RAW 264.7 cells rapidly induce cyclooxygenase-2 (COX-2) in response to lipopolysaccharide treatment. Part of the increased COX-2 expression occurred through post-transcriptional mechanisms mediated through specific regions of the 3'-untranslated region (UTR) of the message. The proximal region of the 3'-UTR of COX-2 contains a highly conserved AU-rich element that was able to confer lipopolysaccharide regulation of a chimeric reporter-gene. Electrophoretic mobility shift assays demonstrated that the RNA-binding proteins TIAR, AUF1, HuR, and TIA-1 all form an RNA-protein complex with the first 60 nucleotides of the 3'-UTR of COX-2. Biotinylated RNA probes were used to isolate additional proteins that bind the 3'-UTR of COX-2. We identified several RNA-binding proteins including TIAR, AUF1, CBF-A, RBM3, heterogeneous nuclear ribonucleoprotein (hnRNP) A3, and hnRNP A2/B1. We identified four alternatively spliced isoforms of AUF1 which migrated at multiple isoelectric points. Likewise, we identified alternatively spliced isoforms of CBF-A, hnRNP A3, and hnRNP A2/B1. Western analysis of two-dimensional gels identified multiple isoforms of TIA-1, TIAR, and AUF1 at pl values that spanned nearly 3 pH units. Thus, through a combination of alternative splicing and post-translational modification cells are able to increase greatly the repertoire of protein species expressed at a given time or in response to extracellular stimuli.

Macrophages play a pivotal role in potentiating the proinflammatory response. Activation of macrophages with bacterial lipopolysaccharides (LPS) leads to production and secretion of various cytokines and prostaglandins. Prostaglandin production requires induction of cyclooxygenase-2 (COX-2), which catalyzes the conversion of arachidonic acid to prostaglandin H₂, the common precursor to all prostaglandins, thromboxanes, and prostacyclins (for review, see Ref. 1). The LPS-dependent induction of COX-2 occurs transcriptionally and post-transcriptionally (2–4). Specifically, LPS has been shown to regulate COX-2 message stability through mitogen-activated protein kinase p38 (5, 6).

The stability of mRNA is determined in many cases by interactions between specific RNA-binding proteins and cis-acting sequences located in the 3'-untranslated region (3'-UTR) of the message (7, 8). One of the best characterized cis-acting sequences is the adenylate/uridylate-rich element (ARE). AREs have been identified in numerous mRNAs (6, 9, 10), including COX-2. AREs can range in size and generally contain one or more copies of the pentameric sequence AUUUA. The COX-2 message contains up to 22 copies of AUUUA in the 3'-UTR, many of which are clustered in the proximal 10% of the 3'-UTR. Murine COX-2 mRNA contains 7 AUUUA repeats within the first 60 nucleotides of the 3'-UTR. We have shown previously that this region plays an important role in regulating message stability and translational efficacy in rat mesangial cells stimulated with interleukin-1β (11).

Several RNA-binding proteins have been identified which recognize ARE-containing sequences. Binding of some proteins can lead to increased message expression, as seen for ELAV family proteins (HuR, HuR/Hel-N1, Huc, and HuD) (12, 13). Binding of other proteins, such as TIA-1, TIAR, and tristetraprolin, promotes decreased message expression (14–18). Still other proteins like AUF1 (also known as hnRNP D) may play a role in both degradation and stabilization of target messages (7, 19). Adding to the complexity of these interactions is the fact that many of these proteins exist as multiple isoforms that arise because of alternative splicing events, all of which may be subject to post-translational modifications.

As a first approach to understanding the mechanism whereby LPS regulates COX-2 expression, we used the proximal region of the 3'-UTR of COX-2 as a target to identify sequences required for LPS stimulation and proteins that bind this region of the 3'-UTR. The proximal 60 nucleotides of the 3'-UTR of COX-2 contained a LPS response element that bound a large number of RNA-binding proteins. Many of these proteins were present as alternatively spliced isoforms and appear to be post-translationally modified.

EXPERIMENTAL PROCEDURES

Cell Culture—The RAW 264.7 macrophage cell line was maintained in a 95% air, 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium plus 10% (v/v) heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 15 µM HEPES. Cells were stimulated with LPS (Escherichia coli 0111:B4, Sigma) at 100 ng/ml for the indicated times.
Plasmids—Reporter-gene constructs were generated as described previously (11). Briefly, various regions of the 3'-UTR of COX-2 were amplified by PCR using primers terminating in XbaI recognition sequences. PCR products were ligated in the unique XbaI site of the pGL3 control vector (Promega Corp.), located in the 3'-UTR of the firefly luciferase gene. Vectors used for synthesis of RNA probes for electro-
Protein complexes with the proximal region of the 3′-UTR of COX-2 and cellular fractions of RAW 264.7 cells. Radio-

labeled RNA probes were incubated with either cytosolic or nuclear fractions isolated from RAW 264.7 cells treated with

or without LPS for 1 h. Complexes were separated on nondenaturing gels and visualized by autoradiography. Complexes

were identified by changes in electrophoretic mobility compared with free probe.

Luciferase Assay—Luciferase activity was determined using a luciferase assay system, following the manufacturer’s protocol (Promega Corp.). Briefly, cell monolayers in 6-well cluster dishes were removed by scraping into 100 μl of reporter lysis buffer. Cells were vortexed and cellular debris removed by centrifugation (30 s at 12,000 g). Luciferase activity was measured using a Lumat LB 9507 luminometer (E G & G Wallac) as described previously (11).

Cell Fractionation—All fractionation buffers were kept on ice and contained 0.25 mM Fafobac, 2 μM leupeptin, 0.3 μM aprotinin, and 0.1 mM sodium orthovanadate. Cells were washed in ice-cold phosphate-buffered saline, scraped off the flask into buffer A (10 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM MgCl2, 5% glycerol), and incubated on ice for 10 min. After incubation, Nonidet P-40 was added (final concentration 0.25%), and the cells were vortexed for 10 s. Nuclei were isolated by centrifugation (1,500 g for 5 min at 4 °C). The supernatant (nuclear protein) was removed and 7 mg of cytosolic protein were obtained. Protein concentrations were measured using the RC DC protein assay (Bio-Rad).

Biotinylated RNA oligonucleotides corresponding to either the 3′-UTR of the COX-2 gene or a 63-nucleotide region of the TA cloning vector (Invitrogen) were made by in vitro transcription using a modified protocol of the T7-MEGAscript high yield kit (Ambion). A final reaction volume of 20 μl contained the following: 2 μl of 10× transcription buffer; 2 μl each of 75 mM ATP, GTP, UTP, 1 μl of 55 mM CTP; 7.5 μl of 10 mM biotinylated CTP (Invitrogen); 10 μg of COX-2 DNA template; and 2 μl of T7 MEGAscript enzyme mix. Transcription was carried out overnight at 37 °C and the reaction stopped by adding 2 μl of RNase-free DNase and incubating for 15 min at 37 °C. Unincorporated nucleotides were removed with mini Quick Spin RNA columns centrifuged at 1500 × g for 4 min. The final concentration of RNA was determined spectrophotometrically by measuring absorbance at 260 nm.

EMSAs and Antibody Supershifts—Typically, 5–10 μg of cell fraction protein was incubated for 30 min at 37 °C in EMSA binding buffer (10 mM HEPES pH 7.6, 5 mM MgCl2, 40 mM KC1, 1 mM DTT, 5% glycerol, 5 mg/ml heparin) with 1–5 pmol of 32P-labeled RNA probe in a final volume of 20 μl. For supershift experiments, binding mixtures included 0.2 μg of affinity-purified IgG for anti-HuR, -TIA-1 and -TIAR (Santa Cruz Biotechnology); or 1 μl of anti-AUF1 (20); or 1 μl of normal goat serum (Sigma). RNase T1 (10 units) was added to EMSAs or supershift samples and incubated for 15 min at 37 °C. Loading buffer (5 μl) containing 80% glycerol, 0.1% bromphenol blue in 50 mM Tris-HCl, pH 7.5 was added, and samples were electrophoresed (250 V for 3 h) on 4% polyacrylamide gels (pre-run 1 h at 200 V) containing 44 mM Tris-HCl, pH 8.3, 44 mM boric acid, 1 mM EDTA, 4% acrylamide:bisacrylamide (29:1), and 2.5% glycerol. EMSAs were visualized by autoradiography.

Binding Reaction—Approximately 20 units of RNase inhibitor was added to 5 mg of nuclear or cytosolic protein and incubated with 120–150 μg of biotinylated RNA at room temperature for 2 h with constant rotation. Streptavidin-agarose beads (600 μl) were washed three times with 1 ml of EMSA binding buffer, added to the protein/biotinylated RNA mixture, and incubated for an additional 2 h at room temperature with constant agitation. The beads were then washed four times with 1 ml of EMSA binding buffer at room temperature, and bound proteins were eluted from the RNA with a high ionic strength salt solution (1 M NaCl in EMSA binding buffer) or with rehydration buffer.

Sample Preparation—For protein identification experiments, samples were desalted using a Millipore Centricon centrifugal filter device with a molecular mass cutoff of 10 kDa. Protein samples were brought to a volume of 2 ml in a 4% CHAPS solution, applied to the Centricon membrane, and centrifuged at 1,500 × g for 4 h. The concentrated sample was again diluted to 2 ml in 4% CHAPS, applied to the membrane, and centrifuged at 1,500 × g for 8 h to a final volume of ~30 μl. Protein concentrations were measured using the RC DC protein assay (Bio-Rad).
For Western analysis, samples were desalted and concentrated by acetone precipitation. Acetone, stored at −80 °C, was added at a ratio of 5:1 (v/v), vortexed briefly, and incubated for 30 min at −80 °C. The precipitate was collected by centrifugation for 5 min at 16,000 × g. The supernatant was removed, and the protein pellet was air dried for 5 min. Protein was resuspended in 250 μl of rehydration buffer prior to isoelectric focusing.

Isoelectric Focusing—Samples were added to the rehydration solution (8 M urea, 0.15 mg/ml DeStreak reagent (Amersham Biosciences), 4% CHAPS, 0.2% carrier ampholytes, pH 7–10, 0.0007% bromphenol blue) in a ratio of 9:1 of rehydration solution to sample. Samples were applied to 11-cm immobilized pH gradient strips, pH 7–10 (Bio-Rad) and incubated at room temperature for 1 h. Strips were passively rehydrated overnight (16 h) while covered in mineral oil. After rehydration, the strips were transferred to a focusing tray with prewetted wicks and covered with fresh mineral oil. The strips were focused for a total of 17,000 volt-hours with rapid ramping on the Bio-Rad Protean IEF Cell.

Second Dimension—After focusing the strips were equilibrated in equilibration buffer I (6 M urea, 2% SDS, 0.05 M Tris-HCl, 20% glycerol, and 2% DTT) for 10 min and equilibration buffer II (6 M urea, 2% SDS, 0.05 M Tris-HCl, 20% glycerol, and 2.5% iodoacetamide) for 10 min, both
were covered with an overlay of agarose and run for 200 V.

Proteins were transferred to Immobilon-P membranes (Millipore Corp.) and subjected to two-dimensional electrophoresis as described above. Proteins were visualized with Coomassie Blue and identified by mass fingerprint analysis and electrospray mass spectrometry sequencing (see Table I).

Western Blot Analysis—Nuclear and cytoplasmic fractions were isolated from RAW 264.7 cells treated with and without LPS for 1 h. Proteins that bound to biotinylated RNA were eluted with rehydration buffer and subjected to two-dimensional electrophoresis as described above. Proteins were transferred to Immobilon-P membranes (Millipore Corp.) for 1 h at 200 V, blocked with 5% nonfat milk, and probed with monoclonal anti-HuR antibody, polyclonal anti-TIA-1 (Santa Cruz Biotechnology); polyclonal anti-TIAR (BD Biosciences); or polyclonal anti-HuR antibody, polyclonal anti-TIA-1 (Santa Cruz Biotechnology); polyclonal anti-TIAR (BD Biosciences); or polyclonal anti-HuR antibody, polyclonal anti-TIA-1 (BD Biosciences); or polyclonal anti-HuR antibody, polyclonal anti-TIA-1 (BD Biosciences); or polyclonal anti-HuR antibody. Peroxidase-linked secondary antibodies (Amersham Biosciences) were used at a 1:10,000 dilution. Signal detection was carried out using enhanced chemiluminescence system (ECL, Amersham Biosciences).

RESULTS

The First 60 Nucleotides of the 3′-UTR of COX-2 Contain a Negative Control Element That Is Regulated by LPS—Various regions of the 3′-UTR of COX-2 were inserted into the 3′-UTR of the luciferase reporter-gene (Fig. 1A) and transiently expressed in RAW 264.7 cells. When the full-length 3′-UTR (1–2232) was placed in the reporter message, luciferase activity decreased more than 90% compared with luciferase alone (Fig. 1B). Luciferase activity decreased 60% with insertion of only the first 60 nucleotides of the 3′-UTR of murine COX-2. This region of the 3′-UTR contains multiple copies of the AUUUA consensus sequence reported to confer decreased stability and/or translational efficiency of COX-2 mRNA (11). Sequential deletion of the proximal portion of this region of the 3′-UTR resulted in first an increase in reporter-gene expression and then a further decrease in expression (Fig. 1B), suggesting that multiple control elements were operative. When RAW cells were exposed to LPS, reporter-gene activity from a message-containing luciferase control without any COX-2 sequence increased less than 2-fold within a 6-h treatment time (Fig. 1C).

However, the activity of the reporter-gene containing the full-length 3′-UTR increased more than 10-fold (Fig. 1C) under the same treatment conditions. Again, a large portion of the increase occurred in the presence of the proximal region of the 3′-UTR alone, where the 60-nucleotide insert caused a 6-fold increase in luciferase activity within 6 h of stimulation (Fig. 1C) and more than 10-fold over a 10-h stimulation (results not shown). The LPS responsiveness of the reporter-gene was lost when the first 23 nucleotides of the 3′-UTR were deleted. Thus, the first 60 nucleotides of the 3′-UTR of COX-2 contain major control elements that confer decreased expression under non-stimulated conditions and increased expression in response to LPS stimulation.

The First 60 Nucleotides of the 3′-UTR of COX-2 Form Multiple RNA-Protein Complexes That Contain Known RNA-binding Proteins—To understand more fully how the proximal region of the 3′-UTR of COX-2 regulates message expression, we set out to identify protein factors that bind to this region of the message. In the first set of experiments we used EMSAs to look for known RNA-binding proteins that regulate message expression. When in vitro transcribed RNA representing the first 60 nucleotides of the 3′-UTR of COX-2 was incubated with either nuclear fractions or cytosolic fractions from RAW 264.7 cells, there were shifts in the mobility of the RNA probe indicating the formation of multiple RNA-protein complexes. EMSA results identified at least five distinct complexes (Fig. 2). These complexes formed using both nuclear (lanes labeled N) and cytosolic (lanes labeled C) fractions and were not grossly altered by treatment with LPS. However, truncating the RNA probe resulted in decreased intensity of some complexes (Fig. 2, C4 and C5) and increased intensity of others (Fig. 2, C2 and C3). These changes were more prominent with the nuclear protein fractions. The fact that the mobility of the complexes did not change as a result of the truncation suggests that the composition of the complexes was not altered drastically, and the change in intensity occurred because of a change in complex stability.

The identity of some of the proteins present in the complexes was determined by adding antibodies raised against known RNA-binding proteins and looking for a loss in complex intensity and/or formation of a higher molecular mass complex (supershift). Using this approach we demonstrated that the RNA-binding proteins TIAR, AUF1, HuR and TIA-1 were able to bind to the proximal 60 nucleotides of the 3′-UTR of COX-2.

When using nuclear fractions (Fig. 3A), two higher molecular mass complexes formed (supershifts S2 and S3). Both supershifts were present with the 1–60 probe, whereas only the higher supershift (S3) remained when the probe was truncated. In all cases, the supershift was accompanied with a severely decreased intensity in complex C3. Likewise, addition of anti-HuR antibody resulted in formation of a supershift (S1) and decreased intensity of complexes C1 and C2 formed with cytosolic proteins (Fig. 3A). The supershift was more apparent in the 60-nucleotide probe and decreased as the probe was truncated, whereas the loss of complexes C1 and C2 occurred with both probes. Addition of antibody directed against AUF1 caused no major change in the intensity of the RNA-protein complexes nor caused the appearance of a supershift when used in conjunction with cytosolic protein fractions (Fig. 3A).

When using nuclear fractions (Fig. 3B), supershift results with TIAR-1, TIAR, and HuR antibodies were similar to those using proteins isolated from cytosolic fractions (Fig. 3A). The exceptions were that the TIAR-1 and TIAR antibodies formed a single higher molecular mass complex (Fig. 3B, S2), and the...
HuR-dependent supershift was greatly diminished (Fig. 3B, S1). In contrast, addition of anti-AUF1 antibody to binding reactions containing nuclear protein fractions resulted in nearly a complete disappearance of all RNA gel shifts (Fig. 3B). These results were reproducible and dependent on the concentration of antibody added, suggesting that AUF1 was present in all the nuclear RNA-protein complexes. In all cases, the appearance of a supershift and disappearance of complex(es) was not affected by treating cells with LPS prior to cellular fractionations.

Additional Proteins That Bind the 3'-UTR of COX-2—As a second approach to identify proteins that bind to the 3'-UTR of COX-2, biotinylated RNA probes were incubated with nuclear protein fractions, and bound proteins were isolated with streptavidin-coated agarose beads. Proteins were eluted from the RNA with a high ionic strength buffer (1 M NaCl), separated on two-dimensional gels, and stained with Coomassie Blue. The indicated spots (Fig. 4) were excised, digested with trypsin, and subjected to peptide mass fingerprinting by MALDI and confirmed by liquid chromatography coupled to electrospray mass spectrometry. The identification of the spots is summarized in Table I. The 19 spots we identified were represented by six different proteins. Two of these proteins were identified previously in the EMSA experiments described above; namely, TIAR and AUF1. The additional proteins were CPF-A (hnRNP A/B), RBM3, hnRNP A2/B1, and hnRNP A3.

CPF-A was identified as an ARE-binding protein that exists as two isoforms, p37 and p42, which arise because of alternative splicing (21). We identified five spots that matched the sequence of CPF-A, three migrated at the lower molecular mass (p37), and two at the higher molecular mass (p42). The liquid chromatography electrospray mass spectrometry data from spots 1 and 4 contained sequences that span the alternatively spliced exon junction confirming that these proteins were

![Fig. 5. Two-dimensional Western analysis of AUF1.](image)

![Fig. 6. Two-dimensional Western analysis of TIAR.](image)
Proteins That Bind an LPS Response Element of COX-2

Fig. 7. Two-dimensional Western analysis of TIA-1. Nuclear protein fractions isolated from RAW 264.7 cells that bound the proximal 60 nucleotides of the 3′-UTR of COX-2 were resolved on two-dimensional gels ranging from pH 7 to 10. Blots using proteins from nonstimulated cells (NS) or cells treated with LPS for 1 h (LPS) were probed with anti-TIA-1 antibody.

Fig. 8. Two-dimensional Western analysis of HuR. Nuclear protein fractions isolated from RAW 264.7 cells that bound the proximal 60 nucleotides of the 3′-UTR of COX-2 were resolved on two-dimensional gels ranging from pH 7 to 10. Blots using proteins from nonstimulated cells (NS) or cells treated with LPS for 1 h (LPS) were probed with anti-HuR antibody.

In addition to different molecular masses, proteins present in multiple spots migrated at different isoelectric points. Some of these differences may occur because of changes in primary sequences as a consequence of alternative splicing. In other cases, isoforms of a protein of the same apparent molecular mass and most likely the same primary sequence were present at multiple isoelectric points. The range of isoelectric points for an individual protein varied from approximately half a pH unit for AUF1 isoforms p45 (spots 3 and 11) and p42 (spots 10 and 12), to 1–2 pH units for CBF-A isoforms p37 (spots 16, 1, and 4) and p42 (spots 2 and 17) and RBM3 (spots 6, 7, and 8). These changes in isoelectric points are believed to be the result of post-translational modifications.

To determine whether additional isoforms of RNA-binding proteins were bound to the 3′-UTR of COX-2, we performed Western analysis using a series of two-dimensional gels with proteins eluted from RNA affinity probes. Western blots were probed with antibodies raised against RNA-binding proteins AUF1, TIAR, TIA-1, and HuR. AUF1 antibody recognized multiple isoforms that span the entire pH range of 7–10 (Fig. 5). Several different molecular mass species appeared which migrated between the 35- and 50-kDa standards. AUF1 and CBF-A have a high degree of homology, and it has been reported that AUF1 antibodies cross-react with CBF-A (21), thus it is likely that the reactive proteins were a combination of AUF1 and CBF-A isoforms.

In contrast to the AUF1 result, antibodies directed against TIAR and TIA reacted with a limited number of protein species (Figs. 6 and 7). TIAR antibodies reacted with isoforms migrating at three different pH values and two different molecular masses. TIAR-1-specific antibodies recognized two molecular mass species migrating at different pH values. The two different molecular mass proteins for TIAR and TIA-1 were consistent with the two known alternatively spliced isoforms (23). The change in isoelectric points may represent post-translational modifications of the proteins. Finally, HuR was detected as a single spot that migrated at an isoelectric point nearly equal to 10 (Fig. 8).

RNA affinity probes were also made using an unrelated DNA template derived from the TA cloning vector that contained no...
**Proteins That Bind an LPS Response Element of COX-2**

Are.

Western analysis of protein fractions from cells treated with LPS indicated subtle changes in the molecular species of the RNA-binding proteins that recognize the 3'-UTR of COX-2. Probing blots with anti-TIAR antibodies demonstrated that three isoforms of the protein, differing in their isoelectric point, were bound to the RNA probe (Fig. 6). The two isoforms of lower pI reacted with the antibody at a similar intensity when using protein from nonstimulated and LPS-treated cells. However, the reactive species present at the highest pI was present at a much higher level in the LPS treatment group. Also, the higher molecular mass isoform was identified only with the LPS-treated protein fraction. AUF1 also showed a difference in reactive protein species bound to RNA probes when using nuclear fractions from nonstimulated and LPS-treated cells. However, the reactive species present at the highest pI was present at a much higher level in the LPS treatment group. 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The proximal region of the 3'-UTR of the COX-2 message plays a pivotal role in regulating protein expression. The first 60 nucleotides of the message contain a highly conserved ARE composed of multiple iterations of the sequence AUUUA. This region of the COX-2 message was sufficient to cause decreased expression of a heterologous reporter-gene. When expressed in a murine macrophage cell line (RAW 264.7), reporter-gene activity increased after treatment with LPS. This response was dependent on the 3'-UTR sequences from COX-2 message which were present in the reporter-gene message. Inclusion of the full-length 3'-UTR or the proximal 60 nucleotides alone caused the reporter-gene to be regulated by LPS. Furthermore, removal of the first 60 nucleotides from the full-length 3'-UTR attenuated the LPS response and truncation of the 60 nucleotide region such that the first group of AUUUA repeats was removed caused a complete loss of LPS induction. The results suggest that the proximal region of the 3'-UTR of COX-2 plays two roles in regulating gene expression. First, this region was required for decreased gene expression under basal conditions, and second, it was required for full induction of gene expression after exposure to LPS.

Several RNA-binding proteins have been reported to bind the 3'-UTR of COX-2: AUF1 (25), HuR (26, 27), CPF-A (21), CUGBP2 (28), TTP (17), and hnRNP A0 (29). Most of these were reported to bind to the proximal region of the 3'-UTR equivalent to the region used in our study, except for TTP which bound a more distal site (the report on hnRNP A0 did not identify the specific binding site). Using a combination of EM-SAs, proteomics, and Western analysis we identified many of these proteins as well as others that bind to the proximal 60 nucleotides of the 3'-UTR of COX-2. The function of many of the proteins we identified is known. Some have been reported to regulate message stability, HuR (26, 27), AUF1 (25), and CPF-A (21) and others such as TIAR and TIA-1 are believed to help identify key proteins required for LPS regulation of COX-2.

Additionally, we identified RBM3 as a COX-2 ARE-binding protein. The exact function of this protein is not yet known, but RBM3 contains a RNA binding motif and has been reported to be up-regulated during cold-shock (32, 33) and identified as a cytokine-induced gene in human premyeloid cells (34).

The complexity of proteins that bind to the 3'-UTR of COX-2 was further exemplified by the proteomic results. Our proteomic analysis demonstrated that multiple isoforms of AUF1, CPF-A, RBM3, hnRNP A2/B1, and hnRNP A3 bind to this region of the 3'-UTR of COX-2. The different isoforms of a given protein were identified by shifts in isoelectric point and/or apparent molecular mass. Changes in molecular mass can arise because of alternative splicing events. In fact, electrospray mass spectrometric data confirmed the presence of alternative splice forms for AUF1 and CBF-A. HnRNP A2/B1 and hnRNP A3 were identified at two spots that migrated at different molecular masses, suggesting that alternatively spliced iso-
forms of these proteins also exist. Alternative splice variants of TIA-1 and TIA-1 have been reported previously (23). Thus, the synthesis of alternatively spliced isoforms appears to be a common theme for RNA-binding proteins.

Changes in a protein's pI may be caused by post-translational modifications, such as phosphorylation, sulfation, and acetylation. We have shown that the majority of the proteins identified in this study migrated to multiple isoelectric points. AUF-1, CFP-A, and RMB3 all were identified by MALDI and electrospray mass spectrometry at different isoelectric points and the same apparent molecular mass. The range of pI values represented by each protein varied but extended from 0.5 to 2 pH units. Western analysis of two-dimensional gels demonstrated that AUF-1 antibodies showed reactive forms of these proteins across the entire gel (pH 7–10). In this case it is likely that the reactive proteins were a composite of AUF-1 and CBF-A because it was reported that AUF-1 antibodies can cross-react with CBF-A because of their high degree of homology (21).

EMSA experiments demonstrated that RNA-binding proteins HuR, TIA-1, TIA-1, and AUF1 could bind to the first 60 nucleotides of the 3'-UTR of COX-2. The results of these experiments differed using proteins isolated from nuclear fractions compared with cytosolic fractions. When using nuclear protein fractions, the addition of antibodies directed toward HuR, TIA-1, or TIA-1 resulted in a single supershift in mobility of the RNA probe which was accompanied by decreased intensity of specific RNA-protein complexes. In contrast, when using cytosolic protein fractions, the supershift formed with antibodies raised against TIA-1 and TIA-1 was present as a doublet. When the RNA probe was truncated, the faster migrating complex was lost. The single supershift using nuclear proteins appeared to migrate at the position of the lower band of the doublet seen with the cytosolic protein fractions. One explanation for these results is that TIA-1 and TIA-1 were modified prior to exiting the nucleus. This modification could affect the affinity of these proteins with other protein partners and thereby change the molecular mass of the supershifted complex. Truncation of the probe promoted the formation of the slower moving complex.

The most striking difference between nuclear and cytosolic proteins was seen with AUF1 EMSA experiments. There was no discernible change in the gel shift pattern when AUF1 antibody was added to cytosolic protein-binding reactions. However, there was nearly complete disruption of all RNA-protein complexes when AUF1 antibody was included in the nuclear protein reactions. The reason for the difference is not clear. Either AUF1 in cytosolic fractions did not bind to the RNA probe, or cytosolic protein complexes had a different composition or conformation such that the anti-AUF1 antibody no longer recognized the reactive epitope. Two-dimensional Western experiments showed that AUF-1 antibodies detected proteins from cytosolic fractions that were bound to the proximal region of the 3'-UTR of COX-2 (results not shown), suggesting that the inability to detect supershifts or disrupt RNA-protein complexes with cytosolic protein fraction was caused by sequestration of the antigenic determinant of AUF1.

In addition to altered binding properties dependent on the cellular location of proteins, we measured subtle changes in protein migration in two-dimensional gels as a result of stimulation with LPS. Western analysis indicated a change in reactivity of some isoforms of RNA-binding proteins and the appearance and disappearance of others. LPS is known to activate multiple protein kinase cascades and may lead to the phosphorylation of many of the RNA-binding proteins and alter their functional properties. Indeed, it has been shown that phosphorylation of various hnRNP proteins resulted in altered affinity of the proteins for either RNA targets and/or other proteins (29, 35, 36). Recently, it has been reported that dephosphorylation of the p40 isoform of AUF1 coincided with rapid induction and increased stability of ARE-containing messages (37). Furthermore, the phosphorylation status of p40AUF1 altered its affinity for ARE-containing messages and altered the RNA conformation (38). The changes in protein migration we have found for AUF1, as well as the other proteins, may be the result of altered phosphorylation states. Changes in pI are not limited to phosphorylation events and may arise because of other post-translational events such as sulfation and acetylation, which would lead to a change in protein charge. The cause of the differences in protein migration after stimulation with LPS is not yet known. Furthermore, it is difficult to determine the exact nature of post-translational modifications using immunological methods and requires the use of more sensitive and quantitative techniques.

The results presented in this report demonstrate a wide range of isoforms of RNA-binding proteins that were present in RAW cells which were able to bind to a specific sequence of the 3'-UTR of COX-2. EMSA results suggest that many of these proteins form stable multimeric complexes that are able to bind to RNA. Results using control RNA affinity probes suggest that binding of these proteins was sequence-specific and increases the number of members that belong to the family of ARE-binding proteins. Further work remains to be done to determine whether the same RNA-protein interactions shown in this report also occur in vivo and to characterize the biological significance of such interactions.

Activation of cells with LPS results in modification of the RNA-protein complexes. The precise nature of the modifications is not yet known but represents potential post-translational alterations that may regulate RNA-protein and/or protein-protein interactions. It is these interactions that ultimately determine whether the message is transported out of the nucleus and directed toward the translational machinery or targeted for degradation.

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REFERENCES

1. Smith, W. L., Dewitt, D. L., and Garavito, R. M. (2000) Annu. Rev. Biochem. 69, 145–182
2. Barrios-Rodiles, M., Tiraloche, G., and Chadee, K. (1999) J. Immunol. 163, 963–969
3. Eliforsos, A. G., Dumitru, C. D., Wang, C. C., Cho, J., and Tsichlis, P. N. (2002) EMBO J. 21, 4831–4840
4. Wadleigh, D. J., Reddy, S. T., Kopp, E., Ghosh, S., and Herschman, H. R. (2000) J. Biol. Chem. 275, 6259–6266
5. Dean, J. L. E., Brook, M., Clark, A. R., and Saklatvala, J. (1999) J. Biol. Chem. 274, 264–269
6. Frevel, M. A., Bakheet, S., Silva, A. M., Hissong, J. G., Khabar, K. S. A., and Williams, B. R. G. (2003) Mol. Cell. Biol. 23, 425–436
7. Guhanayogi, J., and Brewer, G. (2003) Gene (Amst.) 265, 11–23
8. Gryzynskyw, E. A., Wilczynska, A., and Siedlecki, J. A. (2001) Biochem. Biophys. Res. Commun. 288, 291–295
9. Bakheet, T., Frevel, M., Williams, B. R., Greer, W., and Khabar, K. S. (2001) Nucleic Acids Res. 29, 2546–2554
10. Tebo, J., Der, S., Frevel, M., Khabar, K. S. A., Williams, B. R. G., and Hamilton, T. A. (2003) J. Biol. Chem. 278, 12985–12993
11. Col, S. J., and Morrison, A. R. (2003) J. Biol. Chem. 278, 23179–23185
12. Fan, X. C., and Steitz, J. A. (1998) EMBO J. 17, 3448–3460
13. Peng, S. S.-Y., Chen, C.-Y. A., Xu, N., and Shyu, A.-B. (1998) EMBO J. 17, 3461–3470
14. Pecyk, M., Wex, S., Beck, A. R. P., Kedersha, N., Gupta, M., Maritim, B., Chen, S., Guedan, C., Kruys, V., Streuli, M., and Anderson, P. (2000) EMBO J. 19, 4154–4163
15. Guedan, C., Droogmans, L., Chalon, P., Huez, G., Caput, D., and Kruys, V. (1999) J. Biol. Chem. 274, 2322–2332
16. Mahtani, K. R., Brook, M., Dean, J. L. E., Sully, G., Saklatvala, J., and Clark, A. R. (2001) Mol. Cell. Biol. 21, 6481–6489
17. Sawao, H., Dixon, D. A., Oates, J. A., and Boutaud, O. (2003) J. Biol. Chem. 278, 13928–13935
18. Lai, W. S., Kennington, E. A., and Blackshear, P. J. (2003) Mol. Cell. Biol. 23, 3798–3812
19. DeMaria, C. T., and Brewer, G. (1996) J. Biol. Chem. 271, 12179–12184
20. Zhang, W., Wagner, B. J., Ehrenman, K., Schaefer, A. W., and DeMaria, C. T.,
Proteins That Bind an LPS Response Element of COX-2

21. Crater, D., DeHaven, K., Long, L., and Brewer, G. (1993) Mol. Cell Biol. 13, 7652–7665
22. Dean, J. L., Sully, G., Wait, R., Rawlinson, L., Clark, A. R., and Saklatvala, J. (2002) Biochem. J. 366, 709–719
23. Wagner, B. J., DeMaria, C. T., Sun, Y., Wilson, G. M., and Brewer, G. (1998) Genomics 48, 195–202
24. Beck, A. R., Medley, Q. G., O’Brien, S., Anderson, P., and Streuli, M. (1996) Nucleic Acids Res. 24, 3829–3835
25. Kotlyarov, A., Neininger, A., Schubert, C., Eckert, R., Birchmeier, C., Volk, H. D., and Gaestel, M. (1999) Nat. Cell Biol. 1, 94–97
26. Beck, A. R., Medley, Q. G., ’O’Brien, S., Anderson, P., and Streuli, M. (1996) Nucleic Acids Res. 24, 3829–3835
27. Lasa, M., Mahtani, K. R., Finch, A., Brewer, G., Saklatvala, J., and Clark, A. R. (2000) Mol. Cell. Biol. 20, 4265–4274
28. Dixon, D. A., Tolley, N. D., King, P. H., Nabors, L. B., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (2001) J. Clin. Invest. 108, 1657–1665
29. Sengupta, S., Jang, B. C., Wu, M. T., Paik, J. H., Furneaux, H., and Hla, T. (2003) J. Biol. Chem. 278, 25227–25233
30. Mukhopadhyay, D., Houzhen, C. W., Kennedy, S., Dieckgraefe, B. K., and Anant, S. (2003) Mol. Cell 11, 113–126
31. Rousseau, S., Morrice, N., Pegge, M., Campbell, D. G., Gaestel, M., and Cohen, P. (2002) EMBO J. 21, 5505–5514
32. Wilson, G. M., Lu, J., Sutphen, K., Sun, Y., Huyhn, Y., and Brewer, G. (2003) J. Biol. Chem. 278, 33029–33038
33. Wilson, G. M., Lu, J., Sutphen, K., Suarez, Y., Sinha, S., Brewer, B., Villanueva-Feliciano, E. C., Ylas, R. M., Charles, S., and Brewer, G. (2003) J. Biol. Chem. 278, 33039–33048