCAGCT  600
(181) G  L  A  L  G  G  G  L  E  L  A  L  A  C  D  I  R  V  A  A   (200)

 601  TCCTCTGCAAAAATGGGCCTGGTTGAAACAAAATTGGCGATTATTCCTGGTGGAGGGGGG  660
(201) S  S  A  K  M  G  L  V  E  T  K  L  A  I  I  P  G  G  G  G   (220)

 661  ACACAGCGATTGCCACGCGCCATTGGAATGTCCCTGGCCAAGGAGCTCATATTCTCTGCG  720
(221) T  Q  R  L  P  R  A  I  G  M  S  L  A  K  E  L  I  F  S  A   (240)

 721  CGAGTCCTCGATGGCAAAGAAGCCAAAGCAGTGGGCTTAATCAGCCACGTTCTGGAACAG  780
(241) R  V  L  D  G  K  E  A  K  A  V  G  L  I  S  H  V  L  E  Q   (260)
                                                peptide C
 781  AACCAGGAGGGAGACGCGGCCTACAGGAAGGCCTTGGACCTGGCGAGAGAGTTTTACCT  840
(261) N  Q  E  G  D  A  A  Y  R  K  A  L  D  L  A  R  E  F  L  P   (280)

 841  CAGGGACCTGTTGCAATGAGAGTGGCAAAATTAGCAATTAATCAAGGGATGGAGGTCGAT  900
(281) Q  G  P  V  A  M  R  V  A  K  L  A  I  N  Q  G  M  E  V  D   (300)

 901  TTAGTAACAGGGTTAGCCATAGAAGAAGCTTGTTATGCTCAGACCATTCCAACAAAAGAC  960
(301) L  V  T  G  L  A  I  E  E  A  C  Y  A  Q  T  I  P  T  K  D   (320)

 961  AGACTTGAAGGTCTTCTTGCTTTTAAAGAGAAAAGGCCCCCTCGCTATAAAGGAGAATAA 1020
(321) R  L  E  G  L  L  A  F  K  E  K  R  P  P  R  Y  K  G  E  *   (339)

1021  AAGGAACAGAAATTCTTAAGATGCCAATGTAATAAATGTACTTCCTGGAAGTGTCTTTCG 1080
1081  GATCCACTATATGCCTCAGCACATGGAACCTTAATGACCAAAGTGAAGAGCAGATTATTC 1140
1141  ATACGGTGTAATAAGCGTCTGGAATGGACCCATCCGTGTACTTCATTCAAATGTGTAAAT 1200
1201  GTCATATTCATTCAGATTTATAAAGCTAGTAGTGTATAGTCAGAAACAGAATCAAAGTTA 1260
1261  GATATACATTTTTAAATATTTACTGCATATGAGGCTTTCTGTTAATTTTTTAATGTGAAT 1320
1321  AATTTATATATTGCACATTCTAGGAATAATATTGATTGTATGTCTACTGTGCTGCATTAA 1380
1381  GAAAATAAAATTTCTATATACCAAAAATGTGAAGTTATACCAAATAAAGTTTCTAAGTGA 1440
1441  TTAATGCATACGAACAGCTACATATACATATATCTAAACCTGAAAAATGAATTGATATTC 1500
1501  TGAGTGAAAACTACCTAATATAAATAAAATTAGTGAAAAGAAAA                 1544
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B

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                101                                                150
   AUH HUM      NSLSKNLIKM LSKAVDALKS DKKVRTIIIR SEVPGIFCAG ADLKE.RAKM
  ECHM RAT      NALCNGLIEE LNQALETFEE DPAVGAIVLT GGEKA.FAAG ADIKE.MQNR
   ECH RAT      NAVSPTVIRE VRNGLQKAGS DHTVKAIVIC GA.NGNFCAG ADIHGFSA..
 FAOB PSEFR     NKFNRLTLNE LRQAVDAIKA DASVKGVIVS SG.KDVFIVG ADITEFVENF
                151                                                200
   AUH HUM      SSSEVGPFVS KIR..AVIND IANLPVPTIA AIDGLALGGG LELALACDIR
  ECHM RAT      TFQDC..YSG KFL..SHWDH ITRIKKPVIA AVNGYALGGG CELAMMCDII
   ECH RAT      .......FTP GLALGSLVDE IQRYQKPVLA AIQGVALGGG LELALGCHYR
 FAOB PSEFR     KLPDAELIAG NLEANKIFSD FEDLNVPTVA AINGIALGGG LEMCLAADFR
                201                                                250
   AUH HUM      VAASSAKMGL VETKLAIIPG GGGTQRLPRA IGMSLAKELI FSARVLDGKE
  ECHM RAT      YAGEKAQFGQ PEILLGTIPG AGGTQRLTRA VGKSLAMEMV LTGDRISAQD
   ECH RAT      IANAKARVGL PEVTLGILPG ARGTQLLPRV VGVPVALDLI TSGKYLSADE
 FAOB PSEFR     VMADSAKIGL PEVKLGIYPG FGGTVRLPRL IGVDNAVEWI ASGKENRAED
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FIG. 2. (*A*) cDNA and deduced amino acid sequence of the *AUH* clone IMR1. Numbering starts from the putative initiation methionine from clone IMR1. PCR clone HB8 includes the sequence between nt 155 and 1402. The N terminus of the purified brain protein was located at Ser^{68} (circled, see also text). Asterisk indicates stop codon. Boldface type represents putative poly(A) addition signals, the first presumably used in HB8 and the second used in IMR1. Peptide sequences obtained from the affinity-purified AUHp32 are underlined and unidentified residues are indicated by X. (*B*) Amino acid sequence homology between AUH and enoyl-CoA hydratases. The amino acid sequence of human AUH was aligned with that of rat mitochondrial hydratase (ECHM), rat peroxisomal hydratase (ECH), and *Pseudomonas fragi* fatty oxidation complex α subunit (FAOB) by using the PILEUP program. Identical amino acids are in boldface type. Putative hydratase active site (27) is localized between residues 181 and 221.

were started by adding the substrate crotonoyl-CoA (100 μM), and the A_{365} was measured. Reagents including bovine liver crotonase (enoyl-CoA hydratase, 1000 units/mg) were from Sigma.

Matrix Immobilization of AUH Protein. AUHp40–MBP fusion protein (10 μg) was mixed with oligo(dT)-cellulose (30 μl) loaded with poly(A)-tailed $(AUUUA)_6$ in buffer B (10 mM Tris·HCl, pH 7.5/115 mM KCl/5 mM $MgCl_2$/5 mM $CaCl_2$; 100 μl) and incubated for 30 min at room temperature. Unbound material was collected after a quick centrifugation at 3000 rpm in an Eppendorf minicentrifuge for 10 sec, the sedimented matrix was rinsed twice with 30 μl of buffer B, and the rinses were combined with the unbound fraction. The matrix was then washed twice with 1 ml of buffer B, followed by two washes with 1 ml of buffer B adjusted to 0.5 M NaCl. In parallel, only 0.1 μg of bovine crotonase, but supplemented with 10 μg of MBP to adjust the protein concentration, was processed in the same manner. Hydratase activity of matrix-bound proteins was measured after sedimenting the insoluble matrix in the reaction mixture by quick centrifugation at indicated time points. This cleared mixture was combined again with the matrix after each measurement, and the reaction was continued.

To monitor retention of proteins at the end of the reaction, the matrix was sedimented, and the bound protein was eluted into 30 μl of SDS-containing Laemmli gel loading buffer. Initial binding efficiency was likewise determined from a duplicate sample immediately after the washing step.

RESULTS

For affinity purification of AU-binding proteins from human brain, we used a cluster of six AUUUA motifs, referred to as $(AUUUA)_6$, linked to oligo(dT)-cellulose. As shown in Fig. 1, lane 1, a 32-kDa protein designated AUHp32 was the major species detected. The N-terminal end and four tryptic peptides of AUHp32 were microsequenced. This protein was not the 32-kDa hnRNP A1 protein (25) since in the affinity-purified preparation, an anti-hnRNP monoclonal antibody (19) recognized only the 40-kDa protein, which is likely to be hnRNP C protein (lane 10). AUHp32 was able to bind RNA in a Northwestern blot analysis using labeled ARE-containing RNA from the 3′ UTR of GM-CSF (lane 2). The protein species of 50 kDa was identified by peptide sequencing as La protein, an autoimmune antigen known to bind to poly(U) (26) (J. N. and J. H., unpublished data).

Degenerate oligonucleotides were designed as primers for PCR cloning. From human brain cDNA, a 1245-nt fragment was obtained (clone HB8). In addition, a 1544-nt clone was isolated from a cDNA library of IMR32 neuroblastoma cells (clone IMR1), which included the entire HB8 sequence. The deduced amino acid sequence contained AUHp32 peptide sequences (Fig. 2*A*).

IMR1 composed an open reading frame encoding 339 aa (Fig. 2*A*), which corresponds to a 40-kDa protein (AUHp40). However, when the size of the AUH protein in additional brain extracts was reinvestigated by Western blot analysis using an anti-peptide serum, we consistently found a 32-kDa band but failed to detect a 40-kDa band in independent preparations of crude brain extract (Fig. 1, lanes 7 and 8). The 40-kDa band present in the affinity-purified sample (lane 1) contained hnRNP C protein (lane 10) but was not related to AUHp32 (lane 9). Therefore, we assume that the mature form of AUH in brain is 32 kDa.

The first two N-terminal amino acids of AUHp32 remained unidentified due to a high background in the peptide sequencing reaction. However, since three cDNA clones (data not

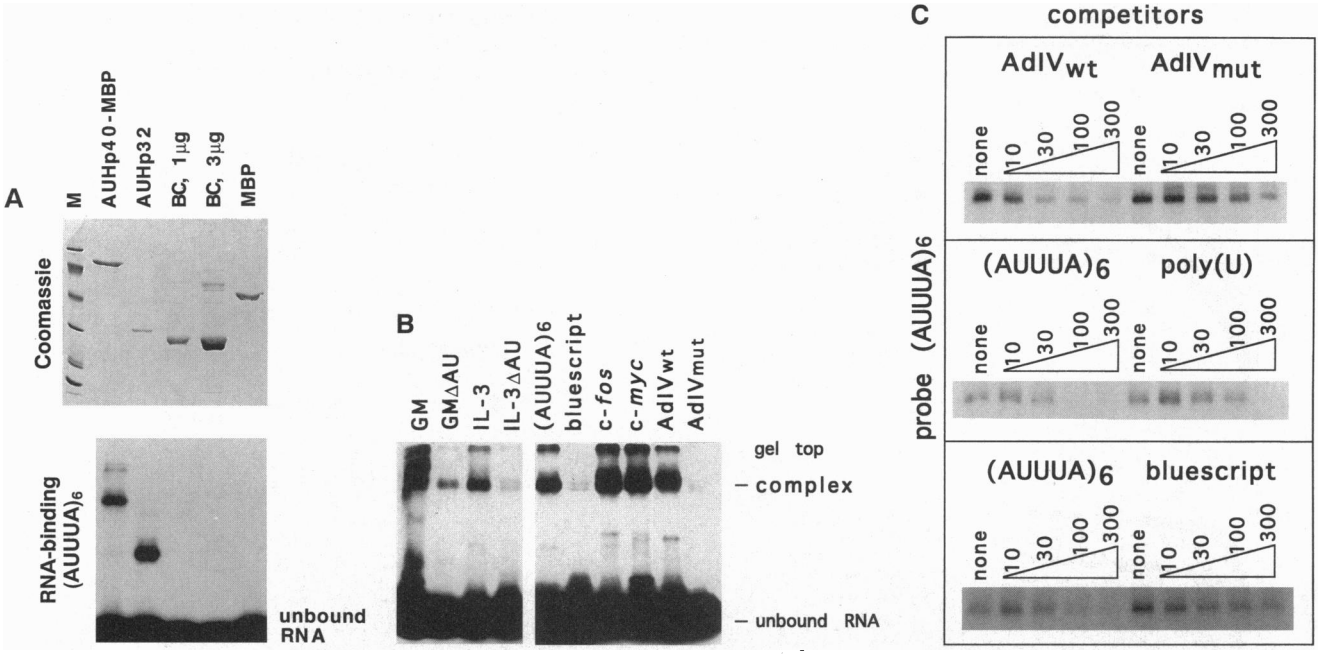

FIG. 3. RNA binding activity of recombinant AUH. (*A*) RNA binding activity demonstrated by UV-cross-linking experiments with labeled $(AUUUA)_6$. (*Upper*) Coomassie blue staining of proteins. (*Lower*) RNA binding of corresponding proteins. One microgram of each protein was used unless otherwise mentioned. Lanes: M, size markers at 98, 67, 45, 31, 21, and 14 kDa from the top (Bio-Rad); BC, bovine crotonase (enoyl-CoA hydratase). (*B*) Specificity of RNA binding. UV-cross-linking experiments were performed with various labeled RNAs and 0.5 μg of purified AUHp40–MBP. Lanes: GM, GM-CSF 3′ UTR (240 nt); GMΔAU, GM-CSF 3′ UTR with deletion of seven out of eight AUUUA motifs (157 nt); IL-3, IL-3 3′ UTR containing sequence downstream of nt 2508 in the IL-3 gene (385 nt); IL-3ΔAU (16), IL-3 3′ UTR with deletion of all eight AUUUA motifs (169 nt); $(AUUUA)_6$, 71 nt including vector sequence; Bluescript (Stratagene), control T7 transcript of Bluescript SK linearized with *Xba* I (89 nt); c-*fos* (3′ UTR, 270 nt); c-*myc* (3′ UTR, 428 nt); AdIVwt, the adenovirus IVa2, which contains one AUUUA motif flanked by U-rich sequences, wild type (87 nt); AdIVmut, AdIV-AGGUA mutant (87 nt). Plasmid constructs for GM, GMΔ, c-*fos*, c-*myc*, AdIVwt, and AdIVmut were kindly provided by E. Vakalopoulou and the sequences were published in ref. 6. Constructs for IL-3 and its mutant were prepared by S. Hahn (University of Basel). (*C*) Competition assays. Competition assay was done with the radioactive $(AUUUA)_6$ probe in the presence of the indicated molar excess of nonradioactive competitors.

shown) specified Ser for both positions 68 and 69, we concluded that the N terminus of the AUHp32 is Ser^{68} (Fig. 2*A*). In HeLa and IMR cells, AUH was expressed as a single mRNA species of 1.8 kb (H. W., unpublished data).

The sequence of AUH, different from the two previously cloned AU-binding proteins, Hel-N1 (12) and AUF1 (11), was not found in the database (February 12, 1994). Computer analysis (MOTIFS/GCG) failed to reveal motifs characteristic for RNA binding (28) as present in small nuclear RNP, hnRNP, AUF1, and Hel-N1 proteins. Significant homology, however, to enoyl-CoA hydratase was revealed by the BLASTP program (Fig. 2*B*). A 104-aa stretch containing residues 163–266 exhibited a 42% identity (65% similarity) with rat peroxisomal hydratase. This stretch included a postulated enzyme active center conserved among the hydratase family (residues 181–221) (27). This suggested that the AUH gene product may have both RNA binding and hydratase activities.

To test this proposition, we examined RNA binding activity of purified recombinant AUHp40 and AUHp32 by using a UV-cross-linking assay. Both recombinant AUHp32 and AUHp40–MBP bound efficiently to $(AUUUA)_6$, whereas MBP, the fusion partner of AUHp40, did not (Fig. 3*A*). Contrary to AUH, a commercial preparation of bovine crotonase (enoyl-CoA hydratase) did not bind to $(AUUUA)_6$ under the same conditions.

The specificity of binding was studied *in vitro* by UV-cross-linking assays using transcripts with or without AREs and the recombinant AUHp40–MBP (Fig. 3*B*). The protein bound to the 3′ UTRs from GM-CSF, IL-3, c-*fos*, c-*myc*, and adenoviral AdIVwt transcripts containing one AUUUA motif (6) and to $(AUUUA)_6$ but failed to bind to IL-3ΔAU, AdIVmut (motif mutated to AGGUA), or Bluescript transcripts. Binding to GM-CSFΔAU mutant containing only one AUUUA was reduced.

RNA binding specificity was further examined in competition assays. While AdIVwt competed as efficiently as $(AUUUA)_6$, poly(U) was less efficient, and no significant competition was observed with either AdIVmut or Bluescript. Thus, the evidence suggests that *AUH* codes for a protein that, at least *in vitro*, binds specifically to transcripts containing AREs.

Next we examined the recombinant AUH proteins for hydratase activity. Recombinant AUHp32 displayed enoyl-CoA hydratase activity, whereas the control column (nickel nitrilotriacetic acid agarose column) eluate loaded with a nonexpressing *E. coli* extract did not (Fig. 4*A*). AUHp32, however, was obviously less active than bovine crotonase (enoyl-CoA hydratase). Hydratase activity of AUHp40 was also less efficient than bovine crotonase in multiple assays (Fig. 4*B*). Control column (amylose column) eluates containing the same amount of MBP in an equivalent volume showed no activity, and the flowthrough fraction was likewise negative. Thus, it is unlikely that we measured contaminating *E. coli* hydratase activity, as the two recombinant proteins were obtained by different purification protocols. Hence, we concluded that *AUH* codes for a bifunctional protein. Specific activity of AUHp40–MBP on crotonoyl-CoA was estimated to be 0.5–1.0 unit/mg, ≈1000-fold lower than that of bovine crotonase (Fig. 4*C*).

Finally, we compared the behavior of AUHp40–MBP and bovine crotonase during the $(AUUUA)_6$ affinity procedure.

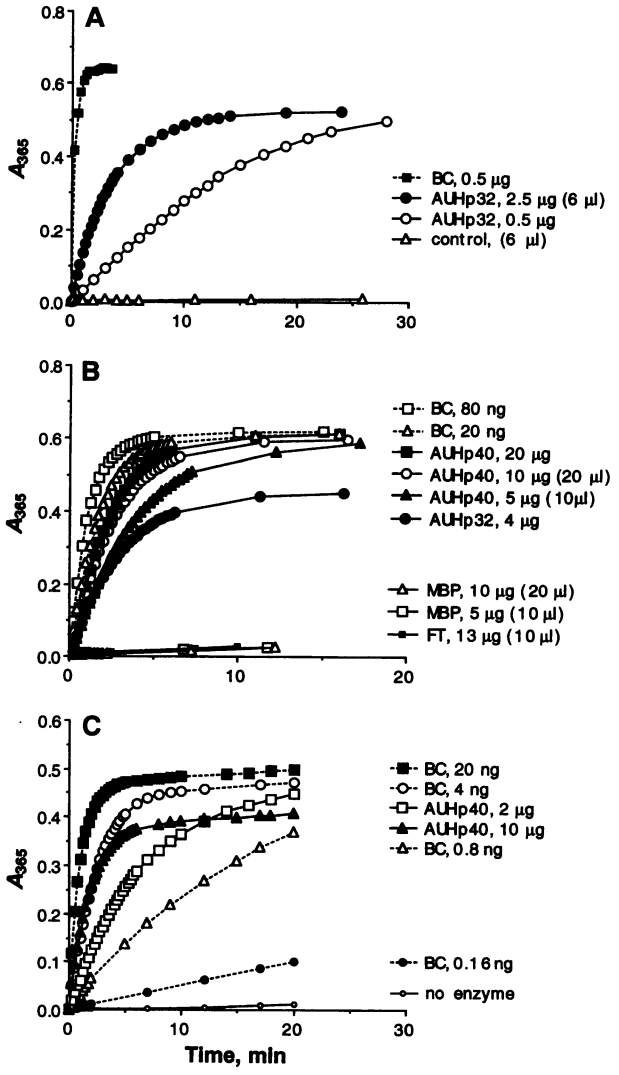

FIG. 4. Enoyl-CoA hydratase activity of AUH proteins. The protein and its concentration used for hydratase assay are indicated. BC, bovine crotonase (enoyl-CoA hydratase); FT, flowthrough fraction from an amylose column loaded with *E. coli* proteins. (*A*) AUHp32. (*B*) AUHp40. (*C*) Titration of BC/AUHp40.

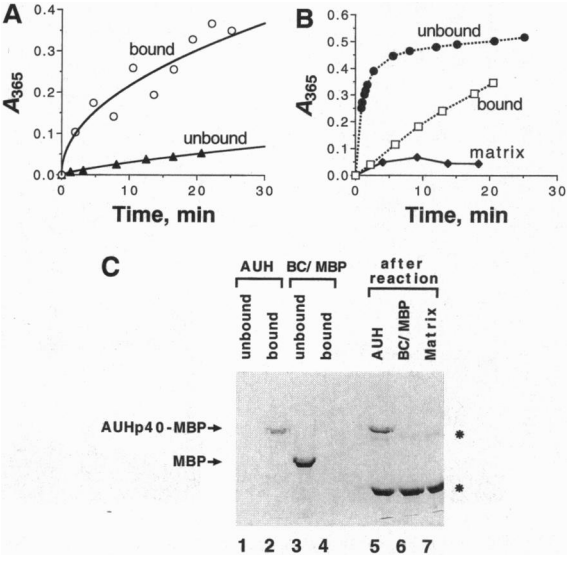

FIG. 5. Hydratase activity of immobilized recombinant AUH protein. Either 10 µg of AUHp40–MBP (*A*) or 10 µg of MBP and 0.1 µg of bovine crotonase (BC/MBP) (*B*) were incubated with an $(AUUUA)_6$ matrix. Unbound and bound fractions were separated and assayed for enoyl-CoA hydratase activity. (*C*) Coomassie blue staining of fractions. Lanes: 1–4, unbound and bound fractions before the hydratase assay; 5–7, retention of bound protein monitored after the completion of hydratase assay. Bands indicated by the asterisks in lanes 5–7 are due to L-hydroxyacyl-CoA dehydrogenase included in this assay.

AUHp40–MBP (10 μg) and, in parallel, bovine crotonase (0.1 μg) supplemented with MBP (10 μg) were loaded on the $(AUUUA)_6$ matrix. As shown in Fig. 5 *A* and *B*, hydratase activity of AUHp40–MBP was almost exclusively recovered in the bound form. Coomassie blue staining demonstrated this binding of AUHp40–MBP was specific since MBP did not bind (Fig. 5*C*, lanes 1–4). The opposite was shown for bovine crotonase, where only ≈1% of hydratase activity (estimated from Fig. 4*C*) was associated with the $(AUUUA)_6$ matrix, as expected from its lack of RNA binding shown in Fig. 3*A*. The enzymatic activity of the bound AUHp40–MBP fraction could mean that actually bound molecules can catalyze the reaction or that molecules dissociate from the matrix and become enzymatically active. We therefore compared the amount of protein bound to the matrix at the beginning and the end of the reaction. AUHp40–MBP did not decline (Fig. 5*C*, compare lanes 5 and 2). This suggested that both enoyl-CoA hydratase and RNA binding activities of AUHp40–MBP could take place simultaneously.

DISCUSSION

We describe here an AU-binding protein, AUH, with an unexpected intrinsic enzymatic activity. AUH lacks canonical RNA recognition motifs, which sets it apart not only from the two previously cloned AU-binding proteins, AUF1 (11) and Hel-N1 (12), but also from hnRNP proteins (28). An unexpected homology, however, was found with enoyl-CoA hydratases. Amino acid sequence comparison of AUH with a recently published human mitochondrial hydratase sequence (29) showed 29% identity within a stretch of 260 aa, mostly centered in the region of a putative hydratase active site.

AUH was able to catalyze the hydration of crotonoyl-CoA but was ≈1000-fold less active than bovine crotonase. This may be because crotonoyl-CoA is not the physiological substrate of the hydration reaction catalyzed by AUH. At this time, the physiological substrate is unknown. AUHp40–MBP was enzymatically active even when linked to a solid matrix (Fig. 5*A*), which suggests that the RNA binding domain is different from the catalytic domain.

RNA binding depended highly on the presence of an ARE. A very similar binding specificity was seen with a 32-kDa protein in HeLa cells reported by Vakalopoulou *et al.* (6). A role for this protein in oncogenesis has been proposed, in that herpes virus Saimiri sequesters it via a viral AU-rich mRNA species, thereby, elevating lymphokine mRNA levels (30).

Our data provide an example of a functional and biochemical chimerism, namely, RNA binding coupled with metabolic enzyme activity (31). The physiological relevance of the connection between AU-mediated mRNA decay and fatty acid degradation remains an enigma at this time. The paradigm of a bifunctional RNA binding protein is the iron-response-element binding protein (IRE-BP), which was recently found to have aconitase activity, an enzyme of the Krebs cycle (32). IRE-BP regulates expression of ferritin and transferrin receptor by binding to the 5′ and 3′ UTRs, respectively, of the corresponding mRNA. Binding of IRE-BP to transferrin receptor mRNA is thought to stabilize this transcript by preventing nuclease attack (33). It is intriguing that both IRE-BP and AUHp32/p40 have hydration enzyme activity and that both bind near, if not at, signals regulating mRNA decay.

While a number of AU-specific RNA binding proteins have been reported (5–10), none has been demonstrated yet to direct mRNA degradation. Availability of recombinant AUF1 (11), Hel-N1 (12), and AUH should allow this issue to be studied.

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