Identification of a novel AU-rich-element-binding protein which is related to AUF1

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The AU-rich element (ARE) is an important instability determinant for a large number of early-response-gene mRNAs. AREs also mediate the stabilization of certain pro-inflammatory mRNAs, such as tumour necrosis factor (TNF)- α and cyclo-oxygenase-2 (COX-2), in response to inflammatory stimuli. To understand how AREs control mRNA stability, it is necessary to identify *trans*-acting factors. We have purified a new ARE-binding protein and identified it as CArG box-binding factor-A (CBF-A). The amino acid sequence of CBF-A is highly similar to that of the ARE-binding protein AUF1. Recombinant CBF-A bound the COX-2 and TNF- α AREs, but not a non-specific control RNA. In contrast, in an electrophoretic-mobility-shift assay (EMSA) of crude RAW 264.7 macrophage-like cell extracts, an antiserum that recognizes both AUF1 and CBF-A failed to supershift complexes formed on the TNF- α ARE, but did supershift a complex specific for the COX-2 ARE. CBF-A exists

as two isoforms, p37 and p42, that differ by a 47-amino-acid insertion close to the C-terminus. By expressing epitope-tagged isoforms of CBF-A it was shown that the p42 isoform binds the COX-2 ARE in EMSA of crude cell extracts. In a HeLa-cell tetracycline-regulated reporter system, overexpression of the p42 CBF-A isoform resulted in stabilization of a COX-2 ARE reporter mRNA. Epitope-tagged p42 CBF-A expressed in HeLa cells co-immunoprecipitated with endogenous COX-2 mRNA, but not glyceraldehyde-3-phosphate dehydrogenase mRNA, as shown by reverse-transcription PCR. The similarity between CBF-A and AUF1 suggests that CBF-A could be re-named AUF2.

Key words: cyclo-oxygenase 2 (COX-2), mRNA stability, post-transcriptional regulation, tumour necrosis factor (TNF), 3'-untranslated region.

INTRODUCTION

It has long been recognized that an AU-rich element (ARE) in the 3'-untranslated region (3'-UTR) of an mRNA targets it for rapid degradation. In the case of proto-oncogenes, AREs are thought to limit their expression to a transient burst that follows transcriptional activation. AREs also exist in the 3'-UTRs of cytokines and chemokines [e.g. interleukin (IL)-1, -2, -6 and -8, tumour necrosis factor (TNF)- α , granulocyte-macrophage colony stimulating factor (GM-CSF), interferon- β and γ , growth-regulated oncogene (GRO)- α , macrophage inflammatory protein-1 α (MIP-1 α)] and mRNAs of inducible enzymes such as cyclo-oxygenase-2 (COX-2) and nitric oxide synthase-2, which produce inflammatory mediators [1]. These mRNAs are induced in a more sustained manner than those of proto-oncogenes and are stabilized by inflammatory stimuli which target the ARE through the p38 mitogen-activated protein kinase (MAPK) signalling pathway. This pathway has been shown to stabilize mRNAs for COX-2 [2,3], TNF- α [4–6], GRO- α , IL-1 β [7], IL-6 [6–8] and MIP-1 α [6]. The two other MAPKs, c-Jun N-terminal kinase and p42 MAPK [or extracellular-signal regulated kinase ('ERK')] have also been shown to stabilize IL-2 and GM-CSF mRNAs respectively [9,10].

Overexpression of active forms of the p38 activator (MAP kinase kinase 6) or the kinase downstream of p38 [MAPK-activated protein kinase-2 (MAPKAPK-2)] stabilizes mRNA reporters containing AREs of IL-6, IL-8, c-Fos, GM-CSF [11], COX-2 [12] and TNF- α [4]. Early studies employing p38 inhibitors implicated the pathway in translation of TNF- α and IL-1 β mRNAs [13,14]. The TNF- α ARE regulates translation of a reporter gene in a murine macrophage line ([15], but see [15a]) and TNF- α mRNA can be induced, but not translated, in MAPKAPK-2-null mice [16]. It is likely that the stabilization and translation of ARE-containing mRNA are intimately linked and could be regulated by a common mechanism.

The stability of ARE-containing mRNA is thought to be controlled by ARE-binding proteins. A large number of proteins can bind to these elements, but only a few have been demonstrated to bind in a specific fashion. These include ARE/poly(U)-binding/degradation factor (AUF1), which is also called heterogeneous nuclear ribonucleoprotein D ('hnRNP D') [17,18], Hu antigen R (HuR; a member of the ELAV family of RNA-binding proteins) [19,20], tristetraprolin [21], Y-box-binding factor ('YB-1') [22], T-cell-restricted intracellular antigen-related ('TIAR') [23] and the related protein T-cell-restricted intracellular antigen-1 ('TIA-1') [24].

Abbreviations used: ARE, AU-rich element; AUF1, ARE/poly(U)-binding/degradation factor 1; CBF-A, CArG box-binding factor-A; COX-2, cyclo-oxygenase-2; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EMSA, electrophoretic-mobility-shift assay; FCS, fetal-calf serum; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Lys; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte-macrophage colony stimulating factor; GRO, growth-regulated oncogene; hnRNP, heterogeneous nuclear ribonucleoprotein; HuR, Hu antigen R; HRP, horseradish peroxidase; IL, interleukin; MALDI, matrix-assisted laser-desorption ionization; MAPK, mitogen-activated protein kinase; MAPKAPK-2, MAPK-activated protein kinase-2; MIP, macrophage inflammatory protein; MS/MS, tandem MS; NCBI, National Center for Biotechnology Information; NP-40, Nonidet P40; RT-PCR, reverse-transcription PCR; TNF, tumour necrosis factor; 3'-UTR and 5'-UTR, 3'- and 5'-untranslated regions.

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The AUF1 exon-7-like sequence for CBF-A has been submitted to the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession number AY137376.

AUF1 was originally identified as a protein which bound to the c-Myc ARE and co-purified with a c-Myc mRNA degradative activity [17,18]. Purified AUF1 was also shown to bind the GMCSF 3'-UTR [17]. Recombinant AUF1 binds the AREs from c-Myc, [25] c-Fos [25], and TNF- α [26] in addition to other mRNAs which are U-rich or contain AUUUA repeat sequences [27]. There is a large body of evidence that it plays a role in mRNA destabilization.

In order to identify proteins which bind strongly to AREs of mRNAs of proteins of the inflammatory response, we have used a strategy involving electrophoretic-mobility-shift assays (EMSAs) for RNA-binding proteins, protein purification and MS. This has led to the isolation and identification of two forms of a murine protein called 'CARG box-binding factor-A' (CBF-A), which has not previously been shown to bind AREs, but which is highly similar to AUF1. Although the protein was identified by its ability to bind the TNF- α ARE *in vitro*, it seems that, in cell extracts, it has more selectivity for the COX-2 ARE.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM) and the antibiotic Geneticin (G418) sulphate were from PAA Laboratories (Linz, Austria). Fetal-calf serum (FCS), pCMV-FLAG2 and anti-FLAG M2 monoclonal antibody were from Sigma-Aldrich Ltd (Poole, Dorset, U.K.) (FLAG is Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys). HeLa tet-off cells were obtained from BD Biosciences Ltd, Oxford, U.K.). SuperFect was purchased from Qiagen Ltd, Crawley, West Sussex, U.K.). pGEX4T1, pGEX4T3, [α - 32 P]-UTP, random hexamers, RNAGuard, Mono-Q and Mono-S columns, poly(U)-Sepharose 4B and an Enhanced Chemiluminescence (ECL[®]) Kit were from Amersham Pharmacia Biotech Ltd (Little Chalfont, Bucks., U.K.). *Taq* polymerase, reverse transcriptase and restriction enzymes were from New England Biolabs Ltd (Hitchin, Herts., U.K.). T7 RNA polymerase and the Ampliscribe kit were from Epicentre Technologies Inc. (Madison, Wisconsin, U.S.A.). RNasin, RNase-free DNase I and modified trypsin were from Promega Ltd (Southampton, UK). RNase T1 was from Roche Diagnostics Ltd (Lewes, East Sussex, UK). Ribonuclease protection assays were performed by using a kit from Ambion Ltd (Huntingdon, Cambs., U.K.). RNase H-free Superscript II reverse transcriptase was from Invitrogen (Paisley, Renfrewshire, Scotland, U.K.). The Pepmap C₁₈ column was from LC Packings (Amsterdam, The Netherlands). PVDF membrane was from DuPont Ltd (Stevenage, Herts., U.K.). Rabbit antisera were made to the CBF-A N-terminal residues EEQPMETT GATEN and the CBF-A C-terminus (QRRGGHQN NYKPY) according to standard procedures at the Babraham Institute (Cambridge, U.K.). A rabbit antiserum to AUF1 (whole protein) was generously given by Dr Gary Brewer (Robert Wood Johnson Medical School, Piscataway, NJ, U.S.A.). 19F12 anti-HuR murine monoclonal antibody was from Molecular Probes Inc. (Eugene, OR, U.S.A.). pTet-BBB was a gift from Dr Ann-Bin Shyu, Houston Medical School, Houston, TX, U.S.A.). A hybridap cDNA clone containing the entire coding sequence of p37 CBF-A was kindly given by Dr Tomas Leanderson (Lund University, Lund, Sweden). The ClustalX (1.8) program [28] is available freely over the internet (<ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/>).

Cell culture

The RAW 264.7 murine macrophage-like cell line, HeLa cells and the HeLa tet-off line were maintained in DMEM supple-

mented with 10% (v/v) FCS. The culture medium for HeLa tet-off cells also included G418 (100 μ g $\cdot$ ml⁻¹). Cells were maintained at 37 °C in 5% CO₂. For overexpression experiments HeLa and HeLa tet-off cells were seeded, transfected by lipofection using Superfect and harvested as described previously [12].

Plasmids

The 75 nt TNF- α ARE [29], c-Myc 3'-UTR [12] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 3'-UTR [29] EMSA riboprobe template plasmids were prepared by PCR of human genomic DNA as described previously. The COX-2 EMSA probe template was constructed by insertion of a double-stranded oligonucleotide (5'-GATCCAAGTCTAATGATCAT-ATTTATTTATTTATATcAACCATcTCTATTAAATTTAAT-TATTTAATAATTTTATATTTAAA-3'; 5'-GATCTTAATA-TAAATATTATTAAATAATTTAAATTAATAGAgATGGT-TgATATAAAATAAAATAATATGATCATTAGACTTG-3') into the pBluescript *Bgl*II site. This contains two mutations shown in lower case where guanine bases in the wild-type sense-strand sequence were substituted for cytosine residues to prevent cleavage of the ARE by RNase T1. pGEX-HuR was prepared by amplifying the HuR coding sequence from I.M.A.G.E. (Integrated Molecular Analysis of Genomes and their Expression) Consortium [30] Clone ID: 121366 using primers 5'-GCCG-GCGAATTCATGTCTAATGGTTATGAAGACCACATG-GCC-3' and 5'-GCCGGCGAATTCCTTATTTGTGGGACTT-GTTGGTTTTGAAGGAAACC-3'. The PCR product was cut with *Eco*RI and cloned into the *Eco*RI site of pGEX4T1. pTet-BBB-COX-2 ARE was prepared as described previously [12] by inserting a 123 nt sequence that spans the main regulatory ARE of the COX-2 3'-UTR into the unique *Bgl*II site in the β -globin 3'-UTR in pTet-BBB. Plasmids containing p37 and p42 CBF-A cDNAs were constructed as follows: The p37 CBF-A construct was prepared by PCR amplification of the entire coding sequence from the hybridap CBF-A clone. The primers used were CBFCDs2 (5'-GCGAATTCATGTCTCGGACGCGGCTGAG-GAGCAG-3') and CBFNE (5'-GCGGCCGCGAATTCCTC-AGTATGGCTTGTAAGTTATTCTGATGACC-3'). The PCR product was cut and cloned into pGEX4T3 using *Eco*RI. The p42 CBF-A coding sequence was cloned by preparing two PCR products. The first was amplified from the hybridap clone using the primers CBFCDs2 and CBF3X (5'-GCGCTTCTCTAG-AACTTTCTTCACAGGATCTTCC-3') to yield product I. The latter primer contains a silent mutation which introduces an *Xba*I site. The second PCR amplification was performed using the primers CBF5X (5'-GCGGTTCTAGAGAAGAAATTTCT-ATACTGTCACTGG-3') and CBF3NE on cDNA prepared from RAW 264.7 cell RNA by reverse transcription (RT) to yield product II. CBF5X also contains a silent mutation which introduces an *Xba*I site. Product I was cut with *Eco*RI and *Xba*I, and product II was cut with *Not*I and *Xba*I. The restricted fragments were joined and inserted into pGEX4T3, which had been restricted with *Eco*RI and *Not*I. CMV-CBF-A-FLAG vectors were prepared by subcloning *Eco*RI fragments from pGEX p37 CBF-A or pGEX p42 CBF-A into the *Eco*RI site of pCMV-FLAG2.

In vitro transcription of riboprobes

α - 32 P-labelled RNA probes were prepared by *in vitro* transcription as described previously [29]. Unlabelled riboprobes for competition assays were prepared using the Ampliscribe kit according to the manufacturer's instructions, with the inclusion of 0.25 μ Ci of [α - 32 P]UTP (800 Ci $\cdot$ mmol⁻¹) to permit RNA

quantification. Probe concentrations were calculated from specific activities determined by liquid-scintillation counting.

RNA EMSA

Typically 10 μ l of extract was incubated for 10 min at 20 °C with 9 μ l of EMSA buffer [20 mM Hepes (pH 7.6)/6 mM MgCl_2 /40 mM KCl/2 mM dithiothreitol (DTT)/10% (v/v) glycerol] and 1 μ l of α - 32 P-labelled RNA probe (20 fmol; approx. 500 000 c.p.m.). A 5 μ l portion of EMSA stop solution [RNase T1 (7 units $\cdot$ μ l $^{-1}$)/heparin (7 mg $\cdot$ ml $^{-1}$)/60% (v/v) glycerol/0.1% (w/v) Bromophenol Blue] was then added and the mixture incubated for 5 min at 20 °C. For antibody supershifts, 1 μ l of antiserum or preimmune serum (or 1 μ g of murine monoclonal antibody or murine IgG) was added together with 20 units of the RNase inhibitor RNasin and the mixture incubated for 1 h on ice before RNase T1 treatment. Samples were loaded on to a 4% (w/v) polyacrylamide gel containing 0.5 $\times$ TBE [Tris/borate/EDTA (1 $\times$ TBE is 45 mM Tris/borate/10 mM EDTA)] and 2.5% (v/v) glycerol (which had been pre-electrophoresed for 1 h at 150 V) and run at 150 V for 3.5 h at 4 °C. Gels were dried on 3 MM blotting paper (Whatman) and images were obtained using a Fuji-BAS 2000 phosphorimager (Fuji, Tokyo, Japan). In EMSA with the wild-type COX-2 probe, only extremely weak complexes were formed by crude extracts. This is due to the presence of two guanylate residues within the ARE which are cleavage sites for RNase T1. In order to obtain stable complex formation, these two residues were mutated to cytidine residues. A more complete description of this observation is available from J.L.E.D. on request.

Cell fractionation

This was performed as described by Dean et al. [29]. Briefly, cells were harvested and rinsed in PBS. The cells were lysed with a buffer containing Nonidet P40 [NP-40; 0.5% (v/v)] and nuclei ('N') pelleted by gentle centrifugation (2000 g , 10 min). Proteins were extracted from the nuclei in a high-salt buffer. The post-nuclear supernatant was centrifuged at 100 000 g for 1 h to give supernatant ('S100') and pellet ('P100') fractions.

Purification of RNA-binding proteins

This was performed exactly as described by Dean et al. [29]. Briefly, nuclear extract from 20 confluent 175 cm 2 flasks of RAW 264.7 cells was dialysed against buffer A [20 mM Tris HCl (pH 8.3)/1 mM DTT/1 mM EDTA/0.05% (v/v) Brij 35], filtered (0.2 μ m-pore-size filter) and chromatographed on a 1 ml Mono-Q column. Protein was eluted with a linear 20 ml salt gradient (0–0.5 M NaCl) and fractions (1 ml each) were collected. Fractions 5–10 from the Mono-Q column were pooled and dialysed against buffer B [50 mM malonic acid (pH 6.0)/1 mM EDTA/1 mM DTT/0.05% Brij 35] for 2 h at 20 °C and chromatographed on a 100 μ l Mono-S column developed with a linear 2 ml salt gradient (0–1 M NaCl); fractions (100 μ l each) were collected. A 10 μ l sample of each fraction was removed for assaying, and proteins in individual fractions were then adsorbed on to poly(U)–Sephadex 4B beads. The beads were washed and electrophoresed on an SDS/10% polyacrylamide gel which was silver-stained [31].

Preparation of samples for MS

In-gel digestion with trypsin was performed as described in [31–33] and modified for use with a robotic digestion system [34]

(Investigator ProGest; Genomic Solutions, Huntington, Cambs, U.K.). Silver-stained gel pieces were first washed with 30 μ l of 15 mM potassium ferricyanide/50 mM sodium thiosulphate [35], followed by three rinses with 100 μ l of deionized water. After reduction, alkylation and drying, 10 μ l of modified trypsin was added to the gel pieces at a concentration of 6.5 ng/ μ l in 25 mM NH_4HCO_3 (37 °C for 8 h). Digestion products were recovered by sequential extractions with 25 mM NH_4HCO_3 , 5% formic acid, and acetonitrile.

Matrix-assisted laser-desorption ionization (MALDI) MS

MALDI mass spectra were recorded with a ToFSpec 2E spectrometer (Micromass, Manchester, U.K.), equipped with a 337 nm nitrogen laser. The instrument was operated in the positive-ion reflectron mode at 20 kV accelerating voltage. The matrix was a mixture of α -cyano-4-hydroxycinnamic acid and nitrocellulose, applied to the target as a microcrystalline thin film by a modification of the procedure of Vorm et al. [36]. Spectra were internally calibrated using trypsin autolysis products, and the resulting peptide mass fingerprints were searched against a local copy of the National Center for Biotechnology Information (NCBI) non-redundant database (<http://www3.ncbi.nlm.nih.gov>) using the Protein Probe search engine (Micromass, Manchester, U.K.). One missed cleavage per peptide was allowed, and an initial mass tolerance of 50 p.p.m. was used in all searches. Cysteine residues were assumed to be carbamidomethylated, but other potential modifications were not considered in the first-pass search.

HPLC electrospray tandem MS (MS/MS)

Tandem electrospray mass spectra were recorded using a Q-ToF hybrid quadrupole/orthogonal acceleration time-of-flight spectrometer (Micromass) interfaced to a Micromass CapLC capillary chromatograph. Samples were injected on to a 300 μ m $\times$ 5 mm Pepmap C $_{18}$ column, washed for 3 min with aq. 0.1% formic acid, and eluted into the mass spectrometer with an acetonitrile/0.1% formic acid gradient (5% to 70% acetonitrile over 20 min).

The capillary voltage was set to 3500 V, and data-dependent MS/MS acquisitions were performed on precursors with charge states of 2, 3 or 4 over a survey mass range 540–1000. The collision gas was argon, and the collision voltage was varied between 18 and 45 V depending on the charge and mass of the precursor. Daughter-ion spectra were charge-state de-encrypted and de-isotoped with a maximum entropy algorithm (MaxEnt 3; Micromass), and uninterpreted mass spectra were correlated to entries in SwissProt/TrEMBL using ProteinLynx global server (Version 1, Micromass). Amino acid sequences were deduced semi-manually with the assistance of Micromass's peptide sequencing program PepSeq. The resulting sequences were searched against NCBI's non-redundant database using BLAST [37].

Western blotting

For preparation of whole-cell lysates, cells were washed once with ice-cold 1 $\times$ PBS and lysed in 10 mM Hepes (pH 7.6)/3 mM MgCl_2 /40 mM KCl/2 mM DTT/5% (v/v) glycerol/0.5% (v/v) NP-40/protease inhibitors {1 mM PMSF, 10 μ g $\cdot$ ml $^{-1}$ aprotinin/10 μ M E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane]/1 μ g $\cdot$ ml $^{-1}$ pepstatin}. The extract was then sonicated for three 10 s bursts at 200 W and clarified at 10 000 g for 10 min. Whole-cell lysates or cell fractions containing 20 μ g of protein were electrophoresed on 10% (w/v) polyacrylamide gels and blotted on PVDF membrane. Immunostaining was

visualized by enhanced chemiluminescence (ECL[®], Amersham). The primary antibody was a 1:1000 dilution of antiserum to the N-terminal peptide of CBF-A. The secondary antibody was a horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG at a 1:2000 dilution. Western blots of cells transfected with FLAG-tagged constructs were stained with anti-FLAG M2 murine monoclonal antibody (1:1000 dilution) and HRP-conjugated rabbit anti-mouse secondary antibody (1:2000 dilution).

Ribonuclease protection assay

This was performed as described [12] using a kit, except that GAPDH probe was synthesized using a concentration of 12 μ M unlabelled UTP. Samples were electrophoresed on 6% polyacrylamide gels containing urea, which were dried and visualized and quantified by phosphorimaging.

Immunoprecipitation

HeLa cells in 10 cm-diameter dishes were transfected (as above) with either 10 μ g of pBluescript only or 5 μ g of pBluescript and 5 μ g of CMV-p42CBF-FLAG DNA. Cells were either left untreated or treated with 20 ng $\cdot$ ml⁻¹ IL-1 for 4 h. Cytoplasmic extracts were prepared as described previously [29] by lysing cells with 0.5 ml of lysis buffer [10 mM Hepes (pH 7.6)/3 mM MgCl₂/40 mM KCl/2 mM DTT/5% (v/v) glycerol/0.5% (v/v) NP-40/protease inhibitors]. A portion (0.45 ml) of cytoplasmic extract was mixed with 180 μ l of 1 M NaCl, 400 units of RNasin and 30 μ l of a 50% slurry of Protein G-Sepharose beads which had been pre-absorbed with a non-immune mouse IgG or M2 mouse monoclonal anti-FLAG antibody. Samples were incubated for 2 h at 4 °C on a rotating wheel. The beads were collected by centrifugation at 8000 *g* for 1 min at 4 °C. 100 μ l of the supernatant was mixed with 350 μ l of RLT lysis buffer (Qiagen) and the pellets were washed four times with 0.5 ml of lysis buffer supplemented with 0.2 ml of 1 M NaCl. A 350 μ l portion of RLT lysis buffer was then added to the beads, which were then vortex-mixed for 10 s and pelleted by centrifugation at 8000 *g* for 1 min at 4 °C, and the supernatant was collected. RNA was prepared using the RNeasy kit (Qiagen), and eluted with 25 μ l of RNase-free water.

RT-PCR

A 11 μ l portion of RNA prepared from pellets and supernatants after immunoprecipitation was mixed with 1 μ l of 100 μ g $\cdot$ ml⁻¹ random hexamers and incubated for 15 min at 65 °C followed by 1 min on ice. A 4 μ l portion of 5 $\times$ RT buffer [250 mM Tris/HCl (pH 8.3)/375 mM KCl/15 mM MgCl₂], 2 μ l of 0.1 M DTT, 0.6 μ l of RNAsguard ribonuclease inhibitor and 0.4 μ l of a mixture of 25 mM each of dATP, dCTP, dGTP, dTTP, and 1 μ l of Superscript II RNase H free reverse transcriptase was added. Samples were incubated at 30 °C for 10 min, 42 °C for 50 min and 70 °C for 15 min. PCR was then performed using the Ready To Go kit (Amersham Pharmacia Biotech) on 1 μ l of cDNA using 0.4 μ M each of forward and reverse primers for COX-2 or GAPDH (primer sequences are available from J.L.E.D. on request). PCR was performed in a T3 thermocycler (Biometa, Göttingen, Germany) with incubations of 5 min at 94 °C, followed by either 27 (GAPDH) or 32 (COX-2) cycles of 30 s at 94 °C, 30 s at 58 °C and 45 s at 72 °C. PCR products were run on a 1% agarose gel containing 1 μ g $\cdot$ ml⁻¹ ethidium bromide and visualized on a Gel Doc 1000 UV transilluminator (Bio-Rad, Hemel Hempstead, Herts., U.K.).

RESULTS

Purification of ARE-binding proteins from a RAW 264.7 nuclear extract

A nuclear extract was made from RAW 264.7 cells, a murine macrophage-like cell line. This extract is an abundant source of ARE-binding proteins and forms strong complexes in EMSA with the TNF- α ARE [29]. It was chromatographed on a Mono-Q column and proteins were eluted with a salt gradient. Only the fractions that were eluted between 0.125 and 0.25 M NaCl contained proteins which formed complexes in EMSA with the TNF- α ARE probe. These were pooled, dialysed and chromatographed on a Mono-S cation-exchange column which was developed with a salt gradient. The fractions were again assayed by EMSA and complexes of differing mobility were observed (Figure 1A). A strong low-mobility complex was formed by fractions 6 and 7. Fractions 8 and 9 formed a faster-moving complex, and very-low-mobility complexes were seen in fractions 10–15. Such low-mobility complexes were not observed in EMSA of crude RAW 264.7 nuclei with the TNF- α ARE probe (Figure 4C below) or in the starting material for the Mono-S column (Figure 1A). AREs are generally U-rich and often flanked by U-rich sequences. ARE-binding proteins have a high affinity for poly(U) RNA. ARE-binding proteins in individual fractions were purified further by absorption on poly(U)-Sepharose 4B beads. EMSAs on supernatants of column fractions after absorption showed no complex formation. The beads were washed, boiled in sample buffer, and eluted proteins were separated on SDS/PAGE and stained with silver (Figure 1B). Fractions 6 and 7 contained a number of proteins of between 36 and 45 kDa. Weakly stained proteins with mobilities identical with that of the three largest ones were also seen in fractions 8–12 (Figure 1B). The prominent protein labelled G in fraction 9 (Figure 1B) has previously been identified as HuR [29] and is responsible for the strong faster complex seen in EMSA of that fraction (Figure 1A).

Identification of ARE-binding proteins by MS

Silver-stained bands from the gel (Figure 1B) were excised, the proteins in the gel slices were digested with trypsin, and the peptides analysed by MALDI and electrospray MS/MS. Proteins were identified by correlating the masses of tryptic peptides to the predicted values calculated from SwissProt/TREMBL. All identifications were confirmed by MS/MS sequencing (Table 1). Band F in lane 4 of Figure 1(B) was the AUF1-like protein JKTBP [JKT-binding protein, where JKT is an oligodeoxynucleotide (JKT41) derived from intron 9 of human myeloperoxidase] (results not shown). Bands A, B and D in fractions 6 and 7 were AUF1. MS/MS was used to determine 75, 55 and 32 residues of amino acid sequence from bands A, B and D respectively (Table 1). These bands may represent three isoforms arising from alternative splicing (see below). Bands C and E were identified as CBF-A by MALDI MS, and six peptides from band C (comprising 60 amino acid residues), and four peptides from band E (39 residues), were sequenced.

CBF-A and AUF1 have highly similar amino acid sequences and both proteins exist as different isoforms

Although CBF-A was originally described as a DNA-binding protein that represses transcription [38], its amino acid sequence is highly similar to that of AUF1 (Figure 3 below). Alternative splicing of exons 2 and 7 of the AUF1 gene (Figure 2) gives rise to four isoforms of apparent molecular masses ranging from

Figure 1 Purification of ARE-binding proteins by Mono-S chromatography and adsorption on to poly(U)-Sephacrose 4B

(A) ARE-binding proteins were partially purified from a nuclear extract of RAW 264.7 cells on a Mono-Q column which was developed with a salt gradient. The fractions forming the major complexes in EMSA with the TNF- α ARE were pooled and chromatographed on a Mono-S column. Proteins were eluted with a 0–1 M NaCl gradient across 20 fractions. The starting material ('S') and run-through ('R') were assayed together with Mono-S column fractions by EMSA with the TNF- α ARE probe. RNase T₁ digested free probe is also shown ('F'). (B) Silver-stained SDS/PAGE of proteins adsorbed on to poly(U)-Sephacrose 4B from individual Mono-S fractions 1–14. The beads were washed and proteins were eluted in SDS/PAGE sample buffer. Bands labelled A–G were excised for identification by MS. Similar results were seen in three independent experiments.

Table 1 Peptides sequenced by MS/MS from the excised gel (Figure 1B, bands A–E)

Gel band	Precursor mass (charge)	Peptide
A (AUF1)	584.4 (2+)	FGEVVDCTLK
	744.9 (2+)	IFVGGLSPDTPEEK
	577.6 (3+)	GFCEFITFKEEPPVK
	586.7 (3+)	IFVGGLSPDTPEEKIR
	658.4 (3+)	IDASKNEEDEGHSSNSPR
	641.1 (3+)	FGEVVDCTLKLDIPITGR
B (AUF1)	584.3 (2+)	FGEVVDCTLK
	745.0 (2+)	IFVGGLSPDTPEEK
	577.9 (3+)	GFCEFITFKEEPPVK
	641.1 (3+)	FGEVVDCTLKLDIPITGR
C (CBF-A)	584.4 (2+)	FGEVVDCTIK
	750.4 (2+)	EVYQQQYQSGGGR
	752.5 (2+)	IFVGGLNPEATEEK
	592.1 (3+)	IFVGGLNPEATEEKIR
	732.4 (3+)	EYFGQFGEIEAIELPIDPK
	822.6 (3+)	IREYFGQFGEIEAIELPIDPK
D (AUF1)	584.4 (2+)	FGEVVDCTLK
	745.0 (2+)	IFVGGLSPDTPEEK
	586.7 (3+)	IFVGGLSPDTPEEKIR
	640.7 (3+)	FGEVVDCTLKLDIPITGR
E (CBF-A)	584.4 (2+)	FGEVVDCTIK
	750.4 (2+)	EVYQQQYQSGGGR
	752.5 (2+)	IFVGGLNPEATEEK
	591.7 (3+)	IFVGGLNPEATEEKIR

37 to 45 kDa [39,40]. The bands, namely A, B and D, are likely to correspond to three of these. The CBF-A sequence in the GenBank® database comprises 285 amino acids. The rat and

human orthologues of CBF-A are the rat-type A/B hnRNP-like protein p40 [41] and human apobec-1 binding protein ('ABBP-1') [42]. These both contain an additional sequence of 47 amino acids that is almost identical with that encoded by the alternatively spliced exon 7 of AUF1. It was possible that the CBF-A proteins of apparent molecular mass 37 kDa (p37) and 42 kDa (p42) purified from RAW 264.7 cells arose from differential splicing of an exon 7-like sequence within the primary transcript. To test this hypothesis, RT-PCR was performed using RAW 264.7 cell mRNA and primer pairs directed against the N-terminal or C-terminal CBF-A coding regions. Primers directed against the N-terminal coding region generated a single product, indicating that alternative splicing does not occur within this region. This suggests that CBF-A transcripts in RAW264.7 cells lack sequences corresponding to exon 2 of AUF1. Primers directed against the C-terminal coding region generated two products, which were cloned and sequenced. The shorter PCR product was identical with the published CBF-A sequence, whereas the longer product contained an additional 141 bp which encodes an amino acid sequence closely resembling that of AUF1 exon 7 (Figure 2) and identical with the additional sequence in rat CBF-A. This suggests that the 37 kDa and 42 kDa forms of CBF-A arise from differential splicing of an exon which corresponds to exon 7 of the AUF1 gene. This is consistent with the results of MALDI MS, since the spectrum of p37 CBF-A contains a signal at m/z 1169.5, corresponding to the peptide GSGGGQGSGTNYGK, which is attributable to tryptic cleavage at Arg²⁵⁸ and Lys²⁷¹ flanking the 'exon 7' boundary. This peptide is not observed in the mass spectrum of p42 CBF-A because the insertion of exon 7 makes its formation impossible. The MS data provided no evidence for exon 2-derived sequences in any of the CBF-A bands, though its presence in AUF1 band A was clearly shown by MS/MS sequencing of the peptide IDASKNEEDEGHSSNSPR (triply charged ion at m/z 658.4;

MSEEQFGGDGAAAAATAVGGSSAGEQEGAMVAATQAAAA
 ..DAAE--EQPMETTG.TEN.HEAAP..EAPVEPSA....
 AGSGAGTGGGTASGGTEGSSAESEGAIDASKNEEDEGhs
 PAAS..S....TTAPS-.NQNGA..DQ.N.....A.--
 nssprhseaataqreewKMFIGGLSWDTTKKDLKDYFSKF
 -----...V.....S.....T..
 GEVVDCTLKLDPTITGRSRGFGFVLFKESESVDKVMQKEH
I.M..N.....I...D.S..E..L.....
 KLNGKVIDPKRAKAMKTKEPVKKIFVGGLSPDTPEEKIRE
 R.D.R.....K.M...-D.....N.EAT.....
 YFGGFGEVESIELPMDNKTNKRGGFCFITFKEEEPVKKIM
 ...Q...I.A....I.P.L.....V.....D....VL
 EKKYHNVLGSKCEIKVAMSKEQYQQQQQ--WGSRGGFAGRA
 ...F.T.SG.....QP..V.....YGS.G..NRNRGN
 RGRGGGpsqnwnqgysnywnqgygnygynsqgyg-gygyg
 ..S...Q..s.....g.....----yq....p.....
 dytgynnyyygyg---dysnQQSGYGKVSRRGGHQNSYKPY
 ..sp.g-.....pgy...qGSTN...SQ.....N....

Figure 2 Alignment of CBF-A and AUF1 amino acid sequences

The amino acid sequence for human p45 AUF1 was aligned with that of murine p42 CBF-A with the program ClustalX (1.8) using the default parameters supplied with the program. The p42 CBF-A sequence was created by inserting the 47-amino-acid sequence coded for by the sequence cloned by RT-PCR (see the Experimental section). The alternatively spliced exons 2 (19 amino acids) and 7 (49 amino acids) of AUF1 are indicated by lower-case letters. The 47-amino-acid sequence, which is present only in p42 CBF-A and is highly similar to exon 7 of AUF1, is indicated by lower-case letters. The amino acids in CBF-A which are identical with those of AUF1 are shown as dots. The underlined sequences denote CBF-A peptides that were used to raise antisera (see the Experimental section).

Table 1) which spans the exon boundary. This peptide was not observed in either the electrospray or MALDI spectra of bands B and D, since it cannot be formed in the presence of exon 2.

Western blotting cell extracts with an antiserum to the unique N-terminal peptide of CBF-A showed two bands (37 kDa and 42 kDa) in all cell types tested (Figure 3A). Staining was not seen with pre-immune serum, and was blocked by adding the N-terminal peptide to the incubation with immune serum. Subcellular fractions of RAW 264.7 and HeLa cells were Western-blotted. Both forms were present in similar amounts in nuclear extracts, but only the smaller form was detected in the cytoplasmic fraction (Figure 3B). Following centrifugation of the cytoplasmic fraction at 100 000 *g*, most of the smaller form was in the pellet (P100 fraction) which also contained a small amount of the larger CBF-A (Figure 3B).

Purified CBF-A binds with high affinity to the TNF- α ARE

In order to confirm that CBF-A binds to the TNF- α probe used to monitor purification, antibody supershift experiments were performed. The antiserum to the N-terminal peptide of CBF-A (which appears highly specific for CBF-A in Western blotting)

Figure 3 Western blotting of CBF-A forms in different cell types and subcellular distribution

(A) Whole-cell lysates (20 μ g of protein) from different cell types were Western-blotted with antiserum to the N-terminal peptide of CBF-A: lane 1, RAW 264.7; lane 2, elutriated human monocytes; lane 3, KIT-225 (human T-cell line); lane 4, 293; lane 5, HeLa; lane 6, elutriated human lymphocytes. (B) Subcellular fractions (10 μ g of protein) of RAW 264.7 and HeLa cells were prepared as described in the Experimental section and Western-blotted for CBF-A. Cells were lysed with a buffer containing 0.5% NP-40 and centrifuged at 500 *g* for 5 min. Proteins were extracted from the nuclei ('N') in a high-salt buffer. The postnuclear supernatant or cytoplasmic fraction ('C') was centrifuged at 100 000 *g* for 1 h to yield supernatant ('S100') and pellet ('P100') fractions. The results shown in (A) and (B) are representative of two different experiments.

did not supershift the complex in EMSA with the TNF- α ARE probe (results not shown). Possibly the N-terminus of CBF-A is inaccessible to antibodies, or these antibodies are of low affinity. Inclusion of an antiserum to the C-terminal peptide of CBF-A, or an antiserum to AUF1 whole protein [18] in EMSA resulted in a shift in mobility of the slow-moving complex formed by Mono-S fraction 6 (Figure 1A) on the TNF- α ARE probe (Figure 4A). However, neither of these antisera appears to discriminate between CBF-A and AUF1 in Western blots (results not shown). It also proved impossible to separate the two proteins by further chromatography. To examine whether CBF-A actually binds the TNF- α ARE recombinant protein was made and complex formation analysed by EMSA (Figure 4B). Recombinant HuR was prepared as a positive control. The titration of the recombinant protein in EMSA resulted in a similar banding pattern for both CBF-A and HuR. At high protein concentrations both RNA-binding proteins formed a single slow-moving complex, whose mobility increased slightly as the protein concentration was reduced. At low protein concentrations of CBF-A and HuR, weaker, higher-mobility diffuse complexes were seen, which, in the case of HuR, appeared to resolve into two separate complexes. The K_d of p37 CBF-A for the TNF- α ARE probe was 75 ± 10 nM, whereas that for HuR was 97 ± 5 nM. p42 CBF-A also formed complexes with the probe, but following electrophoresis they remained trapped in the wells of the gel (results not shown). Possibly the presence of the highly hydrophobic sequence (corresponding to AUF1 exon 7) caused the bacterially expressed p42 protein to be insoluble.

Figure 4(C) shows an antibody-supershift experiment using RAW 264.7 cell nuclear extract. This extract formed four major complexes, a pair of low-mobility complexes (which are found in nuclear and S100 fractions [29]) and two higher-mobility complexes. An antibody against HuR strongly supershifted the higher-mobility complexes (Figure 4C, lane 5), as shown previously [29]. Surprisingly, the inclusion of antisera to either

Figure 4 Purified CBF-A binds the TNF- α ARE with high affinity

(A) EMSA was performed using TNF- α ARE probe with an aliquot of fraction 6 from the Mono-S column (see Figure 1a) without (lane 2), with 1 μ l of the pre-immune serum (lane 3) or 1 μ l of a non-immune rabbit antiserum (lane 4), 1 μ l of antiserum to the CBF-A C-terminal peptide (lane 5) or with 1 μ l of an antiserum to AUF1 whole protein (lane 6). An aliquot of free-probe; with no added sample or serum was also run (lane 1). Identical results were obtained in three independent experiments. (B) Thrombin-cleaved GST-p37 CBF-A and GST-HuR were prepared as described in the Experimental section. Increasing amounts (8, 20, 40, 60, 80, 200, 400, 600 or 800 ng) of p37 CBF-A or recombinant HuR (16, 40, 80, 120, 160, 400, 800, 1200 or 1600 ng) were mixed with a fixed amount of the TNF- α ARE probe (20 fmol) and subjected to EMSA. The largest free probe fragment (FPF) produced by RNase T1 digestion was quantified and plotted against log (protein concentration). K_d values for the TNF- α ARE were calculated from the mid-point of the curves. HuR: $K_d = 97 \pm 5$ nM; p37CBF-A: $K_d = 75 \pm 10$ nM. (C) A 10 μ g portion of protein of a RAW 264.7 nuclear extract was assayed by EMSA with the TNF- α ARE probe either alone (lane 1) or with the inclusion of 1 μ l of a non-immune rabbit antiserum (lane 2), 1 μ l of an antiserum to AUF1 whole protein (lane 3), 1 μ l of antiserum to the CBF-A C-terminal peptide (lane 4) or 1 μ g of 19F12 monoclonal antibody to HuR (lane 5).

CBF-A or AUF1 in EMSAs performed with crude RAW 264.7 nuclear extracts failed to supershift any of the complexes formed on the TNF- α ARE probe (Figure 4C), for reasons which are unclear. Since the physiological relevance of the interaction between CBF-A and the TNF- α ARE was difficult to assess, attention was turned to the interaction between CBF-A and another ARE, namely that of COX-2.

Recombinant CBF-A and CBF-A in crude extracts binds the COX-2 ARE

CBF-A (and AUF1) were identified as ARE-binding proteins purified from extracts of RAW 264.7 cells. We examined whether they were present in the complexes formed by the COX-2 ARE probe with proteins in crude cell extracts. Proteins in RAW 264.7 cell nuclear extracts formed a low-mobility smeared complex, and a pair of discrete, higher-mobility complexes with the COX-2 RNA probe. The CBF-A antiserum strongly supershifted all complexes made with this probe, except that the upper discrete complex was only partially supershifted (Figure 5A, lane 3). The complexes formed by RAW 264.7 cell extract were specific for the COX-2 ARE, since they were specifically competed for by the COX-2 ARE probe but not by the GAPDH 3'-UTR probe (Figure 5B). The AUF1 antiserum strongly supershifted the complexes made with the COX-2 probe (Figure 5A, lane 4), and these complexes were also supershifted by the monoclonal antibody to HuR (Figure 5A, lane 5).

As described above, the antisera used in the supershifts in EMSA do not distinguish between CBF-A and AUF1; therefore recombinant p37 CBF-A was tested for its ability to bind the COX-2 ARE probe. It formed a single intense complex of intermediate mobility with the COX-2 ARE in EMSA (Figure 5C), but almost no binding was seen with the GAPDH 3'-UTR (Figure 5C), which lacks an ARE. Recombinant HuR was again included as a positive control. Recombinant HuR formed a strong, single low-mobility complex at high protein concentration

with the COX-2 ARE probe and a weaker, higher-mobility complex at the lowest concentration. There is another band below this complex on the gel which was also seen with the lowest concentration of CBF-A used, which is a free probe fragment (results not shown). Similarly to CBF-A, HuR failed to form a strong complex with the GAPDH 3'-UTR (Figure 5C). Recombinant CBF-A also formed a single, discrete complex on the c-Myc 3'-UTR, which contains an ARE (Figure 5C).

The p42 isoform of CBF-A binds the COX-2 ARE

It was possible that both forms of CBF-A in the crude extracts bound the COX-2 ARE probe. To differentiate between binding of the two isoforms, they were expressed as FLAG-tagged proteins in HeLa cells, cell extracts were prepared and complex formation with the COX-2 ARE probe was analysed by use of antibodies to the tag. Nuclear extracts from HeLa cells expressing p37 or p42 CBF-A-FLAG formed complexes of mobility similar to that seen with RAW 264.7 cell nuclear extracts (Figures 5A and 5B) or with HeLa cells transfected with empty vector (Figure 5D, lanes 1–3). However, the low-mobility complex formed by extracts from cells expressing p42 CBF-A-FLAG was strongly supershifted by the anti-FLAG antibody (Figure 5D, lane 9). The complexes formed by extracts of cells expressing p37 CBF-A-FLAG were not supershifted (Figure 5D, lane 6), although Western blotting the tagged proteins showed that the expression levels were similar (Figure 5E). The results show that the larger isoform of CBF-A has high affinity for the COX-2 ARE in crude cell extracts.

Overexpression of p42 CBF-A stabilizes a COX-2 ARE mRNA reporter

Having established that p42 CBF-A binds to the COX-2 ARE *in vitro*, we decided to examine the stability of a reporter mRNA carrying the COX-2 ARE in cells overexpressing this isoform of

Figure 5 CBF-A binds the COX-2 ARE in EMSA

(A) AUF1/CBF-A form a complex in EMSA of crude RAW 264.7 extracts with the COX-2 ARE probe. A 10 μ g portion of protein of a RAW 264.7 nuclear extract was assayed by EMSA with the COX-2 ARE probe, either alone (lane 1) or with the inclusion of 1 μ l of a non-immune rabbit antiserum (lane 2), 1 μ l of an antiserum to AUF1 whole protein (lane 3), 1 μ l of antiserum to the CBF-A C-terminal peptide (lane 4) or 1 μ g of 19F12 monoclonal antibody to HuR (lane 5). (B) AUF1/CBF-A binds specifically to the COX-2 ARE. A 2 μ g portion of protein of a RAW 264.7 nuclear extract was assayed by competition in EMSA with 50 fmol of labelled COX-2 ARE probe and unlabelled COX-2 ARE probe (1-, 10- and 100-fold molar excess) or unlabelled GAPDH 3'-UTR probe (1-, 10- and 100-fold molar excess) as indicated. The results in each panel are representative of at least two independent experiments. (C) Binding of recombinant CBF-A to different RNA probes in EMSA. Increasing amounts (80, 200 or 800 ng) of thrombin-cleaved recombinant GST-p37 CBF-A or -HuR (40, 100 or 400 ng) were mixed with 20 fmol of a COX-2 ARE probe or 20 fmol of a GAPDH 3'-UTR probe and subjected to EMSA. EMSA with 400 and 800 ng of CBF-A with 20 fmol of the c-Myc 3'-UTR probe is also shown. (D) Anti-FLAG supershifts in EMSA with the COX-2 ARE. HeLa tet-off cells in 10 cm² dishes were transfected with 10 μ g empty vector (CMV-FLAG; lanes 1–3), CMV-p37 CBF-A-FLAG (lanes 4–6), and CMV-p42 CBF-A-FLAG (lanes 7–9). A 10 μ g portion of a nuclear extract from transfected cells was mixed with approx. 20 fmol of COX-2 ARE probe and RNasin. Non-immune mouse immunoglobulins (4 μ g; lanes 2, 5 and 8) or anti-FLAG M2 monoclonal antibody (4 μ g; lanes 3, 6 and 9) was added and the samples were incubated on ice for 1 h. The samples were subjected to RNase T1 digestion and electrophoresed. (E) Anti-FLAG Western blot of nuclear extracts from cells expressing p37 CBF-A-FLAG, p42 CBF-A-FLAG or HuR-FLAG. Western blot

Figure 6 Transient overexpression of p42 CBF-A stabilizes a COX-2 ARE mRNA reporter

(A) HeLa tet-off cells in six-well plates were transiently transfected with 200 ng of pTet-BBB-COX-2 ARE together with either 500 ng of empty expression vector or 500 ng of CMV-p42 CBF-A-FLAG. Tetracycline was added after 20 h and the cells were harvested at the times indicated and analysed for BBB-COX-2 ARE and GAPDH mRNA. A typical result out of three independent experiments is shown. (B) Graph of log (BBB-COX-2 ARE mRNA levels) against time after addition of tetracycline in the presence (●) or absence (○) of p42 CBF-A overexpression. The error bars indicate the S.D. from a single experiment performed in triplicate. Where the error bars are not shown, they are smaller than the symbol.

CBF-A. For these experiments a cell line stably transfected with a tetracycline-sensitive transcription factor (HeLa tet-off) was transiently transfected with an mRNA reporter together with empty vector or p42 CBF-A expression vector. In this system the transcription of transiently transfected mRNA reporter is rapidly switched off following addition of tetracycline to the culture medium, allowing mRNA stability to be measured. The mRNA reporter used was a plasmid containing rabbit β -globin genomic DNA (BBB) spanning the 5'-UTR, open reading frame and 3'-UTR. Control BBB and BBB-COX-2 ARE differ by the insertion of 123 nt from the major AU-rich region in the COX-2 3'-UTR into the 3'-UTR of the mRNA reporter. In three independent experiments, overexpression of p42 CBF-A resulted in stabilization of the COX-2 ARE reporter, with no effect on the stability of the BBB control construct. The results of a typical experiment are shown in Figure 6. For these experiments, Western blotting with antiserum to the unique CBF-A N-terminus showed the overexpressed FLAG-tagged protein as a band of slightly lower mobility than, but of roughly equal intensity to, that of the

with M2 monoclonal anti-FLAG antibody of 10 μ g of nuclear extract from cells transfected with CMV-FLAG (lane 1), CMV-p37 CBF-A-FLAG (lane 2), and CMV-p42 CBF-A-FLAG (lane 3).

Figure 7 p42 CBF-A binds COX-2 mRNA *in vivo*

HeLa cells were transfected with pBluescript carrier DNA or with pBluescript carrier DNA together with CMV-p42 CBF-A-FLAG DNA (as shown). Cells were either left untreated or treated with 20 ng · ml⁻¹ IL-1 for 4 h (as indicated). The cells were lysed and immunoprecipitation was performed on the cytoplasmic fraction (as described in the Experimental section) with either a non-immune (NI) mouse IgG or with mouse monoclonal M2 anti-FLAG antibody. RNA was prepared from the supernatants (SPNT) and pellets following immunoprecipitation and subjected to RT-PCR. The PCR cycle number was adjusted to ensure that amplifications were within the linear range. The 611 bp PCR product for COX-2 formed after 32 cycles is shown (upper panel) together with the 671 bp product formed for GAPDH after 27 cycles (lower panel). IP Ab is the immunoprecipitating antibody.

endogenous 42 kDa form (results not shown). Given a transfection efficiency of 40 % (results not shown), this represents approx. 3.5-fold overexpression of p42 CBF-A.

p42 CBF-A-FLAG binds endogenous COX-2 mRNA in IL-1-treated HeLa cells

The association of endogenous CBF-A with COX-2 mRNA could not be tested, because our CBF-A-specific antibody did not efficiently immunoprecipitate the protein (results not shown). Therefore we transiently expressed an epitope-tagged version of p42 CBF-A in HeLa cells, performed immunoprecipitations using an antibody against the tag, and used RT-PCR to detect endogenous COX-2 and GAPDH mRNAs in both pellet and supernatant. All PCR reactions were within linear amplification range (results not shown). COX-2 mRNA was strongly induced by IL-1 treatment and was detected in the CBF-A immunoprecipitate (Figure 7). As COX-2 mRNA was also detected in the supernatant, it is clear that not all of the endogenous transcript is associated with CBF-A. COX-2 mRNA was not immunoprecipitated by a control, non-immune antibody, and GAPDH mRNA did not co-immunoprecipitate with CBF-A. Hence endogenous COX-2 mRNA is specifically associated with CBF-A *in vivo*.

DISCUSSION

We have shown that RAW 264.7 cells express 37 and 42 kDa isoforms of the murine protein CBF-A that are generated by alternative splicing of a 141 nt exon lying in the 3' region of the open reading frame. The human orthologue of p37 CBF-A is known as hnRNP A/B [43], C-type hnRNP [44] or hnRNP E0 [45], whereas the human orthologue of p42 CBF-A is known as apobec-1-binding protein [42]. These proteins have been described as transcriptional regulators of the Ig κ -chain gene [46], the Ha-ras gene [47] and genes containing the CARG-box [38], a 10 nt DNA sequence [CC(A+T-rich)₆GG] present in serum response elements [48] and promoter regions of many muscle-

specific genes [38] and references cited therein). However, they appear to bind single-stranded DNA with higher affinity than double-stranded DNA [38,46]. They have also been shown to bind telomeric DNA sequences [45], hnRNA [49], G-rich or U-rich RNA [44], pre-mRNA 3' splice site UUAGG [45] or the apolipoprotein B mRNA editing site [42]. Affinity for AREs of the type described here was not previously examined. The amino acid sequence of CBF-A is highly similar to that of AUF1, a known ARE-binding protein which is thought to be involved in the regulation of mRNA decay. It is reported here that CBF-A is also very similar to AUF1 in its binding specificity and in its biochemical and functional properties.

AUF1 exists as four isoforms, termed p37, p40, p42 and p45 according to their mobility on SDS/PAGE. These arise through alternative splicing of exons 2 and 7 of the AUF1 gene [39,40]. We found two forms of CBF-A, the larger of which contained an AUF1 exon-7-like sequence. The functional difference between p37 and p42 isoforms is unclear, but they may have different affinities for the COX-2 ARE and this may be important for the roles of the different isoforms *in vivo*. The main difference between CBF-A and AUF1 is in the sequences of their N-terminal 37 amino acids: this corresponds to exon 1 of the AUF1 gene. This region is thought to be important for oligomerization of AUF1 [26]. Perhaps the difference in the N-termini favours self-oligomerization of CBF-A as opposed to the formation of CBF-A–AUF1 hetero-oligomers. The C-termini of AUF1 and CBF-A are very similar to each other and to that of a related protein, JKTBP. This sequence has been suggested to control nucleocytoplasmic shuttling of JKTBP [50], and the presence of both CBF-A isoforms in nuclear and P100 fractions would be consistent with nucleocytoplasmic shuttling.

Both partially purified and bacterially expressed CBF-A bind specifically, and with high affinity, to the TNF- α ARE. However, using an antibody that recognizes both CBF-A and AUF1 it was not possible to supershift any of the complexes seen in band-shift assays of crude RAW 264.7 cell extracts. In these crude extracts the predominant complexes formed were supershifted by an anti-HuR antibody [29]. Although recombinant AUF1 binds the TNF- α ARE *in vitro* [51], we and others [52] have not been able to detect AUF1 in complexes formed by the TNF- α 3'-UTR with macrophage cell extracts. These results suggest that, in cell extracts, the TNF- α ARE binds HuR in preference to CBF-A and AUF1. It is possible that the K_d values obtained with the recombinant proteins were misleading, and that the endogenous cellular forms of HuR and CBF-A differ markedly in their affinity for the TNF- α ARE.

In contrast, CBF-A does bind the COX-2 ARE, both in recombinant form and in crude extracts. The COX-2 ARE, like that for TNF- α , also binds HuR. The main regulatory ARE for COX-2 contains six AUUUA motifs, of which only three overlap, whereas the corresponding region for TNF- α has six AUUUA motifs with five overlapping. The less compact structure of the COX-2 ARE may allow multiple proteins (such as CBF-A and HuR) to bind this element simultaneously.

AUF1 has been suggested to be a destabilizing protein [25,53]. Immunodepletion of AUF1 from cell extracts causes a significant reduction in the normally rapid decay of an AU-rich RNA *in vitro* [53], and over-expression of AUF1 isoforms in K562 cells causes a dramatic decrease in the haemin-induced stabilization of c-Fos and GMCSF reporter mRNAs [54]. However, no direct role for AUF1 in mRNA degradation has been established. Addition of purified or recombinant AUF1 to *in vitro* decay reactions depleted of AUF1 by immunodepletion or with poly (U)–Sepharose beads has no effect on AU-rich RNA decay rates [18,55].

Although much of the evidence is consistent with a destabilizing role for AUF1, Shyu and co-workers have recently found that overexpression of AUF1 in NIH3T3 cells stabilizes ARE-containing mRNA, further complicating the picture [56]. We found that overexpression of the 42 kDa form of CBF-A stabilized a COX-2 ARE mRNA reporter. The effect of overexpression could reflect a normal stabilizing function of CBF-A. Alternatively it is possible that overexpressed protein could compete for CBF-A-interacting proteins involved in destabilization and so inhibit a normal destabilizing function. The mRNA stabilization following CBF-A overexpression is similar to that seen following AUF1 overexpression [56]. AUF1-like proteins are abundant, and the normal equilibrium between ARE-binding proteins and AREs in the cell may be only slightly perturbed by overexpression.

The overexpression data provide evidence that CBF-A binds the COX-2 ARE *in vivo*. This finding was further substantiated by the observation that CBF-A specifically interacts with endogenous COX-2 mRNA in cells, as shown by immunoprecipitation of epitope-tagged p42 CBF-A and RT-PCR. However, the precise functions of CBF-A and AUF1 in ARE-mediated decay and its regulation *in vivo* have yet to be elucidated, and it will probably be necessary to carry out gene knock-out or gene knock-down experiments. Given the similarity between the CBF-A and AUF1 sequences, their physiological roles are likely to be related. It also seems reasonable to suggest that CBF-A could be re-named AUF2.

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