Functionally Independent AU-rich Sequence Motifs Regulate KC (CXCL1) mRNA*

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Certain pro-inflammatory chemokine mRNAs containing adenine/uridine-rich sequence elements (AREs) in their 3′ untranslated regions (3′-UTRs) are known to exhibit constitutive instability and sensitivity to pro-inflammatory stimuli resulting in the stabilization of the message. Using tetR-regulated transcription we now show that the 3′-UTR of the mouse CXCL1 (KC) mRNA contains at least two ARE motifs that are structurally and functionally distinct. A fragment of 77 nucleotides containing 4 clustered AUUUA pentamers located at the 5′-end of the KC 3′-UTR is only modestly unstable yet promotes markedly enhanced, post-transcriptional protein production in response to either interleukin-1α (IL-1α) or lipopolysaccharide (LPS), suggesting translational regulation. In contrast, a fragment containing 3 isolated AUUUA pentamers corresponding to the residual 3′ 400 nucleotides of the KC 3′-UTR confers both instability and is stabilized in response to IL-1α. Although the clustered AUUUA pentamers in the upstream region are required for stimulus sensitivity, mutation of all three pentamers in the downstream region has little or no effect on either instability or stimulus sensitivity. The upstream region is comparably stabilized in response to either IL-1α or LPS, whereas the AUUUA-independent downstream determinant is differentially more sensitive to IL-1α. Finally, using UV-induced RNA cross-linking, these functionally independent sequences exhibit different patterns of interaction with RNA-binding proteins. Collectively, these findings document the presence of multiple independent determinants of KC mRNA function and demonstrate that these operate via distinct mechanisms.

Tissue inflammation is orchestrated via the production of multiple cytokines and chemokines that control the trafficking of leukocytes to sites of tissue injury and infection (1–4). The production of these secreted mediators is stringently regulated at multiple mechanistic levels, including gene transcription, mRNA translation, and ultimately selective degradation of specific mRNAs (5–8). Hence the pattern of gene expression depends critically upon appropriate engagement of each of these regulatory steps and deficiencies at any specific stage have been demonstrated to profoundly impact normal function (9, 10).

mRNA degradation is now widely recognized as an important regulatory step in controlling gene expression, and this is particularly true for short lived mRNAs such as those encoding cytokine and chemokine proteins (6, 7, 11, 12). The instability of such mRNAs is determined by sequence motifs frequently located in the 3′ untranslated region (3′-UTR) of the message. The best studied of these sequences are known as adenine/uridine rich elements (AREs) and have been demonstrated to confer marked instability and, in some cases, potent sensitivity for stabilization in response to extracellular stimuli (6, 8, 11, 13). Moreover, ARE sequences have been reported to regulate translational efficiency that also exhibits potent stimulus sensitivity (14–16). More than 1000 ARE-containing mRNAs have been defined within the human genome (17). Not surprisingly, these exhibit marked structural heterogeneity, and at least three broad classes have been identified (18). Class I AREs contain multiple independent repeats of the AUUUA pentamer. Class II AREs are defined on the basis of their content of multiple overlapping or closely juxtaposed AUUUA motifs. Class III AREs contain no pentamer motifs but do contain stretches of AU or U rich sequence. A number of recent reports suggest that there is substantial functional heterogeneity among ARE-containing mRNAs (19–23). Certainly such mRNAs exhibit a broad range of decay rates and likewise show considerable variability with respect to their sensitivity for modulation of decay in response to extracellular stimulation.

The functional activity of ARE sequences reflects their interaction with proteins exhibiting appropriate sequence recognition specificity. A number of ARE-binding proteins have been identified over the last 15 years, and these have been correlated with either altered decay or translation of target mRNAs (24–28). Despite much interest, however, clear cause and effect relationships between individual ARE-binding proteins and the behavior of specific target mRNAs have been established in only a few cases (29–33). In light of the number of ARE-containing mRNAs, the structural heterogeneity they exhibit, and the number of RNA-binding proteins showing preference for AU-rich sequences, it is likely that ARE sequences confer a broad array of behaviors and provide significant diversity to the regulation of gene expression.

In the present study, we have evaluated the functional heterogeneity of sequences within the mRNA encoding the mouse chemokine CXCL1 or KC. The KC gene (scyb1) encodes a potent neutrophil chemoattractant and serves as a functional homologue of human IL-8 in the mouse (1–4). This mRNA is highly unstable in leukocytes, endothelial cells, fibroblasts, and epithelial cells and can be stabilized very effectively in response to several pro-inflammatory stimuli, including lipopo-

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* This work was supported by United States Public Health Services Grants CA39621 and AI50739. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: UTR, untranslated region; KC, mouse CXCL1; ARE, Adenosine Uridine rich elements; Dox, doxycycline; MOPS, 3-(N-morpholino)propanesulfonic acid; IL-8, interleukin-8; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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lysaccharide (LPS) and IL-1α (34–36). Although we have previously reported that a clustered set of four overlapping AUUUA pentamers is a critical determinant of this behavior, the 3′-UTR of KC contains three isolated pentamer motifs as well as additional AU-rich sequence (37). Functional dissociation of the full 3′-UTR of KC mRNA reveals two independent determinants that confer mechanistically different post-transcriptional regulation of KC gene expression.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Dulbecco’s modified Eagle’s medium, Dulbecco’s phosphate-buffered saline, and antibiotics were obtained from Central Cell Services of the Lerner Research Institute. Neomycin sulfate (G418), puroycin, formamide, dextran sulfate, MOPS, diethylpyrocarbonate, actinomycin D, protease inhibitor mixture, and lipopolysaccharide (prepared from the *Escherichia coli* serotype 0111:B4) were purchased from Sigma. Fetal bovine serum was purchased from BioWhittaker (Walkersville, MA). Doxycycline (Dox) and the vector pTRE2 were obtained from Clontech Laboratories (Palo Alto, CA). Random priming kits were purchased from Stratagene (Cedar Creek, TX). RNase-free DNase I (Promega, Madison, WI). Nycodenz transfer membrane was purchased from Micron Separation (Westboro, MA). SuperFect Transfection Reagent was obtained from Qiagen (Valencia, CA), and Triz-Reactant was purchased from Molecular Research Center (Cincinnati, OH). Maxiscript In Vitro Transcription kit, cap analogue (7meGpppG), and salmon sperm DNA were obtained from Ambion Inc. (Austin, TX). Recombinant human IL-1α and KC enzyme-linked immunosorbent assay (ELISA) kits were purchased from BD Mesoscale Discovery (Woburn, MA). PerkinElmer Life Sciences was the source of [α-32P]UTP and [α-32P]dCTP. Protogel, Seqegal (acylamidyl, N,N-methylenebisacrylamide, urea) and related buffers were obtained from National Diagnostics Inc. (Atlanta, GA). Protein assay reagents were purchased from Bio-Rad.

**Plasmids**—Radiolabeled cDNA probes for use in Northern hybridization were derived from plasmids containing fragments of GAPDH and KC in the Bluescript vector. Plasmids used to drive expression of different versions of KC were prepared in pTRE2 (Clontech Inc.). The parent clone was created by insertion of the full KC 5′-UTR and coding region (residues 1 through 359) into the BamHI/NcoI sites of pTRE2, and the 3′-UTR was provided from the rabbit β-globin gene. Additional constructs were created by excising the rabbit β-globin region with XbaI and SapI, and different versions of the KC 3′-UTR sequence were inserted in the remaining EcoRV site. The full-length KC 3′-UTR (designated FL) contained residues 360–1234. The cluster only (CLU) clone was constructed to the 3′-UTR of KC containing the cluster only (CLU) fragment (residues 360–1234) inserted after the TATA box. To create a cluster and promoter construct (designated FL(CLUmt)), the 5′-UTR of KC containing the cluster only (CLU) fragment was inserted 5′ to the TATA box.

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pared by deleting all 7 AUUUA pentamers from KC cDNA (construct ∆5 in Fig. 1); when examined under the same conditions, this mRNA, containing only a truncated version of the 3'-UTR, was stable and insensitive to stimulation. These results were confirmed by measuring the amount of KC protein produced before and after the addition of Dox (Fig. 2).

The role of clustered AUUUA pentameric sequence motifs in determining both mRNA instability as well as sensitivity for stabilization in response to extracellular stimulation has been demonstrated in multiple prior studies (13, 21–23). We have previously reported that the pair of overlapping AUUUA-containing clusters located in the 3'-UTR of the mouse KC mRNA are important for both instability and stimulus-induced stabilization or destabilization in mouse macrophages (34, 35). In the present study we address the role of the residual three isolated pentamer elements located downstream of the cluster. Two different experimental strategies were employed. In the first, we prepared a KC cDNA plasmid construct containing the full 3'-UTR with mutations destroying the 4 clustered AUUUA pentamers. Surprisingly, KC mRNA derived from this construct was highly unstable and exhibited comparably increased rates of decay in response to IL-1α. However, when examined under the same conditions, this mRNA, containing only a truncated version of the 3'-UTR, was stable and insensitive to stimulation. These findings strongly suggest that the KC 3'-UTR contains two independent sequence determinants controlling post-transcriptional mRNA behavior: the clustered AUUUA pentamers located in the 5'-end and the remaining 3 isolated pentamers (including AU-rich regions) in the more 3' region.

To further document the separate function of the CLU and ∆1 fragments, the CLU fragment was placed independently in a construct terminated by the polyadenylation signal (∆5 fragment) and compared with that of the ∆1 fragment following stable transfection into 293tet-off cells. As expected, each construct exhibited constitutive instability and sensitivity for enhanced stabilization in response to IL-1α (Fig. 4, A and B). Surprisingly, however, we noted that there was a significant quantitative difference in the half-lives of mRNAs derived from the two constructs; whereas the ∆1 fragment was highly unstable, as seen in Fig. 3, the CLU fragment was more stable. Moreover, whereas the ∆1 fragment exhibited strong stabilization in response to IL-1α stimulation, the stabilization of the CLU-derived message was more modest, in keeping with its more limited rate of decay. Of interest, however, was the finding that IL-1 stimulated a comparable increase in post-Dox KC protein secretion from both constructs. This finding suggests that IL-1α may regulate the translation of CLU-containing mRNA, a possibility consistent with prior reports demonstrating that some ARE motifs possess such activity (14–16).

Because both the CLU and the ∆1 fragments contain multiple repeats of the AUUUA motif, we assessed the importance of the pentamers in each fragment for their contribution to instability and/or stimulus sensitivity. Wild type and mutated versions of the CLU or the ∆1 fragments were prepared, and their function
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The KC 3′-UTR containing 7 AUUUUA pentamers confers instability and stimulus sensitivity. A, 293tet-off cells were transfected in separate pools with plasmid constructs FL or Δ5 (see Fig. 1) and after 3 h were separated into 3 individual Petri dishes and cultured overnight. Dox (1 µg/ml) with or without IL-1α (10 ng/ml) was added and total RNA was prepared following further incubation for the indicated times. KC and GAPDH mRNA levels were determined in each sample by northern hybridization as described under “Experimental Procedures.” B, groups of 293tet-off cells transfected as in A were separated into four individual 35-mm Petri dishes. After overnight incubation, the cultures were washed and KC protein secretion before and after the addition of Dox ≥ IL-1α were determined as a quantitative estimate of the rate of KC mRNA degradation as described under “Experimental Procedures.” Similar results were obtained in three separate experiments.

FIG. 2. The KC 3′-UTR containing 7 AUUUUA pentamers confers instability and stimulus sensitivity. A, 293tet-off cells were transfected in separate pools with plasmid constructs FL or Δ5 (see Fig. 1) and after 3 h were separated into 3 individual Petri dishes and cultured overnight. Dox (1 µg/ml) with or without IL-1α (10 ng/ml) was added and total RNA was prepared following further incubation for the indicated times. KC and GAPDH mRNA levels were determined in each sample by northern hybridization as described under “Experimental Procedures.” B, groups of 293tet-off cells transfected as in A were separated into four individual 35-mm Petri dishes. After overnight incubation, the cultures were washed and KC protein secretion before and after the addition of Dox ≥ IL-1α were determined as a quantitative estimate of the rate of KC mRNA degradation as described under “Experimental Procedures.” Similar results were obtained in three separate experiments.

The finding that mutation of all three pentamers in the Δ1 fragment caused little or no change in instability and did not impact stimulus-induced stabilization suggests that there are other regulatory sequence elements located within this fragment. As illustrated in Fig. 1, each of the pentamers in the Δ1 fragment is associated with an AU-rich region either upstream (P1 and P2) or downstream (P3). In addition, there are two separate AU-rich regions (one located between P2 and P3 and one located downstream of P3). We therefore surveyed the mutant form of the Δ1 fragment for functional regions by preparing a series of deletion constructs in which the mutated pentamers or AU-rich regions were sequentially removed. The results show an incremental reduction in instability as each segment is removed and complete loss of instability following the deletion of the last region of AU-rich character (excluding the polyadenylation signal) (Fig. 5). mRNA containing only the last 82 nucleotides (termed “Δ5”) was also shown in Fig. 1 to be stable. Interestingly, although each successive deletion results in an incrementally more stable message, all constructs that were unstable could be stabilized by IL-1α. These findings indicate that there are multiple instability determinants within the Δ1 fragment. Stimulus sensitivity, however, appears to reside in the last AU-rich segment.

To further evaluate the functional distinction between the two separate determinants in the KC 3′-UTR, we compared their sensitivity to either IL-1α or LPS, another inflammatory stimulus known to promote enhanced stability of short-lived ARE-containing mRNAs (38, 46). This was accomplished using a 293tet-off cell line constructed to express TLR4 and MD2, cell surface proteins that together confer sensitivity to LPS (39). Cells transfected with the CLU construct exhibited comparable sensitivity to either IL-1α or LPS in terms of KC protein secretion after Dox (Fig. 6A). In contrast, cells transfected with the Δ1 construct showed greater sensitivity to IL-1α than to LPS. This differential behavior did not reflect differential cell sensitivity to LPS, because treatment with LPS or IL-1α promoted comparable activation of a co-transfected β-dependent luciferase reporter (Fig. 6B). Similar differential sensitivity to the two stimuli was also observed when examined by Northern hybridization (Fig. 6C). Although the CLU-containing mRNA was more stable than that containing the Δ1 fragment, the CLU fragment exhibited comparable (though modest) stabilization in response to either IL-1α or LPS. The mRNA containing the Δ1 fragment was highly unstable, well stabilized by IL-1α but relatively insensitive to stimulation with LPS. The differences in half-life as compared with protein secretion confirm the possibility that the behavior of the CLU fragment involves translational control.

The differential stimulus sensitivity of the two sequence determinants was independently documented in cell-free mRNA degradation assays (41, 42). Using post-polysomal fractions of untreated, IL-1α-treated, or LPS-treated cells, we evaluated the decay of 5′-capped, polyadenylated in vitro transcripts from the CLU or Δ1 fragments. The CLU fragment was readily degraded using extracts obtained from untreated cells and showed comparably enhanced stability in extracts from cells treated with either IL-1α or LPS (Fig. 6D). In contrast, the Δ1 fragment was more unstable in extracts from untreated cells and showed significantly greater stability when assayed with extracts from IL-1α-treated cells as compared with extracts from LPS-treated cells.

The regulatory function of mRNA sequences controlling their rates of decay must operate through recognition by and interaction with factors that mediate this behavior. Indeed, a number of proteins that recognize ARE sequences have been identified, cloned, and evaluated (24–28). Because of the different structural and functional characteristics of the two fragments composing the KC mRNA 3′-UTR, we sought to examine the spectrum of proteins capable of interacting with each sequence. Radiolabeled in vitro transcripts corresponding to the CLU and Δ1 fragments were prepared, and their interaction with proteins present in post-polysomal S100 extracts from 293tet-off cells was assessed by UV radiation-induced cross-linking as described under “Experimental Procedures.” The RNA-tagged proteins were separated by SDS-PAGE and evaluated by autoradiography (Fig. 7). Although a number of cross-linked proteins interact comparably with both fragments, two show sig-
significant and reproducible differences in multiple experiments (see arrows in figure). A protein of ~90 kDa is the most abundant protein binding to both the CLU and Δ1 fragments but exhibits quantitative preference for the CLU fragment. The specificity of this interaction is indicated by a loss of binding interaction with mutant transcripts of the CLU fragment (Fig. 7B). Interestingly, the 90-kDa protein binds to both the wild type and the mutant version of Δ1 fragment. A second protein of ~60 kDa binds selectively to the Δ1 fragment but not the CLU fragment. This protein interaction was retained in the version of Δ1 containing mutated AUUUA pentamers. When the Δ4 fragment was used as a probe for protein binding, the 60-kDa protein interaction was lost (Fig. 7C). The behavior of these two proteins is consistent with their potential roles in mediating either stimulus sensitivity or instability. The binding of the 90-kDa protein is lost upon mutation of the CLU, and this mutation also destroys stimulus sensitivity for this fragment in intact cells and instability when assayed in the cell-free mRNA decay system. The 90-kDa protein binding is not lost in the mutant Δ1 fragment where both instability and stimulus sensitivity are retained. Likewise, the 60-kDa protein shows specificity for the Δ1 fragment that exhibits instability/stimulus sensitivity in both the wt and mutant versions. The loss of binding to the Δ4 fragment suggests that this interaction does not confer stimulus sensitivity. The pattern of protein binding to either fragment was not altered when cross-linking studies were performed using extracts from IL-1α- or LPS-stimulated 293TLR4/MD2tet-off cells (Fig. 7D).

DISCUSSION

The importance of ARE sequences in enhancing mRNA decay kinetics is well established, and such sequences are also known
to confer sensitivity for mRNA stabilization in response to extracellular stimuli (6, 7, 11, 12). AREs are, however, structurally diverse and are recognized with variable specificity by a relatively broad spectrum of RNA-binding proteins (24–28). Although it is likely that this structural diversity translates into substantial functional diversity, the relationship between

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specific sequences, sequence-specific-binding proteins, and the pattern of decay for individual mRNAs is still poorly understood. In the present study we began to explore these issues by examining the relative importance of the 7 AUUUA pentamers found in the mouse KC chemokine mRNA in governing the pattern of decay for individual mRNAs. We now report that KC mRNA contains at least two functionally independent determinants, each of which exhibits sensitivity to extracellular stimuli. These two determinants are functionally distinguished by several criteria indicating that they operate through separate mechanisms. These conclusions are supported by the following findings. 1) Deletion or mutation of the 4 clustered AUUUA pentamers does not affect the capacity of the remaining sequence to confer instability nor reduce its sensitivity to stimulation with IL-1α. The cluster alone, however, confers only modest instability but marked sensitivity to IL-1α or LPS that appears to involve regulation of translation. 2) Although the behavior of the CLU fragment depends upon the clustered AUUUA pentamer content, the function of the second element (Δ1) is only modestly altered by mutation of the AUUUA structures. 3) The two sites exhibit differential sensitivity to two stimuli; LPS and IL-1α comparably modulate function of the CLU fragment, whereas IL-1α is more effective than LPS in promoting enhanced stability of the Δ1 fragment. 4) The two sequence fragments exhibit RNA-binding protein specificities that correlate with their functional differences. Collectively these findings establish the functional heterogeneity of the ARE sequence motifs.

The demonstration of distinct and independent nucleotide sequence motifs regulating mRNA instability, translation, and stimulus-mediated stabilization underscores the diversity of mechanisms that are associated with ARE structure. Prior work has clearly demonstrated that specific mRNAs contain both ARE and non-ARE determinants of instability, and these may be differentially sensitive to stimuli such as IL-1α or LPS (21, 47–49). Furthermore, both the diversity of ARE structure and the number of ARE-containing mRNAs strongly suggest that ARE function and mechanism are likely to be diverse. For example, whereas many ARE-containing mRNAs are unstable, only a subset exhibit sensitivity for stabilization in response to stimulation (17, 19, 20). The present study extends the understanding and scope of mechanistic heterogeneity within ARE-dependent decay. The observation that the Δ1 fragment is more unstable than is the CLU fragment suggests significant functional differences between the two sequences. This is more clearly demonstrated, however, by the finding that both fragments exhibit nearly com-

**Fig. 6.** Δ1 and CLU determinants exhibit differential sensitivity to stabilization by IL-1α and LPS. A, 293TLR4/MD2 tet-off cells were transiently transfected with either the Δ1 or CLU constructs and used to determine the KC protein secretion before and after the addition of Dox with or without the addition of IL-1α (10 ng/ml) or LPS (1 μg/ml) as described in the legend to Fig. 2. Similar results were obtained in three separate experiments. B, the 293tet-off cell lines described in A were co-transfected with a 5×XκB luciferase reporter. After overnight incubation, the cells were stimulated with nothing (NT), IL-1α (10 ng/ml), or LPS (1 μg/ml) for 6 h prior to cell lysis and measurement of luciferase activity. Similar results were obtained in two separate experiments. C, 293tet-off cells stably expressing TLR4/MD2 were transiently transfected with the Δ1 or CLU constructs as described in A and used to determine the decay of mRNA following the addition of Dox with or without the addition of IL-1α or LPS. KC and GAPDH (not shown) mRNA levels were determined by Northern blot hybridization. Similar results were obtained in three separate experiments. D, 32P-radiolabeled substrate RNAs corresponding to either the CLU or Δ1 regions of the KC 3′UTR were prepared by in vitro transcription with a 5′-7-meG cap and a poly(A) tail as described under “Experimental Procedures.” S100 protein extracts were prepared from 293tet-off cells stably expressing both TLR4/MD2 and IL-1R1 receptors either without treatment or following treatment for 2 h with IL-1α (10 ng/ml) or LPS (1 μg/ml). Reactions were carried out in a total volume of 25 μl containing 10 μg of extract protein and 4 × 105 cpm of RNA substrate for the indicated times, and residual substrate RNA was determined as described under “Experimental Procedures.” Similar results were obtained in two separate experiments.
parable increases in protein production following stimulation despite the marked differences in both mRNA stability and stabilization in response to stimulation with IL-1α. This finding strongly suggests that stimulus sensitivity of the CLU fragment operates by modulation of translational efficiency. Stimulus-dependent translational control has been previously documented in the context of the tumor necrosis factor-α mRNA ARE motif (14, 16, 50). Interestingly, both the CLU and the tumor necrosis factor-α AREs possess two overlapping AUUUA pentamer sets (14, 28). These findings strongly suggest that the functions of the two regulatory elements are carried out through distinct molecular mechanisms.

This is further supported by the difference in AUUUA sequence requirements between the CLU and Δ1 portions of the KC 3′-UTR. Moreover, the differential sensitivity of each element to stimulation with either IL-1α or LPS suggests that signaling pathways coupling with these post-transcriptional control mechanisms are distinguishable. Although the CLU element is comparably stabilized by LPS and IL-1α, the Δ1 fragment is more sensitive to IL-1α than to LPS. Although LPS and IL-1 both signal through Toll-Interleukin 1 family receptors that share many features in signal transduction, these pathways appear to couple with at least partially independent downstream mechanisms. The finding that separate stimuli differentially control the function of a single mRNA represents an extension of our earlier studies illustrating that a single stimulus can promote enhanced stability of two different sequences through separate pathways (19).

Although ARE sequences have been known to confer mRNA instability for many years, the definition of specific sequences and their relationship to function remains poorly understood. There are a large number of mRNAs in the human genome that contain ARE sequences, and it is not surprising that the stability of such sequences will be highly diverse (17). Indeed, oligonucleotide and cDNA array analyses examining the behavior of these sequences demonstrate a broad spectrum of stability and sensitivity to extracellular stimulation (19, 20). ARE-containing mRNA sequences fall into one of three classes (I, II, or III) based upon the content and organization of pentameric AUUUA sequences (18). Class II mRNAs, containing multiple overlapping clusters of AUUUA, are found in many pro-inflammatory cytokine genes, and such sequences have been most commonly linked with instability and sensitivity to stabilization in response to agents, including IL-1 and LPS.

These structures have been characterized in detail in several mRNAs, including tumor necrosis factor-α, granulocyte macrophage-colony stimulating factor, Cox2, and IL-8 (13, 22, 23, 51, 52). Although non-class II ARE-containing mRNAs are also known to exhibit stimulus-mediated stabilization, the linkage between any given ARE structure and stimulus sensitivity remains largely undefined. One objective of our study was to determine the relative importance of the pentameric AUUUA structures found in the mouse KC mRNA for its decay and stabilization behavior. Although our findings confirm the importance of AUUUA structure in the context of the isolated CLU fragment, the Δ1 fragment confers both properties without requirement for any pentamer. Although this separate element contains 3 isolated pentamers, these can be disrupted by mutagenesis without significant effect on either instability or sensitivity to stabilization by IL-1. This demonstrates that

**Fig. 7.** Analysis of protein binding to CLU, Δ1, and Δ4 fragments. A. 5′-P-radiolabeled RNA fragments corresponding to wild-type versions of the CLU and Δ1 fragments of the KC 3′-UTR were prepared by *in vitro* transcription, and each fragment was incubated in a total volume of 25 μl containing 20 μg of S100 extract from 293tet-off cells and analyzed by UV-cross-linking and SDS-PAGE as described under "Experimental Procedures." B. 32P-radiolabeled RNA fragments corresponding to wild-type or AUUUA mutant versions of the CLU and Δ1 fragments of the KC 3′-UTR were prepared and used for UV cross-linking analysis as described in A. C. 32P-radiolabeled 5′-capped RNA fragments corresponding to the Δ1 and Δ4 fragments of the KC 3′-UTR were incubated with S100 extracts from 293TLR4/MD2tet-off cells and analyzed for RNA-binding proteins as in A. D. 32P-radiolabeled 5′-capped RNA fragments corresponding to wild-type versions of the CLU and Δ1 fragments of the KC 3′-UTR were incubated with S100 protein extracts prepared from 293TLR4/MD2tet-off cells either untreated or treated with IL-1α (10 ng/ml) or LPS (1 μg/ml) for 2 h. Reactions were subjected to UV-cross-linking and analysis as described in A. Positions of molecular weight markers are indicated on the left of each figure. Arrows indicate the positions of bands that exhibit selective binding with CLU or Δ1 fragments. Similar results were obtained in three separate experiments.
stimulus-specific responses can involve a broad range of ARE structures and do not depend specifically on the AUUUA motif.

It is fully expected that the sequence specific decay behavior of mRNAs will result from their recognition by and interaction with specific protein factors. Indeed, multiple ARE-binding proteins have been identified, and a subset has been studied in detail (16, 24, 25, 27, 28). In some experimental models, ARE-binding proteins have been causally linked with specific message instability and/or stimulus-sensitive behavior (10, 30, 53). Indeed, a recent report suggests that the zinc finger protein Tristetraprolin may be a mediator of LPS- or IL-1-induced stabilization of ARE containing cytokine mRNAs (54). Because Tristetraprolin (and several related isoforms) are not present in the HEK293 cell line employed in the present study (45), there are likely to be additional factors mediating the regulation of KC mRNA decay. By comparing protein binding interactions using these two distinct sequences, we have identified two proteins whose binding specificity exhibit some correlation with function. A major protein with a molecular size of ~90 kDa binds to both CLU and Δ1 sequences, and binding to the CLU is lost when the pentameric structure is mutated. This behavior correlates well with the loss of function. Furthermore, the 90-kDa protein binding interaction with the Δ1 fragment is not lost when the three pentamers in this sequence are mutated, and this is consistent with the retention of both instability and stimulus sensitivity in the mutant fragment. The second protein exhibits a molecular size of ~60 kDa. This binding interaction appears to exhibit specificity for the Δ1 fragment and could contribute to the functional differences in decay behavior seen between the Δ1 and CLU fragments. Because the binding of the 60-kDa protein is lost in the Δ4 fragment, which exhibits much of the stimulus sensitivity of the full Δ1 fragment, it is unlikely that this protein participates in stimulus sensitivity. Rather, because some level of instability is lost by deletion of the region to which the 60-kDa protein binds, this factor may contribute to instability though the magnitude of the effect is modest. Further studies are underway to determine the identity and functional roles for these factors.

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