Developmental Regulation of RNA Transcript Destabilization by A + U-rich Elements is AUF1-dependent*

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The developmental immaturity of neonatal phagocytic function is associated with decreased accumulation and half-life ($t_{1/2}$) of granulocyte/macrophage colony-stimulating factor (GM-CSF) mRNA in mononuclear cells (MNC) from the neonatal umbilical cord compared with adult peripheral blood. The in vivo $t_{1/2}$ of GM-CSF mRNA is 3-fold shorter in neonatal (30 min) than in adult (100 min) MNC. Turnover of mRNA containing a 3'- untranslated region (3'-UTR) A + U-rich element (ARE), which regulates GM-CSF mRNA stability, is accelerated in vitro by protein fractions enriched for AUF1, an ARE-specific binding factor. The data reported here demonstrate that the ARE significantly accelerates in vitro decay of the GM-CSF 3'-UTR in the presence of either neonatal or adult MNC protein. Decay intermediates of the GM-CSF 3'-UTR are generated that are truncated at either end of the ARE. Furthermore, the $t_{1/2}$ of the ARE-containing 3'-UTR is 4-fold shorter in the presence of neonatal (19 min) than adult (79 min) MNC protein, reconstituting developmental regulation in a cell-free system. Finally, accelerated ARE-dependent decay of the GM-CSF 3'-UTR in vitro by neonatal MNC protein is significantly attenuated by immunodepletion of AUF1, providing new evidence that this accelerated turnover is ARE- and AUF1-dependent.

Neonatal myelopoiesis is developmentally immature compared with that of adults (reviewed in Ref. 1). Although both the circulating levels and proliferative capacity of myeloid progenitor cells are elevated in neonatal humans and rats, total body neutrophil storage pools are significantly decreased in neonatal compared with adult rats (reviewed in Ref. 2). Neonatal rats cannot further increase their myeloid progenitor pool size or proliferative rate in response to experimental sepsis, whereas adult animals can. This impaired response leads to a further reduction in their already depleted neutrophil storage pools, a condition also associated with a fatal outcome in septic human newborns. These immaturities, coupled with diminished neonatal phagocytic function (3, 4), appear to predispose newborns to depletion of mature effector phagocytes during overwhelming bacterial sepsis. Prophylactic (5) and therapeutic (6) administration of recombinant granulocyte/macrophage colony-stimulating factor (GM-CSF)$^1$ enhances the survival of neonatal rats during experimental sepsis. Recombinant human GM-CSF has also been shown to increase neutrophil, eosinophil, monocyte, and platelet production in very low-birthweight human newborns (7, 8).

GM-CSF modulates myeloid proliferation, differentiation, and activation (reviewed in Ref. 9). The expression of GM-CSF mRNA is regulated post-transcriptionally by a destabilizing, adenylate + uridylate (A + U)-rich element (ARE) comprising AUUUA motifs (10–12) within the 3'-translated region (3'-UTR). These elements are present in the 3'-UTR of a number of proto-oncogene and cytokine transcripts, including nearly all of the colony-stimulating factors, interleukins, and interferons (reviewed in Ref. 13). AREs are also evolutionarily conserved in the 3'-UTRs of diverse invertebrate and vertebrate transcripts (14). The degradation kinetics of these transcripts is dependent upon the presence of multiple AUUUUA motifs (reviewed in Ref. 15) and, in particular, the nonamer AUUUA (16–18). The human GM-CSF ARE consists of a cluster of eight AUUUUA motifs including three overlapping nonamer sequences (Fig. 1 and Ref. 19), characteristic of class II AREs, which initiate mRNA decay through processive deadenylation (15). ARE-targeted ribonuclease activities (20–24) and many ARE binding factors (24–33) have now been identified in mammalian cells, but the function of most is not yet clear. Two of these factors, AUF1 (34) and HuR (35), have been cloned and found to exhibit apparently opposing functional activities. HuR stabilizes ARE-containing mRNA when overexpressed in transfected fibroblasts (36, 37), whereas AUF1-enriched protein fractions accelerate ARE-dependent in vitro mRNA decay (26). However, the exact mechanism by which transcript turnover occurs, including the explicit function(s) of AUF1, remains to be determined.

GM-CSF is produced by human peripheral blood mononuclear cells (MNC) activated by phorbol myristate acetate (PMA) + phytomagglutinin (PHA) (38). Expression of GM-CSF is differentially regulated depending upon whether its cellular source is neonatal or adult. For example, GM-CSF protein and mRNA are expressed at 7- and 4-fold lower levels in activated neonatal umbilical cord blood-derived MNC than in adult pe-

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** The abbreviations used are: GM-CSF, granulocyte/macrophage colony-stimulating factor; ARE, A + U-rich element; 3'-UTR, 3'-translated region; MNC, mononuclear cell(s); PMA, phorbol myristate acetate; PHA, phytomagglutinin; nt, nucleotide(s); PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
rheological blood-derived MNC (39, 40). Because the rate of gene transcription is comparable in neonatal and adult MNC (41), the 4-fold lower level of GM-CSF mRNA in neonatal MNC is most likely because of its 3-fold shorter half-life ($t_{1/2}$) in neonatal MNC (39). Several other ARE-containing cytokine transcripts, including macrophage colony-stimulating factor (42), granulo-... 

Taken together, these data suggest that increased turnover of cytokine transcripts in neonatal MNC could account for their reduced production and, in part, for the dysregulation of neonatal phagocytic immunity.

To explore the molecular basis for this increased turnover, we previously compared interaction of the GM-CSF 3′-UTR with neonatal versus adult MNC proteins, including AUF1 (46). The results of these studies suggest that increased ARE binding by specific AUF1 isoforms may contribute to the developmentally increased rate of GM-CSF mRNA turnover in neonatal MNC. The present study was designed to determine the functional roles of both the ARE and AUF1 in regulating the turnover of GM-CSF mRNA by neonatal versus adult MNC. A cell-free system (47) was employed to assay mRNA degradation activity in MNC protein extracts. Our data indicate that the GM-CSF ARE directs rapid in vitro RNA decay, characterized by the generation of discrete RNA decay intermediates. Moreover, decay is significantly more rapid in the presence of neonatal compared with adult MNC protein. Finally, immunodepletion of AUF1 from neonatal MNC protein extracts significantly stabilizes RNA containing the GM-CSF ARE. These results indicate that accelerated in vitro RNA decay in the presence of neonatal MNC protein is ARE-directed and AUF1-dependent.

EXPERIMENTAL PROCEDURES

Human MNC Isolation and Cell Culture—Human MNC were isolated from normal buffy-coated adult peripheral blood and cultured as described previously (42). After incubation overnight at 37°C in 5% CO₂, cultures were stimulated for 6 h with 20 ng/ml PMA (Sigma) + 2 µg/ml PHA (Life Technologies, Inc.).

In Vitro RNA Synthesis and 32P Labeling—The human GM-CSF 3′-UTR, a 305-base pair MstI-XhoI fragment derived from pXM:GM-CSF (courtesy of Dr. G. G. Wong, Genetics Institute), was cloned into the pBluescriptII (+) transcription vector (Stratagene) digested with Smal and XhoI (Fig. 1) to create pBSGM305. Internally, 32P-labeled RNA transcripts were synthesized from pBSGM305 by linearization at the restriction sites shown (41). Cultured MNC were obtained from Life Technologies, Inc. and used according to their specifications.

Cytoplasmic extracts were pre treated with 5′-phosphate-buffered saline (PBS; Life Technologies, Inc.) and lysed in 35 µl of cell extraction buffer (10 mM Tris-HCl, pH 7.4 (Sigma), 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM EDTA, 0.1% Nonidet P-40 (Sigma), 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride (Sigma), 0.5 µg/ml pepsin (Sigma), 40 units/ml RNasin (Promega)/10⁶ cells for 60 min on ice. Supernatant aliquots (50–200 µl) were stored in liquid nitrogen after microcentrifugation (12,000 × g for 10 min at 4°C) to remove insoluble cell debris. Total protein yield was determined using the BCA protein assay (Pierce). Protein contents were then normalized to β-actin levels by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to account for increased amounts of hemoglobin from residual red blood cells in neonatal MNC extracts, as described previously (46).

Cell-free RNA Decay Assay—RNA degradation activity in the cytoplasmic extracts was assayed by modification of methods previously established for polysomal extracts, using radioactive substrates (47). Decay reactions containing 2 µg of cytoplasmic protein and 0.5 ng of 32P-labeled 8AU or 6AU RNA (200–1000 kcpm) in 25 µl of decay buffer (50 mM Tris-HCl, pH 7.6, 100 mM KCl, 2 mM Mg(ÅAc), 2 mM dithiothreitol, 0.1 mM spermine, 1 mM ATP, 0.4 mM GTP, 10 mM phosphocreatine (Sigma), 1 µg creatine phosphokinase (Sigma), 1 unit/µl RNasin) were incubated at 37°C for 30 to 120 min, after which reactions were terminated with 400 µl of urea lysis buffer (10 mM Tris-HCl, pH 7.5, 350 mM NaCl, 10 mM EDTA, 2% SDS, 7 M urea). The addition of 20 µg of Escherichia coli transfer RNA (Sigma) as a carrier, penicillin-dependent, and ethanol precipitation, radioactive pellets were resuspended in 10 µl of distilled H₂O + 10 µl of denaturing RNA gel loading buffer (80% formamide, 10 mM EDTA, 1 mg/ml bromophenol blue (Sigma), 1 mg/ml xylene cyanol (Sigma)) and electrophoresed on denaturing 7% urea-5% (1:29 acrylamide:acrylamide gels. Following autoradiography, the relative signal strength of the 32P-labeled RNA was quantified by two-dimensional densitometric scanning with the Bio-Image Model 505S (Millipore) or the Gel-Pro CCD Analyzer (Media Cybernetics) imaging systems. Quantitative decay of 32P-labeled transcripts was calculated as the percentage of signal remaining compared with substrate incubated in decay buffer alone for the entire time course of the respective assay.

Immunodepletion and Immunoblotting of AUF1—AUF1 immunodepletion was accomplished using protein A-Sepharose CL-4B (Sigma) incubated with a 1:10 volume of rabbit anti-human AUF1-immune or preimmune serum (34) for 30 min at room temperature in cytoplasmic extraction buffer. After three washes, equal volumes of MNC extract and antibody-protein A-Sepharose beads were incubated for 60 min at 4°C twice in succession, using microcentrifugation (12,000 × g for 10 min) to sediment beads.

To verify AUF1 depletion, cytoplasmic extract proteins were electrophoresed on denaturing SDS-polyacrylamide gels (10%, 1:29 acrylamide) along with low range, prestained SDS-PAGE standards (Bio-Rad). Proteins were electroblotted onto nitrocellulose membranes using a MiniTrans-Blot Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer’s recommendations. Electroblot membranes were incubated with 5% nonfat milk in PBS for 60 min at room temperature, followed by 1:3000 AUF1-immune serum overnight at 4°C and 1:3000 horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) for 2 h at room temperature. Membranes were washed with antibody-binding buffer (2% nonfat milk in PBS) for 15 min at room temperature between incubations and with 0.1% Triton X-100 in PBS three times for 10-min each before development with luminol/peroxide chemiluminescent substrate (Proteinstar) and exposure to x-ray film.

Statistical Analysis—Data are presented as the mean ± S.E. Probability of significant differences (p < 0.05) was calculated using Student’s t test on Instat for Windows (GraphPad Software). Curve fitting was accomplished by regression analysis using Prism for Windows (GraphPad Software).

RESULTS

Decay of GM-CSF 3′-UTR RNA in Vitro Is ARE-dependent—To determine whether the GM-CSF ARE conferred instability to an RNA substrate in the in vitro system, decay of the 32P-labeled GM-CSF 3′-UTR was evaluated, with (8AU) and without (OU) the ARE (drammatically as dashed lines in Fig. 1). The “Free” samples shown in Fig. 2 (lanes 1 and 4) and throughout the study were incubated in decay buffer alone for the entire time course of the assay. In the presence of adult MNC extract protein, significantly more 32P-8AU than 32P-6AU decayed ($p$ = 0.0076) as a substrate for 45 min (16 ± 5% versus 62 ± 7% of substrate remaining; Fig. 2, lane 2 versus 5). The decay of 32P-8AU compared to 32P-6AU was not as rapid after 45 min in the presence of adult MNC extract protein (66 ± 4% versus 91 ± 7% of substrate remaining; Fig. 2, lane 3 versus 6), but the difference remained significant ($p$ < 0.008). A 32P-labeled, 335-nt transcript derived from the pBluescriptII:
neonatal MNC is reflected by a significantly shorter \( t_{1/2} \) of the GM-CSF 3′-UTR in the presence of neonatal MNC protein.

Decay of 8AU RNA Generates Discrete Intermediates—Lower molecular weight \( ^{32}P \)-labeled products were also observed as a consequence of \( ^{32}P \)-8AU in vitro decay. Quantitative analysis of four time course decay assays with three sets of MNC extracts found that generation of two of these products was reproducibly dependent upon incubation with MNC protein. Decay of the 360-nucleotide (nt) \( ^{32}P \)-8AU substrate by neonatal MNC protein generated significant levels \((p < 0.03)\) of 320- and 245-nt \( ^{32}P \)-labeled products (Fig. 2, lane 2; 8AU − 40 and 8AU − 115, respectively) after 45 min. Although these products did not accumulate to the same extent during the slower decay of \( ^{32}P \)-8AU with adult MNC protein, they remained detectable (Fig. 2, lane 3) with significant levels \((p < 0.02)\) of 8AU − 40 generated after 45 min. To unambiguously locate the cleavage sites responsible for generating these degradation products, in vitro transcribed 8AU was \( ^{32}P \)-labeled exclusively at the 5′-end. Appearance of the 320- and 245-nt products after incubation of this 5′-end \( ^{32}P \)-labeled 8AU substrate with neonatal MNC protein confirmed that 8AU − 40 and 8AU − 115 resulted from cleavage at the 3′-end of the 3′-UTR (Fig. 4, lanes 2–4). Compared with the levels of these products generated by rapid decay in the presence of neonatal MNC protein, accumulation of 8AU − 40 and 8AU − 115 in the presence of adult MNC protein was not significant (Fig. 4, lanes 5–7). The 3′-ends of these decay products map in the A + U-rich region, 40 and 115 nt upstream of the polyadenylation site within 20 nt from either end of the ARE (designated by vertical arrows in Fig. 1). These results suggest that ribonucleolytic cleavage on either side of the ARE plays a role in the rapid turnover of GM-CSF mRNA by neonatal MNC.

Immunodepletion of AUF1 Stabilizes 8AU RNA—The effect of endogenous AUF1 on stability of the GM-CSF 3′-UTR substrates was examined by immunodepleting the MNC extracts of AUF1 prior to assaying in vitro decay. Immunodepletion of AUF1 was verified by immunoblot analysis of MNC extracts (Fig. 5). The in vitro decay of \( ^{32}P \)-8AU was significantly diminished by immunodepletion of AUF1 from neonatal MNC protein extracts (Fig. 6, lanes 2 and 4). Only 11 ± 6% of \( ^{32}P \)-labeled 8AU remained after 30 min incubation in neonatal MNC extracts precipitated with pre-immune serum, whereas 81 ± 14% remained after 30 min incubation in neonatal MNC extracts precipitated with AUF1-immune serum \((p < 0.05, \text{ Table I})\). Conversely, \( ^{32}P \)-labeled 8AU degradation activity of adult MNC extracts was not affected by precipitation with AUF1-immune (84 ± 4% remaining) compared with pre-immune (85 ± 11% remaining) serum (Fig. 6, lanes 3 and 5; Table I). Immunodepletion of AUF1 also had no significant effect on the
versus p was incubated with adult MNC protein. Four AUF1 isoforms, p37AUF1 has the highest ARE binding affinity. The initial 32P-0AU substrate remained was determined by curve fitting and densitometric scanning. Data are presented as the mean ± S.E. percentage of remaining RNA substrate as compared with incubation in decay buffer alone for 120 min in four separate assays with four sets of neonatal and adult MNC extracts. A, 32P-labeled 0AU (○) or 0AU (●) RNA substrate was incubated with neonatal MNC protein. *, p < 0.04 for 0AU versus 0AU. B, 32P-labeled 0AU (△) or 0AU (▲) RNA substrate was incubated with adult MNC protein. *, p < 0.008 for 0AU versus 0AU. C, 32P-0AU RNA substrate was incubated with neonatal (○) or adult (△) MNC protein. The data displayed here are the same as those shown in panels A and B for 32P-0AU decay. *, p < 0.005 for neonatal versus adult MNC protein. RNA half-life (t1/2 = time after which 50% of initial 32P-0AU substrate remained) was determined by curve fit regression analysis.

decays of 32P-0AU (Fig. 6, lanes 6–10; Table I). These results indicate that depletion of AUF1, and possibly AUF1-associated proteins as well, significantly increases the in vitro stability of ARE-containing RNA in the presence of neonatal MNC protein.

DISCUSSION

The present study was designed to test the hypothesis that depletion of AUF1 would attenuate ARE-dependent RNA decay in vitro. The binding affinity of AUF1 for a particular ARE is known to reflect the transcript destabilizing potential of that ARE in vivo (52), and AUF1-enriched protein fractions have been shown to accelerate ARE-dependent in vitro mRNA decay (26). Previous studies have found that the t1/2 of GM-CSF mRNA is shorter in neonatal MNC (30 min) than in adult (100 min) MNC (39). We have also demonstrated previously that the levels of AUF1 binding activity and the p37AUF1 and p40AUF1 isoforms are significantly increased in neonatal MNC extracts (46). Of the four AUF1 isoforms, p37AUF1 has the highest ARE binding affinities. Furthermore, expression of AUF1 mRNA and protein is higher in fetal compared with adult murine liver (54). These findings suggested that increased levels of the p37AUF1 and/or p40AUF1 isoform(s) might promote ARE binding and transcript destabilization in developmentally immature tissues.

To directly measure RNA degradation activity in the same extracts that were analyzed for ARE binding and AUF1 activity (46), the cell-free mRNA decay assay (47) was adapted for use with whole cytoplasmic rather than fractionated polysomal extracts. The cell-free system has been previously demonstrated to reconstitute the rank order, response to stimuli, and decay products observed during in vivo mRNA decay (reviewed in Ref. 55). In the present study, a 32P-labeled GM-CSF 3′-UTR RNA transcript (8AU; Fig. 1) was utilized as a substrate to focus on the ARE region known to interact more strongly with neonatal than with adult MNC extract protein (46). The 3′-UTR ARE can confer instability independent of flanking RNA sequences (reviewed in Ref. 56). Initial characterization of the cell-free RNA decay system also established that RNA length alone does not affect the rate of decay (57), as evidenced by comparable stabilities of the 212-nt 0AU and 335-nt pBlue-scriptII:KS (+) vector transcripts in the presence of either neonatal or adult MNC protein (data not shown). A GM-CSF ARE deletion mutant (58) similar to 0AU, as well as small segments of the tumor necrosis factor-α 3′-UTR ARE (24, 58), have been effective as substrates for assaying ARE-specific decay activity in comparable cell-free systems. Transcript decay is a multistep process involving the degradation of ARE-containing RNA by the ARE-binding proteins AUF1 and TTP (59, 60), which are targeted to ARE-containing transcripts (61). AUF1, required for ARE-mediated destabilization, is encoded by the AUF1 gene and is highly evolutionarily conserved (62).

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step process in which deadenylation precedes decay of the mRNA body (15, 59–61). Therefore, nonadenylated substrates were utilized in order to focus on the subsequent ARE-directed decay of the RNA body. A GM-CSF 3′-UTR substrate with the ARE deleted (0AU; Fig. 1) was employed to assay ARE dependence because its inability to interact with MNC extract protein had been previously characterized (46). Acceleration of in vitro RNA decay by the ARE in the presence of neonatal or adult MNC extract protein demonstrated the validity of this system to assay ARE-dependent turnover (Figs. 2 and 3). A non-specific decrease in 0AU decay activity, coupled with our previous finding of 35-fold less AUF1 binding activity in adult MNC extracts (46), suggests that AUF1-dependent mRNA destabilization is greater in neonatal MNC.

Decay of E. coli mRNA is mediated by a ribonuclease complex known as the degradosome (64, 65). Characterization of the degradosome has demonstrated a central role for the endoribonuclease E (RNase E) component (66, 67). Two apparently distinct human RNase E homologs have also been identified (22, 23). Both these and bovine endoribonuclease V (21) specifically cleave A + U-rich RNA containing AUUUA motifs. Thus, an RNase E-like activity could play a role in cleaving the GM-CSF transcript within the ARE. Although the machinery for degrading the mRNA body in eukaryotes has not been as thoroughly characterized, a multiprotein complex known as the exosome mediates 3′ → 5′ exonuclease activity in yeast (68, 69). At least one exoribonuclease subunit, Rrp4p, has a human homolog that is also found in a multiprotein complex (68) and may be related to an ARE-targeted murine ribonuclease activity (20). Additionally, another multiprotein complex known as the proteosome has recently been implicated in ARE-directed mammalian mRNA decay. Purified murine proteosomes specifically bind and endonucleolytically cleave AUUUA motifs in the tumor necrosis factor-α 3′-UTR (24). Inhibiting human proteosome activity stabilizes GM-CSF ARE-containing transcripts and causes the accumulation of polyubiquitinated AUF1 (70). Because a major function of the proteosome is the degradation of polyubiquitinated proteins, it is possible that proteosomes are targeted to the 3′-UTR by AUF1-bound polyubiquitinated AUF1, after which the proteosome itself can bind and cleave the ARE. In short, it seems likely that both endoribonuclease(s), possibly RNase E-like (22, 23) and associated with proteosomes (70), and exoribonuclease(s) like Rrp4p (68), play a role in degrading the body of ARE-containing human transcripts such as that of GM-CSF. The interplay of human ARE-targeted ribonucleases (22–24, 68, 70) and other ARE

FIG. 6. Effect of AUF1 immunodepletion on in vitro decay of GM-CSF 3′-UTR by MNC protein. Following immunodepletion with preimmune (lanes 2-3 and 7-8) or AUF1-immune (lanes 4-5 and 9-10) serum, cytoplasmic protein from PMA + PHA-stimulated neonatal (Neo, lanes 2, 4, 7, and 9) or adult (Ad, lanes 3, 5, 8, and 10) MNC was incubated with 32P-labeled 8AU (lanes 1-5) or 0AU (lanes 6-10) RNA substrate at 37 °C for 30 min. Purified RNA was fractionated by denaturing 7% urea-5% PAGE, and 32P-labeled products were detected by autoradiography. Lanes 1 and 6 show 32P-labeled 8AU and 0AU RNA substrates, respectively, after incubation in decay buffer alone for 30 min. Sizes indicated are based upon 8AU = 360 nt and 0AU = 212 nt. Decay intermediates are designated as 8AU − 40 and 8AU − 115. Results shown are representative of three separate assays with four sets of neonatal and adult MNC extracts.

Table I

| Substrate | Neonatal MNC protein | Adult MNC protein |
|-----------|----------------------|-------------------|
| %         | a-AUF1 Significance  | %                 | a-AUF1 Significance |
| 8AU       | 11 ± 6               | 85 ± 11           | NS                 |
| 0AU       | 87 ± 15              | 106 ± 1           | 103 ± 3            | NS                 |

Reproducing the relative rates of GM-CSF transcript decay observed during myeloid development demonstrates the ability of the cell-free system to also reconstitute developmental regulation in vitro. Hence, this assay appeared applicable as an in vitro system for identifying the molecular components of accelerated ARE-dependent mRNA turnover in neonatal MNC. This capability was utilized to evaluate the effect of AUF1 immunodepletion on decay of 8AU in the presence of MNC extract protein. The in vitro decay of 8AU was significantly diminished by immunodepletion of AUF1 (Table I) from neonatal but not adult MNC extracts (Fig. 6). The inability of AUF1 immunodepletion to attenuate adult MNC protein 8AU decay activity, coupled with our previous finding of 35-fold less AUF1 binding activity in adult MNC extracts (46), suggests that AUF1-dependent mRNA destabilization is greater in neonatal MNC.

directly to the more rapid turnover of GM-CSF mRNA in neonatal (t1/2 = 30 min) versus adult (t1/2 = 100 min) MNC (39). Reproducing the relative rates of GM-CSF transcript decay observed during myeloid development demonstrates the ability of the cell-free system to also reconstitute developmental regulation in vitro. Hence, this assay appeared applicable as an in vitro system for identifying the molecular components of accelerated ARE-dependent mRNA turnover in neonatal MNC. This capability was utilized to evaluate the effect of AUF1 immunodepletion on decay of 8AU in the presence of MNC extract protein. The in vitro decay of 8AU was significantly diminished by immunodepletion of AUF1 (Table I) from neonatal but not adult MNC extracts (Fig. 6). The inability of AUF1 immunodepletion to attenuate adult MNC protein 8AU decay activity, coupled with our previous finding of 35-fold less AUF1 binding activity in adult MNC extracts (46), suggests that AUF1-dependent mRNA destabilization is greater in neonatal MNC.

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![Image](60x584 to 285x729)

Quantitative analysis of AUF1 immunodepletion effects on GM-CSF 3′-UTR in vitro decay

Following immunodepletion with pre-immune or AUF1-immune (a-AUF1) serum, cytoplasmic protein from PMA + PHA-stimulated neonatal or adult MNC was incubated with 32P-labeled 8AU or 0AU RNA substrate at 37 °C for 30 min and assayed for in vitro decay. Data are presented as the mean ± S.E.% of remaining RNA substrate as compared with incubation in decay buffer alone for 30 min in two separate assays with three sets of neonatal and adult MNC extracts. NS = not significant (p > 0.05).

The more rapid turnover of 8AU with neonatal (t1/2 = 19 min) versus adult (t1/2 = 79 min) MNC protein (Fig. 3C) corresponded
binding factors (24–33) within multimeric complexes (68, 70) remains an active area of investigation.

At least six proteins are known to co-immunoprecipitate with AUF1 (34, 70), four of which have recently been identified as heat shock protein 70, heat shock cognate protein 70, eukaryotic translation initiation factor-4G, and poly(A)-binding protein (70). Previous data demonstrating that either purified (26) or recombinant2 AUF1 can still bind with but can no longer accelerate in vitro decay of ARE-containing mRNA suggests that these protein cofactors are likely contributors to accelerated ARE-dependent RNA turnover in neonatal MNC. In the present study, depletion of AUF1 and, possibly AUF1-associated protein from neonatal MNC extracts, increased ARE-dependent in vitro RNA stability, supporting the proposition that AUF1 plays a role in destabilizing ARE-containing transcripts. ARE-dependent RNA transcript stabilization in vitro by specific immunodepletion, thus, represents a novel and highly significant new finding.

Taken together, our results indicate that accelerated in vitro RNA decay in the presence of neonatal MNC protein is ARE- and AUF1-dependent. Earlier studies also demonstrated an associated increase in the binding of a neonatal MNC protein complex containing AUF1 (46). Further investigation into the possible roles of AUF1 binding cofactors (34, 70) and the enzymes (22–24, 70) responsible for the formation of RNA decay intermediates (60, 62, 63) should clarify the mechanism for ARE-dependent mRNA destabilization in neonatal MNC. This knowledge is essential for understanding the development of phagocytic immunity.

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