Increased Granulocyte-Macrophage Colony-Stimulating Factor mRNA Instability in Cord Versus Adult Mononuclear Cells Is Translation-Dependent and Associated With Increased Levels of A+U-Rich Element Binding Factor

By Jeffrey S. Buzby, Sun min Lee, Patrick Van Winkle, Christine T. DeMaria, Gary Brewer, and Mitchell S. Cairo

The level of granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA is fourfold lower in phorbol myristate acetate (PMA) + phytohemagglutinin (PHA)-activated mononuclear cells (MNC) from newborns compared with adults. The GM-CSF transcription rate is similar in umbilical cord and adult MNC, but transcript half-life is threefold lower in cord activated MNC. Interaction of RNA binding proteins, such as the cloned adenosine + uridine-rich element, binding factor, AUFI, with eight AUUUA motifs in the human GM-CSF mRNA 3'-untranslated region (GM-3'-UTR) has been implicated in regulating transcript stability. Translational inhibition by cycloheximide (CHX) significantly increased GM-CSF mRNA accumulation and half-life by threefold in activated cord MNC, but had a minimal effect in activated adult MNC as compared with PMA + PHA alone. Electrophoretic mobility-shift assays with a 32P-labeled, 305-nucleotide RNA comprising the GM-3'-UTR revealed two RNaseT1-resistant, bound complexes that were almost twice as abundant in cord than in adult MNC extracts. Mobility-shift competition assays and RNaseT1 mapping localized the binding site of both complexes to a 52-nucleotide region containing seven of eight AUUUA motifs. Inclusion of AUFI antisera produced a supershifted complex at 35-fold higher levels in cord than in adult MNC extracts. Extracts from the carcinoma cell line 5637, with extended GM-CSF mRNA half-life, also had very low levels of anti-AUFI supershifted complex. Anti-AUFI immunoblotting showed significantly higher levels of two AUFI protein isoforms and lower levels of one in cord than in adult MNC or 5637 extracts. These results suggest that destabilization of GM-CSF mRNA in cord MNC is translation-dependent and that increased levels of specific AUFI isoforms in cord MNC may target transcripts for increased degradation, which could account in part for dysregulation of neonatal phagocytic immunity.

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may also be less stable in cord MNC. Furthermore, stabilization of M-CSF mRNA by cycloheximide (CHX), a protein synthesis inhibitor, is greater in cord than in adult MNC, suggesting that M-CSF mRNA accumulation in cord MNC may be translation-dependent.  

Although there is evidence that ARE-directed degradation of GM-CSF mRNA is cotranslational,24,25 the molecular components of the degradation process have yet to be fully described. Arguments supporting mechanisms for both cotranslational26 and labile trans-acting, factor-mediated27 degradation of c-fos mRNA have been reported. At least three ARE-directed endoribonuclease activities28-30 and several ARE binding factors31-37 have been reported. One group of these is a family of RNA binding proteins designated AUFl, with 37-, 40-, 42- and 45-kD isoforms, and cloned from cDNA and genomic libraries.38-40 AUFl binds to both the GM-CSF and c-myc AREs and copurifies with a mRNA degradation activity.32 Moreover, the ARE binding affinity of AUFl parallels the transcript destabilizing potency of specific AREs.41 Finally, increased levels of AUFl correlate with the downregulation of a2-adrenergic receptor mRNA,42 suggesting a destabilizing role for AUFl in regulating the decay rates of ARE-containing transcripts.

The present study was designed to investigate the molecular basis for the differential stability of GM-CSF mRNA in neonatal (cord) and adult MNC to further our understanding of a possible mechanism behind the predisposition for neuronal injury in newborns during overwhelming bacterial sepsis. We examine the effects of translation inhibition on intact MNC, compare and characterize GM-3'-UTR binding activities in MNC extracts, and ascertain the involvement of AREs and AUFl in 3'-UTR binding in vitro. We find that there are some factors that interact with the ARE at slightly higher levels in cord versus adult MNC extracts. Moreover, a binding activity revealed by AUFl immune serum is present at much higher levels and specific AUFl protein isoforms are differentially expressed in cord versus adult MNC. The activity of these different AUFl isoforms may account in part for the shorter GM-CSF mRNA half-life in cord MNC.

**MATERIALS AND METHODS**

**Human MNC isolation and cell culture.** Human MNC were isolated from normal donor adult and normal term umbilical cord peripheral blood and cultured as previously described.43 After incubation overnight at 37°C in 5% CO2, cultures were stimulated for 6 hours with 20 ng/mL phorbol myristate acetate (PMA; Sigma, St Louis, MO) plus 2 µg/mL phytohemagglutinin (PHA; Gibco-BRL, Gaithersburg, MD). Inhibition of protein synthesis with 10 µg/mL CHX (Sigma) and transcription with 10 µg/mL actinomycin D (Sigma) were performed as previously described.41 The adherent human bladder carcinoma cell line, 5637 (American Type Culture Collection, Rockville, MD), was cultured in RPMI 1640 medium (Sigma) with 10% fetal bovine serum (Gemini, Calabasas, CA).

**RNA isolation and hybridization.** Total RNA was isolated, electrophoresed, transferred to nitrocellulose membranes, hybridized with 32P-labeled DNA probes, and densitometrically quantified as previously described.44 Templates for 32P-labeled probes were an 800-bp XhoI fragment derived from the human cDNA clone, pXM:GM-CSF (a gift from G.G. Wong, Genetics Institute, Cambridge, MA) for GM-CSF mRNA and a 775-bp PstI-XhoI fragment from pHGAP (American Type Culture Collection) for glyceroldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

**Cytoplasmic protein extraction.** Cytoplasmic extracts were prepared by modification of previously published methods.46 Cultured MNC and 5637 cells were washed with phosphate-buffered saline (PBS) and lysed in cytoplasmic extraction buffer (10 mmol/L Tris hydrochloride, pH 7.4, 1.5 mmol/L MgCl2, 50 mmol/L KCl, 0.2 mmol/L EDTA, 0.1% Nonidet P-40, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, 5 µg/mL Pepstatin A and 40 U/mL RNasin [Promega, Madison, WI], 35 µL per 106 cells) for 60 minutes on ice. Supernatant aliquots (75 to 200 µL) were stored in liquid nitrogen after centrifugation at 3500 x g for 5 minutes. Total protein yield was determined using bicinchoninic acid (Pierce, Rockford, IL). Due to increased amounts of hemoglobin from residual red blood cells in neonatal MNC extracts, protein contents were normalized to β-actin levels. Four to 10 µg protein from each cytoplasmic extract was analyzed by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) 12%, 1-20% acrylamide).45 After staining with Coomassie brilliant blue, gels were dried onto cellophane sheets (Bio-Rad, Hercules, CA) and the amount of β-actin (42-kD) in each extract was determined by two-dimensional densitometry with a BioImage model 505 automated scanning system (Millipore, Bedford, MA).

**Electrophoretic mobility-shift assay.** GM-3'-UTR binding proteins were assayed by modification of the RNaseT1-protection electrophoretic mobility-shift procedure.47 Binding reactions containing 2 to 16 µg cytoplasmic protein and 0.1 to 10 ng 32P-labeled 8AU or 7AU (200 to 1,000 kcpm/ng) RNA in 10 to 20 µL binding buffer (20 mmol/L HEPES, pH 7.8, 100 mmol/L KCl, 2 mmol/L MgCl2, 0.25 mmol/L EDTA, 10 mmol/L β-mercaptoethanol, 0.5 mmol/L PMSF, 5 µg/mL Pepstatin A, 2 U/L RNasin, and 15% glycerol) were incubated on ice for 15 minutes, digested with 200 U RNaseT1 on ice for 10 minutes, and incubated with 5 µg/mL heparin (10,000 U/mL injectable; Elkins-Sinn, Cherry Hill, NJ) on ice for 10 minutes. AUFl-immune and preimmune rabbit sera were prepared as previously described,48 and 1 µL was added after RNaseT1 in supershift assays. Reactions were electrophoresed in Tris-acetate buffer49 at 10 V/cm for 4.5 hours on nondenaturing 4% (1:29 bisacrylamide) polyacrylamide gels, which were then dried and autoradiographed. Relative signal strength of bound complexes was quantified by radiographic scanning of the autoradiograms.

**RNaseT1 mapping of protein complex binding.** The protein complex binding site was mapped using RNaseT1 protection by modification of previously published methods.47 Preparative mobility-shift
electrophoresis of RNaseT1-digested RNA-protein complexes was performed as previously described.48 RNA purified from RNaseT1-digested free and protein-bound mobility-shifted 32P-labeled RNA, along with 32P-labeled RNA size standards, were electrophoresed on denaturing 7-mol/L urea/10% (1:29 bis:acrylamide) polyacrylamide gels, which were then dried and autoradiographed.

Anti-AUFl immunoblotting and immunodepletion. For immunoblots, cytoplasmic extract proteins were electrophoresed on denaturing SDS-polyacrylamide gels (12%, 1.29 bis:acrylamide) along with low-range prestained SDS-PAGE standards (Bio-Rad). Proteins were electroblotted onto nitrocellulose membranes using a Mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer’s recommendations. Electroblot membranes were incubated with 5% nonfat milk in PBS for 60 minutes at room temperature, followed by 1:3,000 AUFl-immune serum overnight at 4°C and 1:3,000 horseradish peroxidase–conjugated goat anti–rabbit IgG (Bio-Rad) for 2 hours at room temperature. Membranes were washed with antibody binding buffer (2% nonfat milk in PBS) for 15 minutes at room temperature between incubations and with 0.1% Triton X-100 in PBS for 10 minutes three times before developing with luminol/peroxide chemiluminescent substrate (Kirkegaard and Perry, Gaithersburg, MD) and exposing to x-ray film. Chemiluminescence was quantified by two-dimensional densitometric scanning of films. AUFl immunodepletion was accomplished using Protein A–Sepharose CL-4B (Sigma) incubated with a 50-fold excess of antibody to AUFl protein in protein to better control for the inherent variability between protein preparations. Cytoplasmic extract proteins were electrophoresed on denaturing SDS-polyacrylamide gels (20% (1:29 bis:acrylamide) polyacrylamide gels, which were then dried and autoradiographed.

**Statistical analysis.** Results are expressed as the mean ± SEM ratio for four sets of adult/cord or stimulated/unstimulated MNC (100 minutes) extracts in triplicate with mobility-shift assays and three sets in duplicate with supershift and immunoblot assays. The probability of significant differences from a theoretical mean ratio of 1.0 was determined using the unpaired Student’s t-test on InStat for Macintosh (GraphPad Software, San Diego, CA). P values less than .05 were considered significant.

**RESULTS**

Translational inhibition increases GM-CSF mRNA accumulation and stability in cord more than in adult MNC.
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(B) Neonatal | Adult

Fig 2. Comparison of CHX effects on GM-CSF mRNA accumulation and stability in cord (neonatal) versus adult MNC. (A) Isolated MNC were cultured in the presence of CHX with or without PMA + PHA for 6 hours (+) or during the final 3 hours (3). (B) Isolated MNC were cultured in the presence of PMA + PHA for 6 hours with (○) or without (■) CHX during the final 3 hours. RNA was extracted at indicated time points after addition of actinomycin D. Transcript levels are relative to the 0-minute time point of each series. (---) Time points where 50% of initial mRNA level is remaining (t1/2). Results are representative of 3 different neonatal versus adult RNA blot hybridizations normalized to the expression of GAPDH mRNA.

lane 1) was digested into fragments of no greater than 50 bases by RNaseT1, which were too small to be seen under these assay conditions. Both complexes were sensitive to 1 mg/mL proteinase K (30 minutes on ice; data not shown). Binding activity was almost twofold higher in newborn than in adult MNC extracts (lanes 2 and 3) for the upper and lower complexes (1.7 ± 0.2 and 1.8 ± 0.1 times greater, P = .0002 and P < .0001, respectively). PMA + PHA stimulation had no significant effect (P > .2) on either binding complex in newborn (lane 2 vs 4) or adult (lane 3 vs 5) MNC extracts. Inhibiting protein synthesis with CHX after stimulating with PMA + PHA reduced levels of the upper and lower complexes by 39% ± 12% (P = .0271) and 33% ± 9% (P = .013), respectively, in the newborn MNC extracts (lane 4 vs 6), but did not significantly affect their levels in the adult MNC extracts (lane 5 vs 7, P > .1). These complexes were further characterized by locating their GM-3'-UTR binding sites and assaying for one potential component, the GM-3'-UTR binding protein, AUF1.

Protein complexes interact with the GM-3'-UTR ARE. Unlabeled synthetic RNA fragments of 8AU (Fig 1) were used as competitors to confirm MNC protein binding specificity and localize the 32P-8AU binding site. At 200:1 molar ratios, the GM-3'-UTR fragments containing two to eight AUUUA motifs (Fig 4A, lanes 2, 3, and 5) competed for the binding of 32P-8AU, whereas binding was not appreciably reduced by OAU (lane 4) or control (lane 6), which lacked AUUUA motifs (Fig 1). Competition may have been dependent on the number of AUUUA motifs present, since approximately 85% of the total binding activity was competed for by unlabeled 8AU at a 20:1 molar ratio, 7AU at a 200:1 molar ratio, and 2AU at a 500:1 molar ratio (Figs 1 and 4B). 32P-7AU also showed a pattern of binding identical to that of 32P-8AU, whereas 32P-control did not bind with MNC protein (data not shown). RNaseT1 mapping was used to confirm binding to the 7AU region. Using 32P-8AU as the binding substrate, 7AU was the primary fragment recovered from both the upper (Fig 5, lane 1) and lower (lane 3) bound complexes. Fragments of less than 51 nt cannot be eliminated by redigestion with RNaseT1 after removal of proteins (lane 4) and are most likely RNaseT1-independent degradation products, since no such fragments are evident after RNaseT1 digestion of unbound 32P-8AU (lane 5) and the next largest

Fig 3. Comparison of GM-3'-UTR binding activity from cord (neonatal) versus adult MNC. Binding activity in equal amounts of cytoplasmic protein, normalized to β-actin, from unstimulated (control), PMA + PHA-stimulated (6-hour), and PMA + PHA (6-hour)/CHX (3-hour)-stimulated newborn (Nb) and adult (Ad) MNC reacted with 32P-8AU and analyzed in electrophoretic mobility-shift assays. Results are representative of 3 sets of Nb versus Ad MNC extracts.
RNaseT1 digestion products of 8AU are only 23 nt long. These results indicated that the GM-3'-UTR ARE, specifically the seven tandemly repeated AUUUA motifs in 7AU, was the primary binding site for proteins in both the upper and lower complexes.

**Fig 4.** Competition for MNC protein extract binding to 32P-8AU. RNaseT1-protected RNA was isolated from the upper (U), lower (L), and supershifted (S) complexes of PMA + PHA-stimulated adult MNC protein extract with 32P-8AU and sized by denaturing PAGE. Supershifted complexes were produced by inclusion of AUFI antisera in binding reactions. RNA isolated from the lower MNC extract complex was also reincubated with RNaseT1 (T1) before denaturing PAGE to confirm the identity of the protected RNA. Free 32P-8AU was digested with RNaseT1 in the absence of protein (lane 5). Exact size standards (nt, nucleotides) were transcribed in vitro from pBluescriptII. Lane 1 was exposed twice as long as the others for signal detection. Results are representative of 2 separate experiments.

**AUFI activity is decreased in adult versus cord MNC extracts.** The presence of AUFI, which has been implicated as an ARE-directed destabilizer, was assayed using supershift and immunoblot assays. Inclusion of AUFI antisera in electrophoretic mobility-shift assays produced a super-shifted complex, present at approximately 35-fold higher levels (36 ± 8, \( P = .0003 \)) in neonatal versus adult MNC extracts (Fig 6, lane 2 v 4), that was not significantly affected by PMA + PHA (\( P > .5 \), lane 2 v 6 and 4 v 8). Inhibiting protein synthesis with CHX after stimulating with PMA + PHA slightly reduced the amount of supershifted complex by 30% ± 20% in neonatal MNC extracts, but did not alter the level in adult MNC extracts, as was also the case for the upper and lower binding complexes (Fig 3). RNaseT1 mapping also showed the 7AU region to be the primary product recovered from the supershifted MNC extract complexes (Fig 5, lane 2). Preimmune rabbit serum had no effect on mobility shifts (Fig 5, lane 2). Preimmune rabbit serum had no effect on mobility shifts (Fig 6, lanes 1, 3, 5, 7, 9, and 11), and AUFI antisera alone did not bind with 32P-8AU (data not shown). Immunodepletion of the MNC extracts eliminated the anti-AUFI supershift, but did not alter the upper or lower complex in subsequent mobility-shift assays (data not shown). Anti-AUFI immunoblotting showed twofold higher levels (2.0 ± 0.3, \( P = .0016 \)) of total anti-AUFI immunoreactive isoforms in newborn than in adult MNC extracts (Fig 7, lane 1 v 2). However, more striking differences were evident when relative levels of each of four anti-AUFI immunoreactive isoforms, three of which had been previously observed in K562 cells, were com-
**AUFI activity is decreased in 5637 cells.** The constitutive half-life of GM-CSF mRNA in the bladder carcinoma cell line 5637 is greatly extended (4 hours) compared with immortalized T-lymphocyte and TNF-α–stimulated fibroblast controls (~30 minutes, comparable to activated cord MNC). 2) Cytoplasmic extracts from 5637 showed the upper and lower complexes with mobility shifts matching those from MNC extracts (Fig 8A). Although the activity in these complexes was similar to the higher levels from neonatal MNC extracts (lane 1 vs 5), the low amount of binding activity supershifted by anti-AUFI paralleled the low levels observed with adult MNC extracts (lane 4 vs 6). AUFI immunoblot comparison of MNC with 5637 cytoplasmic proteins showed that the 5637 extracts contained lower levels of p37 and p40 and elevated levels of p45, also comparable to the adult MNC extracts (Fig 8B). These results suggested a greater similarity in RNA binding activities and levels of specific AUFI isoforms between 5637 and adult MNC, which have a longer GM-CSF mRNA half-life than neonatal MNC.

**DISCUSSION**

Decreased accumulation of GM-CSF mRNA in activated cord MNC is associated with a shorter half-life (30 minutes) compared with adult MNC (100 minutes). 3) Translational inhibition by CHX after 3 hours PMA + PHA stimulation caused a superinduction of GM-CSF mRNA, which was approximately 2.5-fold greater in cord than in adult activated MNC (Fig 2A) and was found to result from increased transcript stabilization by CHX in activated cord MNC (Fig 2B). CHX alone did not induce either GM-CSF (Fig 2A) or M-CSF mRNA expression, and in fact prevented their induction when added simultaneously with activators. This translation-dependent repression presumably acts through a mechanism dissimilar to the stabilization of preinduced mRNA. Increased transcript stabilization in activated cord MNC after CHX treatment suggested that a destabilizer, whose activity depends on continuous protein synthesis, may have been present in the cord MNC. The central role of the 3'-UTR in regulating mRNA stability 11 suggested that trans-acting 3'-UTR binding factors may be targeting the transcripts for degradation in MNC.

Electrophoretic mobility-shift assays showed the presence of two RNA-protein complexes with the GM-3'-UTR substrate (Fig 3), with slightly higher levels in cord than in adult...
mRNA instability and AUFl motifs, and showed no response to PMA stimulation of EL-4 cells. The twofold-increased level of upper and lower complexes in cord versus adult MNC and the 35% decrease following CHX treatment of cord MNC (Fig 3) were inversely related to but not as large as the threefold-decreased half-life and 300% CHX superinduction (Fig 2A) of GM-CSF mRNA in cord MNC. However, elevated levels of the upper and lower complexes in 5637 cells (Fig 8), where GM-CSF mRNA is stabilized, and in cord MNC extracts would appear to argue against destabilization of the mRNA by these complexes. It is possible that in the absence of a competing destabilizer such as AUFl, the components of the upper and lower complexes function as ARE-dependent stabilizers, leading to the greatly extended GM-CSF mRNA half-life in 5637 cells. A 32-kD ARE binding protein, designated AUFB, has been found to reduce the GM-CSF mRNA half-life from 90 minutes to 20 minutes in an in vitro decay assay upon depletion from human PMA + PHA-stimulated MNC polysomes, suggesting an ARE binding stabilizer activity. However, the method of AUFB depletion may have also removed other ARE binding proteins. Therefore, the components and function of the ARE binding upper and lower complexes remain to be defined.

Supershifted complexes formed by the addition of AUFl antisera were observed at 35-fold higher levels in cord than in adult MNC extracts (Fig 6). The GM-3'- UTR binding site of the supershifted complex (Fig 5) was the same as that of the upper and lower bound complexes, but the inability of AUFl antisera to electrophoretically supershift or immunodeplete either of these complexes (Fig 6) precludes identifying the complex of AUFl origin. A similar effect has been reported previously, wherein Fos antiserum formed a supershifted complex using nuclear extracts but failed to decrease the amount of the original bound complex. However, when pure Fos/JunD heterodimers were used, a clear decrease in the original complex was observed. It is possible that other ARE binding factors in the MNC extracts may act to modulate the destabilizing activity. This possibility is supported by the low anti-AUFl supershift activity, high p45 level, and low p40 and p37 levels observed in cord versus adult MNC extracts. It is also possible that the p42 isoform, the levels of which were not appreciably different in cord versus adult MNC extracts, represents an immunologically cross-reactive protein not detected in previous assays. However, the precise function of each AUFl isoform remains to be determined.

**Fig 8. Anti-AUFl mobility supershift and immunoblot assays of 5637 and MNC extract protein. Comparisons using equal amounts of cytoplasmic protein, normalized to β-actin, from 5637 and PHA-stimulated cord (neonatal) and adult MNC are shown. (A) After incubating with [32P]-8AU, the presence of bound AUFl was detected as electrophoretically supershifted complexes with AUFl immune serum (I) (1 μL per reaction). Reactions containing equal volumes of preimmune serum (PI) are also shown. (B) Distribution of AUFl species in protein extracts was determined by anti-AUFl immunoblotting, as in Fig 7. Results are representative of 3 sets of 5637 v cord v adult MNC extracts.
The slightly reduced levels of bound and supershifted complexes and p37 in cord MNC extracts after inhibiting translation with CHX are consistent with a mechanism that requires ongoing protein synthesis to maintain active ARE-directed destabilization involving bound AUFl. However, since CHX does not reduce any of these levels to those of adult MNC extracts but does superinduce GM-CSF mRNA to adult MNC levels, translational inhibition itself may be sufficient to prevent AUFl-mediated destabilization in cord MNC, or there may be other components involved in the CHX superinduction process, such as increased transcription. Similarly, the lack of a significant effect of PMA + PHA on AUFl binding activity or level suggests that GM-CSF mRNA induction by these agonists involves other processes, such as increased transcription.

In summary, significantly higher levels of the p37 and p40 isoforms and ARE binding activity of AUFl were observed in cord MNC versus 5637 and adult MNC extracts. These increases are associated with a significantly shorter GM-CSF mRNA half-life in cord versus adult MNC or 5637 cells (0.5 ± 1.7 hours vs 4 hours), respectively. Furthermore, the p37 and p40 AUFl isoforms are the most abundant polypeptides copurifying with an ARE-dependent in vitro mRNA destabilizing activity. Taken together, these data strongly suggest a role for the increased AUFl p37 and p40 isoform levels in ARE-dependent destabilization of GM-CSF mRNA in cord MNC. Finally, increased levels or activity of destabilizing factors such as AUFl could pleiotropically reduce the stability of other myelopoietic cytokine transcripts with 3′-UTR ARE, such as M-CSF mRNA, in cord MNC. Elucidation of the activities of the different AUFl isoforms should contribute to determining the functional role of AUFl and ARE-directed destabilization in the dysregulation of neonatal phagocytic immunity.

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JS Buzby, SM Lee, P Van Winkle, CT DeMaria, G Brewer and MS Cairo