Title

Widespread Effects of Chemokine 3' Untranslated Regions on mRNA Degradation and Protein Production in Human Cells.

Journal

The Journal of Immunology, 201(3)

ISSN

0022-1767

Authors

Zhao, Wenxue
Erle, David J

Publication Date

2018-08-01

DOI

10.4049/jimmunol.1800114

Peer reviewed

HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2019 August 01.

Published in final edited form as:

J Immunol. 2018 August 01; 201(3): 1053–1061. doi:10.4049/jimmunol.1800114.

Widespread Effects of Chemokine 3'-UTRs on mRNA Degradation and Protein Production in Human Cells

Wenxue Zhao[*,†,1] and **David J. Erle**[*,1]

[*]Lung Biology Center, Department of Medicine, University of California San Francisco, San Francisco, California, USA

[†]School of Basic Medicine (Shenzhen), Sun Yat-Sen University, Guangzhou, P.R. China

Abstract

Chemokines are a large family of chemotactic cytokines that play critical roles in inflammation, development, and diseases. Chemokine expression is highly regulated during development and in response to environmental stimuli. The 3' untranslated regions (3'-UTRs) of mRNA are believed to be important in control of chemokine gene expression. However, the regulatory effects of most chemokine 3'-UTRs have not been characterized previously. In this work, we systematically studied the effects of 43 CC and CXC chemokine 3'-UTRs on gene expression in eight human cell lines and two types of human primary cells. We found that chemokine 3'-UTRs had a wide spectrum of regulatory effects on mRNA abundance and protein production that were tightly correlated to the effects on mRNA stability. In general, 3'-UTRs had remarkably similar effects across all cell types studied. The presence of AU-rich elements, microRNA targets, and Pumilio binding sites were associated with chemokine 3'-UTR activity but did not fully account for all 3'-UTR activity detected using the reporter assay. Mutational analysis illustrated how specific cis-elements contributed to the regulatory effect of chemokine 3'-UTRs. These findings bring new insights into the mechanisms by which chemokine expression is regulated by 3'-UTRs.

Keywords

Chemokine; 3'-UTR; gene expression and regulation; mRNA decay

Introduction

Chemokines are a large family of chemotactic cytokines that can be subdivided based on the distribution of cysteine residues in each protein (1). In humans, there are 28 CC chemokines, 16 CXC chemokines, one CX_3C chemokine and two XC chemokines. Chemokine binding by G protein-coupled chemokine receptors was originally discovered to direct the movement

[1]Correspondence should be addressed to W. Z. (Tel: 415-514-4370, fax: 415-514-4278, wzhao_sysu@163.com) or D.J.E. (Tel: 415-514-4372, fax: 415-514-4278, david.erle@ucsf.edu).

Disclosures

The authors declare no competing financial interests.

Author Contributions

W.Z. and D.J.E. conceived of key aspects of the project and designed the experiments. W.Z. carried out the experimental work, analyzed the data, and wrote the manuscript. D.J.E. edited the manuscript.

of mononuclear cells to sites of inflammation or injury (1, 2). In vitro assays indicated that the movement toward chemokines is gradient-dependent (1). An example is the receptor *CCR3*, which binds several chemokines including *CCL11* and is involved in the recruitment of T_H2 cells during allergic inflammation (3, 4). The *CXCR4* ligand *CXCL12* regulates lymphocyte trafficking within lymph nodes (5). In embryogenesis, chemokine signaling has multiple functions in formation of the cardiovascular, hematopoietic, urogenital, and nervous systems (1). Deficiency of *CXCR4* or *CXCL12* in mice leads to extensive neurodevelopmental defects including neuron malpositioning in the cerebellum and dorsal root ganglia (6, 7). *CXCL1* and its receptor *CXCR2* participate in determining positioning and oligodendrocyte numbers in developing spinal cord (8). Evidence is accumulating that chemokines are also involved in cancer progression. For example, increased *CXCR4* level in the primary breast and colon cancer is associated with lymph node metastasis (9, 10). A recent study demonstrated that *CCL2* and its receptor *CCR2* are coordinately elevated in human and mouse breast cancer cells (11). Other functions of chemokines include roles in lipid metabolism (12), angiogenesis (13) and HIV infection (14).

Chemokine expression is controlled at multiple levels including transcriptional activation and translational regulation (15). The transcription factor *NF-κB* is involved in transcriptional activation of multiple chemokines. For *CXCL8*, *NF-κB* and co-factors including *AP1* and *NF-IL6* are necessary, whereas *CXCL1* transcription is initiated by *NF-κB* interacting with *Sp1* and *CBP* (16, 17). In contrast, posttranslational regulation can convert chemokine precursors into biologically active peptides or modify the biological activity. In neutrophil granulocytes, for example, removal of the first 5 amino acid residues in *CXCL8* results in the increase of biological activity (15, 18). Recently, however, increasing attention has been paid to 3'-UTR mediated regulation of gene expression. 3'-UTRs are critical in determining the fate of the mRNA by serving as binding sites for numerous proteins and noncoding RNAs (19–21), which subsequently change mRNA stability (22, 23), translation (24–26) and localization (27). Types of cis-regulatory elements in 3'-UTRs include AU-rich elements (AREs) (28), microRNA (miRNA) targets (22) and Pumilio binding sites (29). These cis-elements interact with the trans-acting factors and thus affect development (30), metabolism (31) and disease pathogenesis (32). In the chemokine family, the 3'-UTRs of several members such as *CXCL2* and *CXCL8* have been well investigated. Binding of an ARE in the *CXCL8* 3'-UTR to the protein *KSRP* causes rapid mRNA decay during inflammatory reactions (33). Antigen stimulation stabilizes *CXCL2* mRNA via 3'-UTR interactions with the *p38 MAPK* and *p44/42 MAPK* pathways (34). *CXCL1* has also been shown to contain active AREs that affect mRNA stability (35).

Despite this progress, there is a lack of systematic understanding of regulatory effects of chemokine 3'-UTRs on gene expression. In this work, we used a reporter assay to systematically quantify the regulatory effects of 43 CXC and CC chemokine 3'-UTRs on protein production, mRNA level, and mRNA turnover. Since the trans-acting factors may vary with tissue types, we tested these 3'-UTR sequences in eight cell lines and two primary cell types and compared their regulatory activity across cell types. We further analyzed the roles for predicted AREs, miRNA targets and Pumilio binding sites. These cis-elements are associated with the regulatory activity and co-regulate gene expression.

Materials and Methods

Construction of the reporter BTV and cloning of 3’-UTR sequences

Construction of the BTV reporter and the subsequent cloning steps were performed as described previously (36). In brief, we used Phusion® High-Fidelity PCR polymerase (NEB, M0531) to amplify chemokine 3’-UTRs from genomic DNA derived from multiple anonymous donors (Promega, G3041). After digestion with *Mlu*I and *Pac*I, the PCR products were ligated into BTV vector, followed by Sanger sequencing to validate sequences. All cloned chemokine 3’-UTR sequences and 50-mer oligonucleotide sequences containing wild type and mutant pumilio binding motifs are listed in Supplementary Table 1.

Cell culture

To study chemokine 3’-UTRs in immortalized cells, we used stable tTA-expressing cell lines. BEAS-2B.tTA, Jurkat.tTA, and WIDR.tTA cell lines were described previously (36). The other five tTA cell lines were expanded from single clones of cells transduced with a tTA lentivirus as described previously (36). We verified cell identity for Jurkat, BEAS-2B, and H292 cells using Short Tandem Repeat (STR) profiling (Mclab). Other cell line identities were not verified and cells were not tested for mycoplasma. BEAS-2B.tTA cells were maintained in DMEM/F12 (1:1) with 10% FBS, 2 mM glutamine, 0.1 mM non-essential amino acids and penicillin-streptomycin. DLD-1.tTA cells and Jurkat.tTA cells were maintained in RPMI1640 with 10% FBS, 2 mM glutamine, 0.1 mM non-essential amino acids and penicillin-streptomycin. WIDR.tTA cells, H292.tTA cells, HelaS3.tTA cells, MG63.tTA cells, and RD.tTA cells were maintained in DMEM with 10% FBS, 2 mM glutamine, 0.1 mM non-essential amino acids and penicillin-streptomycin. 293T cells were maintained in DMEM with 10% FBS, 2 mM glutamine, and 0.1 mM non-essential amino acids. Normal human primary lung fibroblast cells were purchased from ATCC (PCS-201-013) and cultured in Fibroblast Basal Medium (ATCC, PCS-201-030) with Fibroblast Growth Kit-Low serum (ATCC, PCS-201-041). The human bronchial smooth muscle cells were purchased from Lonza (CC-2576, Lot# 0000581076) and cultured in SmBM Basal Medium (Lonza, CC-3181) with SmGM-2 SingleQuot Kit (Lonza, CC4149).

Lentivirus production and transductions

1 μg each of pMDL, p-RSV, and p-VSV-G plus 3 μg of backbone plasmid in Fugene HD (Roche, 04709691001) was used to transfect 293T cells (>80% confluent in 10-cm dishes) cultured in high glucose DMEM containing 10% FBS, 2 mM glutamine, and 0.1 mM non-essential amino acids (37). Conditioned medium containing virus was harvested 48 h post-transfection and used immediately to infect cells or frozen at −80°C. To infect cells, we added conditioned medium diluted 1:4 with fresh medium plus 8 μg/ml (final) polybrene to cultured cells. The medium was replaced with fresh medium 24 h after infection. Transduced cells were allowed to grow for 72 h and employed for flow cytometry analysis in bulk, without clonal selection. For transduction of primary cells, a mixture containing reporter virus, tTA expressing virus (36), and fresh medium (4:1:5 by volume) was added to the wells in 96-well plates and replaced with fresh medium 24 h later. Cells were allowed to grow for 72 h and used for flow cytometric analysis.

Cell staining and flow cytometry

Cells were incubated with Alexa 647–conjugated anti-LNGFR antibody (UCSF Monoclonal Antibody Core, ME20-4), 1:500 in PBS containing 10% FBS, for 30 min on ice. After washing twice with PBS, cells were re-suspended in PBS and fixed with 1% paraformaldehyde. Data were collected from ≥ 2000 $LNGFR^+$ cells using a FACSCanto II (Becton Dickinson). Mean values were determined from triplicate experiments.

RNA isolation

Cellular total RNA was extracted with RNeasy Mini Kit (Qiagen, 74104). DNase I was used to eliminate possible DNA contamination in all RNA samples.

Quantification of mRNA level with Quantitative Real Time PCR

SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, 18080-051) was used for cDNA synthesis. The abundance of *EGFP* in cDNA was detected with Fast SYBR® Green Master Mix (Applied Bio., #4385612) on ABI prism 7900HT Sequence Detection System. Relative RNA levels were evaluated using the ΔΔCt method (38) and normalized to *LNGFR* for steady state mRNA, and to *GAPDH* in mRNA decay assays. Primers for *EGFP*: GAAGTCGTGCTGCTTCATGT and TGACCCTGAAGTTCATCTGC; primers for *GAPDH*: TTCCAATATGATTCCACCCA and GATCTCGCTCCTGGAAGATG; primers for *LNGFR*: GCACCTCCAGAACAAGACCT and TGGAGCAATAGACAGGGATG.

RNA stability assay

Transduced BEAS-2B.tTA cells were expanded and divided equally into multiple dishes. On the following day, doxycycline solution was added to the medium to 1 μg/ml (final) and cells were harvested 0–8 h later. The total RNA was isolated from the cellular lysate, followed by reverse transcription for cDNA synthesis. The *EGFP* mRNA remaining at each time point was measured with quantitative real time PCR as stated above and mRNA decay rates were calculated by exponential fitting.

Co-transfection of let-7 sensor and let-7 inhibitor

BEAS-2B.tTA cells were plated in 24-well plates (60,000 cells/well). On the following day, 150 ng plasmid (BTV cloned with two repeats of the perfect let-7 target) and 5 pmol of let-7 inhibitor (sequence 5’ ACCATACAACCTACT, Exiqon) was transfected using Lipofectamine 2000 (Invitrogen, 11668-027) according to the manufacturer’s instructions. Gene expression was detected by measuring *EGFP* intensity with flow cytometry 48 h after transfection.

AREs, miRNA targets, and Pumilio binding site prediction

AREs were identified using the ARED database (39); typical Pumilio binding sites and nontypical Pumilio motifs were defined as described previously (40, 41). Selected 50-mer sequences including predicted Pumilio motifs tested in Fig. 4F are included in Supplementary Table 1; conserved miRNA targets were identified using TargetScan 7.1 (42). The complete list of predicted elements are shown in Supplementary Table 1.

Results

Chemokine 3'-UTRs have widespread effects on protein production

To quantify the regulatory effects of chemokine 3'-UTRs, we used the BTV lentiviral reporter to directly measure the effect of the 3'-UTRs on *EGFP* transgene expression in BEAS-2B cells (Fig. 1A, 1B). We first validated this reporter by testing previously studied 3'-UTR sequences. An ARE-containing fragment from the *CXCL8* 3'-UTR (33) led to the expected decrease in reporter expression (Fig. 1C). A synthetic sequence containing a let-7 target (43) also decreased expression and this effect was eliminated by mutating the miRNA target site (Fig. 1C). We then measured the effects of 43 chemokine 3'-UTRs on *EGFP* protein in BEAS-2B cells. We included 3'-UTRs from all 40 CC and CXC chemokines with 3'-UTRs longer than 100 nt, and for three of these chemokines (*CXCL12*, *CCL16*, and *CCL23*) we included two different 3'-UTRs that can arise from alternative polyadenylation. We did not include 3'-UTRs from *CCL24* (no annotated 3'-UTR sequence) or *CCL27* (short 3'-UTR, length 77 nt). One example of a highly active chemokine 3'-UTR, *CCL3*, which decreased expression to 4% of the level seen with the control reporter without a test 3'-UTR sequence, is shown in Fig. 1C. The set of chemokine 3'-UTRs showed a wide spectrum of effects on reporter expression ranging from 3% (for *CXCL2*) to 124% (for *CCL16*.1) (Fig. 1D). Twenty 3'-UTRs decreased *EGFP* protein level to < 50%. Another sixteen had modest down-regulatory effects, with expression ranging from 51%–90%. CCL14.2 and CCL16.1 3'-UTRs showed modest up-regulatory effects (122% and 124% respectively). In some cases, polyadenylation variants showed much different effects. For example, a longer isoform of *CXCL12* (*CXCL12*.1, 3139 nt, 10% expression) reduced expression more than a shorter isoform (*CXCL12*.2, 1537 nt, 48%) and a longer *CCL16* isoform (*CCL16*.2, 1041 nt, 41%) reduced expression whereas a shorter isoform (*CCL16*.1, 120 nt, 124%) increased expression (Fig. 1D). These results suggest that alternative polyadenylation of chemokine mRNAs likely have important effects on regulation of expression.

Most chemokine 3'-UTR activity was conserved across cell types

Since 3'-UTR regulatory activity depends upon trans-acting factors that may vary across cell types, we measured the effects of each chemokine 3'-UTR in seven other cell lines. Most 3'-UTRs had generally similar activity across cell types (Fig. 2A). For example, the *CXCL2, CXCL3, CXCL12.1, CCL3, CXCL8, CXCL14.1, CXCL6, CCL4*, and *CCL8* 3'-UTRs decreased reporter gene expression in all eight cell lines ($p < 0.01$ compared with the empty vector control by Dunnett's test) whereas the *CCL16*.1 3'-UTR did not significantly decrease expression in any cell line. Reporter protein levels in each cell line were highly correlated with levels in other cell lines (Fig. 2B, 2C). However, some 3'-UTRs had different effects between cell types. For example, *CCL18* 3'-UTR significantly decreased gene expression only in DLD-1 and Jurkat cells ($p < 0.001$ compared with the empty vector control by Dunnett's test) and the inhibitory effects of this 3'-UTR were significantly greater in Jurkat cells than in BEAS-2B, H292, HeLa S3, MG63, RD, and WIDR cells (all $p < 0.02$ by Tukey-Kramer test). *CCL7* 3'-UTR decreased expression in all cell types ($p < 0.0005$ compared with the empty vector control by Dunnett's test), but expression was higher in RD cells (mean 65%) and H292 cells (58%) than in any of the 6 other cells types (range 27–40%, $p < 0.05$ compared with both RD and H292 by Tukey-Kramer test). To determine

whether chemokine 3'-UTRs had similar activities in primary cells, we tested the 3'-UTR reporters in two human primary cell types. Across the entire set of 43 chemokine 3'-UTRs, reporter protein levels in primary lung fibroblast cells were highly correlated to mean protein levels in the 8 cell lines ($R^2 = 0.95$, Fig. 2D). In addition, we studied 15 3'-UTRs in primary human bronchial smooth muscle cells and also found a strong correlation ($R^2 = 0.83$, Fig. 2E). Taken together, these studies in multiple cell lines and in primary cells show substantial functional similarities across cell types and also provide examples of the dependence of 3'-UTR activity on cellular context.

Chemokine 3'-UTRs regulate steady state mRNA and mRNA stability

3'-UTRs have been reported to regulate protein production by modulating mRNA stability (23) and protein translation (44). Since effects on mRNA stability result in changes in steady state mRNA levels, we began by systematically investigating the effects of chemokine 3'-UTRs on reporter mRNA levels in BEAS-2B cells. Most 3'-UTRs led to reduced abundance of mRNA; some such as the *CXCL12*.1 (longer isoform of *CXCL12*) and *CXCL2* 3'-UTRs resulted in a very low level of mRNA, ~5% relative to empty reporter BTV. Some 3'-UTRs had little or no effect on mRNA levels and one, the *CCL1* 3'-UTR, increased *EGFP* mRNA level (30% higher than control, Fig. 3A). These results showed that chemokine 3'-UTRs had a wide spectrum of effects on mRNA abundance. Strikingly, by comparing mRNA and protein levels, we found that protein level was highly correlated with the mRNA level ($R^2 = 0.91$, $p < 0.0001$) (Fig. 3B), indicating that chemokine 3'-UTR effects on protein production are tightly coupled to effects on steady state mRNA levels.

To directly determine whether chemokine 3'UTRs regulate mRNA stability, we measured the half-lives of reporter transcripts containing each of the chemokine 3'-UTRs after inhibiting reporter transcription by adding doxycycline. For example, Fig. 3C shows that the *CXCL8* 3'-UTR caused more rapid mRNA decay compared with a control construct lacking a 3'-UTR test sequence. We found half-lives varied widely, ranging from <1 h to >5 h and were correlated with mRNA abundance ($R^2 = 0.75$, $p < 0.0001$, Fig. 3D) and protein level ($R^2 = 0.75$, $p < 0.0001$, Fig. 3E). These results indicate that effects on steady state levels of mRNA and protein are generally coupled to changes in mRNA stability. However, two 3'-UTRs (*CCL22* and *CXCL9*) showed a long half-life but much lower abundance of mRNA. Furthermore, although *CCL1* 3'-UTR increased steady state mRNA level compared the BTV control, this 3'-UTR had no detectable effect on mRNA half-life (4.2 h for *CCL1* versus 4.3 h for BTV). This suggests that some 3'-UTRs regulated reporter mRNA level by affecting mRNA production (e.g., transcription) rather than mRNA stability.

Multiple classes of cis-elements are associated with the regulatory activity of chemokine 3'-UTRs

3'-UTR activity is often governed by cis-elements that interact with trans-acting factors. AREs, miRNA targets and Pumilio binding sites in 3'-UTRs are each known to regulate mRNA stability. We identified predicted cis-elements of these three classes in all chemokine 3'-UTRs. We found that the presence of one or more AREs, miRNA targets, or Pumilio binding sites was associated with significantly lower levels of reporter protein production in BEAS-2B cells (Fig. 4A–C). Some 3'-UTRs containing predicted cis-elements did not have

reduced expression, which illustrates the current limitations of computational methods for functional annotation of regulatory sequences. 3'-UTRs that lacked any of these three types of predicted elements had modest effects on *EGFP* expression, with none reducing expression by >50% (Fig. 4D). We (36) and others (45) have previously established that AU-rich elements in 3'-UTRs have major roles in destabilizing chemokine mRNAs. To test the regulatory activity of predicted Pumilio binding sites, we tested five 50-nt predicted Pumilio binding site-containing segments from the *CXCL8*, *CXCL10*, *CXCL11*, *CXCL14*, and *CCL21* 3'-UTRs. In each case, we compared the activity of the 50 nt segment with the activity of a sequence containing mutations in the predicted Pumilio binding site (Fig. 4E). Mutagenesis of the predicted Pumilio binding sites significantly increased reporter protein levels in BEAS-2B cells (Fig. 4F), demonstrating that these sites have regulatory activity.

A let-7 target and an AU-rich element in CCL3 co-regulate gene expression

In order to understand how multiple cis-elements in one 3'-UTR co-regulate gene expression, we chose *CCL3* 3'-UTR for further study. *CCL3* is secreted by activated inflammatory cells, acts as a chemoattractant for monocytes and lymphocytes (46), and is involved in diseases such as asthma, arteriosclerosis, and rheumatoid arthritis (47). The reporter assay showed that the *CCL3* 3'-UTR had a very strong down-regulatory effect in all eight cell types (10 ± 5% relative to BTV) (Fig. 2A). Using ARED (39) and TargetScan (42), we found a predicted ARE and a predicted let-7 target in the *CCL3* 3'-UTR. Both of these predicted elements are evolutionarily conserved (Fig. 5A). Deletion of the ARE caused a 4-fold increase in *EGFP* expression and mutation of let-7 target resulted in a 2-fold increase in *EGFP* expression (Fig. 5B). ARE deletion combined with let-7 target mutation led to a 6-fold increase in expression (Fig. 5B). To confirm the dependence of the activity of *CCL3* 3'-UTR on the microRNA let-7, we co-transfected BEAS-2B cells with the reporter and a let-7 inhibitor. We found that the let-7 inhibitor decreased the inhibitory effect of the *CCL3* 3'-UTR to a similar extent as did mutation of the *CCL3* 3'-UTR (Fig. 5C), demonstrating that the activity of the predicted let-7 target was let-7-dependent. Both AREs (28) and miRNA targets (22) frequently destabilize mRNAs, and our results indicate that the destabilizing effect of the *CCL3* 3'-UTR (Fig. 3) is due in large part to its ARE and let-7 target sequences. Since the effect of the let-7 target in the *CCL3* 3'-UTR has not been reported previously, we measured the activity of the let-7 target on mRNA decay. Mutation of the let-7 seed sequence significantly extended mRNA half-life (1.7 h) compared with the wild type (0.9 h) ($p < 0.005$) (Fig. 5D). Previous studies of multiple miRNAs (25, 48), including let-7 (49), show that miRNA-induced mRNA destabilization is frequently coupled to translational inhbition, and our finding that the effect of let-7 target mutation on reporter protein levels (270% relative increase) is somewhat larger than the effect on stability (89% relative increase) suggests that the let-7 target in the *CCL3* 3'-UTR may affect translation as well as stability. Our results show that conserved elements from two different classes (an ARE and a miRNA target) co-regulate expression of the *CCL3* chemokine gene.

Discussion

In this work, we systematically studied the regulatory effects of chemokine 3'-UTRs on gene expression. Previous studies have shown that 3'-UTRs play important roles in

controlling mRNA stability, subcellular localization, and translation (22, 23, 27). 3'-UTR mediated regulation of gene expression is important during development and in disease pathogenesis (30, 32). The regulatory activity of 3'-UTRs is dependent on cis-elements and trans-acting factors. Computational approaches based on identifying sequence motifs can be used to predict active cis-elements. Well-known cis-elements include AREs (28), Pumilio binding sites (29) and miRNA targets (22, 36). ARE motifs are found in 5–8% of coding genes and can either increase or decrease gene expression, dependent on RNA binding proteins (50). Over 50% of coding genes are predicted to be under the control of miRNAs, which inhibit translation and destabilize mRNA (51). However, computational approaches have limited ability to predict functional activity since the activity of cis-elements is cellular context-dependent and it is not known whether individual elements are active or not (52). In this study, for example, some chemokine 3'-UTRs containing predicted AREs, miRNA targets or Pumilio binding sites did not show significant effects on *EGFP* expression (Fig. 4D). Another limitation of computational approaches is that many active elements identified in functional studies do not contain known RNA binding protein or miRNA target motifs (36). Therefore, direct experimental evaluation of the effects of 3'-UTRs can provide information that cannot be provided by current computational methods.

The reporter assay allowed us to systematically quantify the regulatory effects of chemokine 3'-UTRs. We found that chemokine 3'-UTRs have a wide spectrum of regulatory effects on gene expression, ranging from 3% to 124% of control reporter protein expression. Most chemokine 3'-UTRs had similar regulatory activity across the set of cell lines and primary cells we studied, suggesting that common trans-regulatory networks operate in these cells despite their different origins. By correlating predicted cis-elements (AREs, Pumilio binding sites, and miRNA targets) with regulatory activity, we found that the presence of any or all of these types of cis-elements was associated with chemokine 3'-UTR activity. We directly tested the role of several predicted cis-elements using mutagenesis. This approach demonstrated the activity of individual elements and showed how two distinct elements in one chemokine 3'-UTR work together to regulate expression. We directly demonstrated the importance of one trans-factor, the miRNA let-7, in *CCL3* 3'-UTR-mediated post-transcriptional regulation. Many trans-factors are likely to be involved given the large number of miRNAs and RNA binding proteins (e.g., 3 Pumilio proteins and >20 ARE binding proteins (53)) found in human cells, and further studies will be required to determine the contributions of these trans-factors. Several 3'-UTRs which do not contain predicted miRNA targets, AREs, and Pumilio binding sites still altered reporter expression (Fig. 4D), suggesting that other 3'-UTR cis-elements and trans-factors can also contribute to post-transcriptional chemokine gene regulation. We further found the regulatory effect on protein is tightly coupled with mRNA level and mRNA decay rate. In general, a longer mRNA half-life was associated with a higher level of mRNA at steady state. Surprisingly, *CCL22* and *CXCL9* 3'-UTRs had similar mRNA half-lives as the control reporter with no 3'-UTR test sequence despite having much lower levels of steady state mRNA. One possible mechanism is that these 3'-UTRs can regulate gene transcription. We recently showed that targeting 3'-UTRs of some chemokine genes with CRISPR-Cas9 decreased the transcription of these genes (54), suggesting that the 3'-UTR or the corresponding DNA sequence could regulate gene transcription as well as post-transcriptional regulation.

Chemokines are a class of regulators that play a central role in many homeostatic and pathological conditions. Existing evidence indicates that chemokine regulation is complex and involves multiple steps, including transcription, post-translational modifications, and interactions with extracellular matrix (15, 55). Transcription factors that bind to chemokine promoters and enhancers influence the initiation and cessation of chemokine expression. Post-translational processing can convert an immature precursor to a functional chemokine. An increasing collection of studies suggest that post-transcriptional regulation plays an important role in control of chemokine gene expression. In particular, the mechanisms controlling mRNA stability appear to be crucially involved in determining the timing and the levels of chemokine gene expression during an inflammatory response. In this work, we found many chemokine 3'-UTRs are active in regulation of gene expression. These 3'-UTRs contain multiple cis-elements such as miRNA targets, AREs, and Pumilio binding sites and likely other active elements that could potentially serve as targets of immunomodulatory therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Walter Eckalbar for assistance with data analysis.

This work was supported by NIH grants R01HL124285 and R01GM110251 (to D.J.E.).

References

1. Ransohoff RM. Chemokines and chemokine receptors: standing at the crossroads of immunobiology and neurobiology. Immunity. 2009; 31:711–721. [PubMed: 19836265]
2. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. The New England journal of medicine. 2006; 354:610–621. [PubMed: 16467548]
3. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. Nature immunology. 2001; 2:123–128. [PubMed: 11175804]
4. Gerber BO, Zanni MP, Uguccioni M, Loetscher M, Mackay CR, Pichler WJ, Yawalkar N, Baggiolini M, Moser B. Functional expression of the eotaxin receptor CCR3 in T lymphocytes co-localizing with eosinophils. Current biology : CB. 1997; 7:836–843. [PubMed: 9480044]
5. Okada T, Ngo VN, Ekland EH, Forster R, Lipp M, Littman DR, Cyster JG. Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. The Journal of experimental medicine. 2002; 196:65–75. [PubMed: 12093871]
6. Li M, Ransohoff RM. Multiple roles of chemokine CXCL12 in the central nervous system: a migration from immunology to neurobiology. Progress in neurobiology. 2008; 84:116–131. [PubMed: 18177992]
7. Lazarini F, Tham TN, Casanova P, Arenzana-Seisdedos F, Dubois-Dalcq M. Role of the alpha-chemokine stromal cell-derived factor (SDF-1) in the developing and mature central nervous system. Glia. 2003; 42:139–148. [PubMed: 12655598]
8. Tsai HH, Frost E, To V, Robinson S, Ffrench-Constant C, Geertman R, Ransohoff RM, Miller RH. The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. Cell. 2002; 110:373–383. [PubMed: 12176324]
9. Su YC, Wu MT, Huang CJ, Hou MF, Yang SF, Chai CY. Expression of CXCR4 is associated with axillary lymph node status in patients with early breast cancer. Breast. 2006; 15:533–539. [PubMed: 16239110]

10. Schimanski CC, Schwald S, Simiantonaki N, Jayasinghe C, Gonner U, Wilsberg V, Junginger T, Berger MR, Galle PR, Moehler M. Effect of chemokine receptors CXCR4 and CCR7 on the metastatic behavior of human colorectal cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2005; 11:1743–1750. [PubMed: 15755995]
11. Fang WB, Jokar I, Zou A, Lambert D, Dendukuri P, Cheng N. CCL2/CCR2 chemokine signaling coordinates survival and motility of breast cancer cells through Smad3 protein- and p42/44 mitogen-activated protein kinase (MAPK)-dependent mechanisms. The Journal of biological chemistry. 2012; 287:36593–36608. [PubMed: 22927430]
12. Loughrey B, McGinty A, Young I, McCance, Powell L. Increased circulating CC chemokine levels in the metabolic syndrome are reduced by low dose atorvastatin treatment: evidence from a randomised controlled trial. Clinical endocrinology. 2012
13. Caccuri F, Giagulli C, Bugatti A, Benetti A, Alessandri G, Ribatti D, Marsico S, Apostoli P, Slevin MA, Rusnati M, Guzman CA, Fiorentini S, Caruso A. HIV-1 matrix protein p17 promotes angiogenesis via chemokine receptors CXCR1 and CXCR2. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109:14580–14585. [PubMed: 22904195]
14. Kalinkovich A, Weisman Z, Bentwich Z. Chemokines and chemokine receptors: role in HIV infection. Immunology letters. 1999; 68:281–287. [PubMed: 10424433]
15. Mortier A, Van Damme J, Proost P. Regulation of chemokine activity by posttranslational modification. Pharmacology & therapeutics. 2008; 120:197–217. [PubMed: 18793669]
16. Amiri KI, Richmond A. Fine tuning the transcriptional regulation of the CXCL1 chemokine. Progress in nucleic acid research and molecular biology. 2003; 74:1–36. [PubMed: 14510072]
17. Wolf JS, Chen Z, Dong G, Sunwoo JB, Bancroft CC, Capo DE, Yeh NT, Mukaida N, Van Waes C. IL (interleukin)-1alpha promotes nuclear factor-kappaB and AP-1-induced IL-8 expression, cell survival, and proliferation in head and neck squamous cell carcinomas. Clinical cancer research : an official journal of the American Association for Cancer Research. 2001; 7:1812–1820. [PubMed: 11410524]
18. Ohashi K, Naruto M, Nakaki T, Sano E. Identification of interleukin-8 converting enzyme as cathepsin L. Biochimica et biophysica acta. 2003; 1649:30–39. [PubMed: 12818188]
19. Barrett LW, Fletcher S, Wilton SD. Regulation of eukaryotic gene expression by the untranslated gene regions and other non-coding elements. Cellular and molecular life sciences : CMLS. 2012; 69:3613–3634. [PubMed: 22538991]
20. Conne B, Stutz A, Vassalli JD. The 3' untranslated region of messenger RNA: A molecular 'hotspot' for pathology? Nature medicine. 2000; 6:637–641.
21. Sandberg R, Neilson JR, Sarma A, Sharp PA, Burge CB. Proliferating cells express mRNAs with shortened 3' untranslated regions and fewer microRNA target sites. Science. 2008; 320:1643–1647. [PubMed: 18566288]
22. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature. 2010; 466:835–840. [PubMed: 20703300]
23. Al-Souhibani N, Al-Ahmadi W, Hesketh JE, Blackshear PJ, Khabar KS. The RNA-binding zinc-finger protein tristetraprolin regulates AU-rich mRNAs involved in breast cancer-related processes. Oncogene. 2010; 29:4205–4215. [PubMed: 20498646]
24. McDermott R, Gabikian P, Sarvaiya P, Ulasov I, Lesniak MS. MicroRNAs in brain metastases: big things come in small packages. J Mol Med (Berl). 2012
25. Djuranovic S, Nahvi A, Green R. miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. Science. 2012; 336:237–240. [PubMed: 22499947]
26. Braun F, Durand S, Condon C. Initiating ribosomes and a 5′/3′-UTR interaction control ribonuclease action to tightly couple B. subtilis hbs mRNA stability with translation. Nucleic acids research. 2017; 45:11386–11400. [PubMed: 28977557]
27. Mignone F, Gissi C, Liuni S, Pesole G. Untranslated regions of mRNAs. Genome biology. 2002; 3 REVIEWS0004.
28. Cairrao F, Halees AS, Khabar KS, Morello D, Vanzo N. AU-rich elements regulate Drosophila gene expression. Molecular and cellular biology. 2009; 29:2636–2643. [PubMed: 19273595]
29. Qiu C, Kershner A, Wang Y, Holley CP, Wilinski D, Keles S, Kimble J, Wickens M, Hall TM. Divergence of Pumilio/fem-3 mRNA binding factor (PUF) protein specificity through variations in

an RNA-binding pocket. The Journal of biological chemistry. 2012; 287:6949–6957. [PubMed: 22205700]

30. Arumugam K, Wang Y, Hardy LL, MacNicol MC, MacNicol AM. Enforcing temporal control of maternal mRNA translation during oocyte cell-cycle progression. The EMBO journal. 2010; 29:387–397. [PubMed: 19959990]
31. Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, Zeller KI, De Marzo AM, Van Eyk JE, Mendell JT, Dang CV. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature. 2009; 458:762–765. [PubMed: 19219026]
32. Kulkarni S, Savan R, Qi Y, Gao X, Yuki Y, Bass SE, Martin MP, Hunt P, Deeks SG, Telenti A, Pereyra F, Goldstein D, Wolinsky S, Walker B, Young HA, Carrington M. Differential microRNA regulation of HLA-C expression and its association with HIV control. Nature. 2011; 472:495–498. [PubMed: 21499264]
33. Winzen R, Thakur BK, Dittrich-Breiholz O, Shah M, Redich N, Dhamija S, Kracht M, Holtmann H. Functional analysis of KSRP interaction with the AU-rich element of interleukin-8 and identification of inflammatory mRNA targets. Molecular and cellular biology. 2007; 27:8388–8400. [PubMed: 17908789]
34. Numahata K, Komagata T, Hirasawa N, Someya K, Xiao YQ, Ohuchi K. Analysis of the mechanism regulating the stability of rat macrophage inflammatory protein-2 mRNA in RBL-2H3 cells. Journal of cellular biochemistry. 2003; 90:976–986. [PubMed: 14624457]
35. Hamilton TA, Novotny M, Datta S, Mandal P, Hartupee J, Tebo J, Li X. Chemokine and chemoattractant receptor expression: post-transcriptional regulation. Journal of leukocyte biology. 2007; 82:213–219. [PubMed: 17409125]
36. Zhao W, Pollack JL, Blagev DP, Zaitlen N, McManus MT, Erle DJ. Massively parallel functional annotation of 3' untranslated regions. Nature biotechnology. 2014; 32:387–391.
37. Wang X, McManus M. Lentivirus production. Journal of visualized experiments : JoVE. 2009
38. Yuan JS, Reed A, Chen F, Stewart CN Jr. Statistical analysis of real-time PCR data. BMC bioinformatics. 2006; 7:85. [PubMed: 16504059]
39. Bakheet T, Williams BR, Khabar KS. ARED 2.0: an update of AU-rich element mRNA database. Nucleic acids research. 2003; 31:421–423. [PubMed: 12520039]
40. Campbell ZT, Valley CT, Wickens M. A protein-RNA specificity code enables targeted activation of an endogenous human transcript. Nat Struct Mol Biol. 2014; 21:732–738. [PubMed: 24997599]
41. Galgano A, Forrer M, Jaskiewicz L, Kanitz A, Zavolan M, Gerber AP. Comparative analysis of mRNA targets for human PUF-family proteins suggests extensive interaction with the miRNA regulatory system. PLoS One. 2008; 3:e3164. [PubMed: 18776931]
42. Garcia DM, Baek D, Shin C, Bell GW, Grimson A, Bartel DP. Weak seed-pairing stability and high target-site abundance decrease the proficiency of lsy-6 and other microRNAs. Nature structural & molecular biology. 2011; 18:1139–1146.
43. Gurtan AM, Ravi A, Rahl PB, Bosson AD, Jnbaptiste CK, Bhutkar A, Whittaker CA, Young RA, Sharp PA. Let-7 represses Nr6a1 and a mid-gestation developmental program in adult fibroblasts. Genes & development. 2013; 27:941–954. [PubMed: 23630078]
44. Yamashita A, Takeuchi O. Translational control of mRNAs by 3'-Untranslated region binding proteins. BMB Rep. 2017; 50:194–200. [PubMed: 28287067]
45. Matoulkova E, Michalova E, Vojtesek B, Hrstka R. The role of the 3' untranslated region in post-transcriptional regulation of protein expression in mammalian cells. RNA biology. 2012; 9:563–576. [PubMed: 22614827]
46. Sivina M, Hartmann E, Kipps TJ, Rassenti L, Krupnik D, Lerner S, LaPushin R, Xiao L, Huang X, Werner L, Neuberg D, Kantarjian H, O'Brien S, Wierda WG, Keating MJ, Rosenwald A, Burger JA. CCL3 (MIP-1alpha) plasma levels and the risk for disease progression in chronic lymphocytic leukemia. Blood. 2011; 117:1662–1669. [PubMed: 21115978]
47. Kang JG, Amar MJ, Remaley AT, Kwon J, Blackshear PJ, Wang PY, Hwang PM. Zinc finger protein tristetraprolin interacts with CCL3 mRNA and regulates tissue inflammation. J Immunol. 2011; 187:2696–2701. [PubMed: 21784977]
48. Bazzini AA, Lee MT, Giraldez AJ. Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish. Science. 2012; 336:233–237. [PubMed: 22422859]

49. Wakiyama M, Takimoto K, Ohara O, Yokoyama S. Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. Genes & development. 2007; 21:1857–1862. [PubMed: 17671087]
50. Bakheet T, Williams BR, Khabar KS. ARED 3.0: the large and diverse AU-rich transcriptome. Nucleic acids research. 2006; 34:D111–114. [PubMed: 16381826]
51. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nature reviews. Genetics. 2010; 11:597–610.
52. He B, Tan K. Understanding transcriptional regulatory networks using computational models. Current opinion in genetics & development. 2016; 37:101–108. [PubMed: 26950762]
53. Shen ZJ, Malter JS. Regulation of AU-Rich Element RNA Binding Proteins by Phosphorylation and the Prolyl Isomerase Pin1. Biomolecules. 2015; 5:412–434. [PubMed: 25874604]
54. Zhao W, Siegel D, Biton A, Tonqueze OL, Zaitlen N, Ahituv N, Erle DJ. CRISPR-Cas9-mediated functional dissection of 3'-UTRs. Nucleic acids research. 2017; 45:10800–10810. [PubMed: 28985357]
55. Fan J, Heller NM, Gorospe M, Atasoy U, Stellato C. The role of post-transcriptional regulation in chemokine gene expression in inflammation and allergy. The European respiratory journal. 2005; 26:933–947. [PubMed: 16264057]

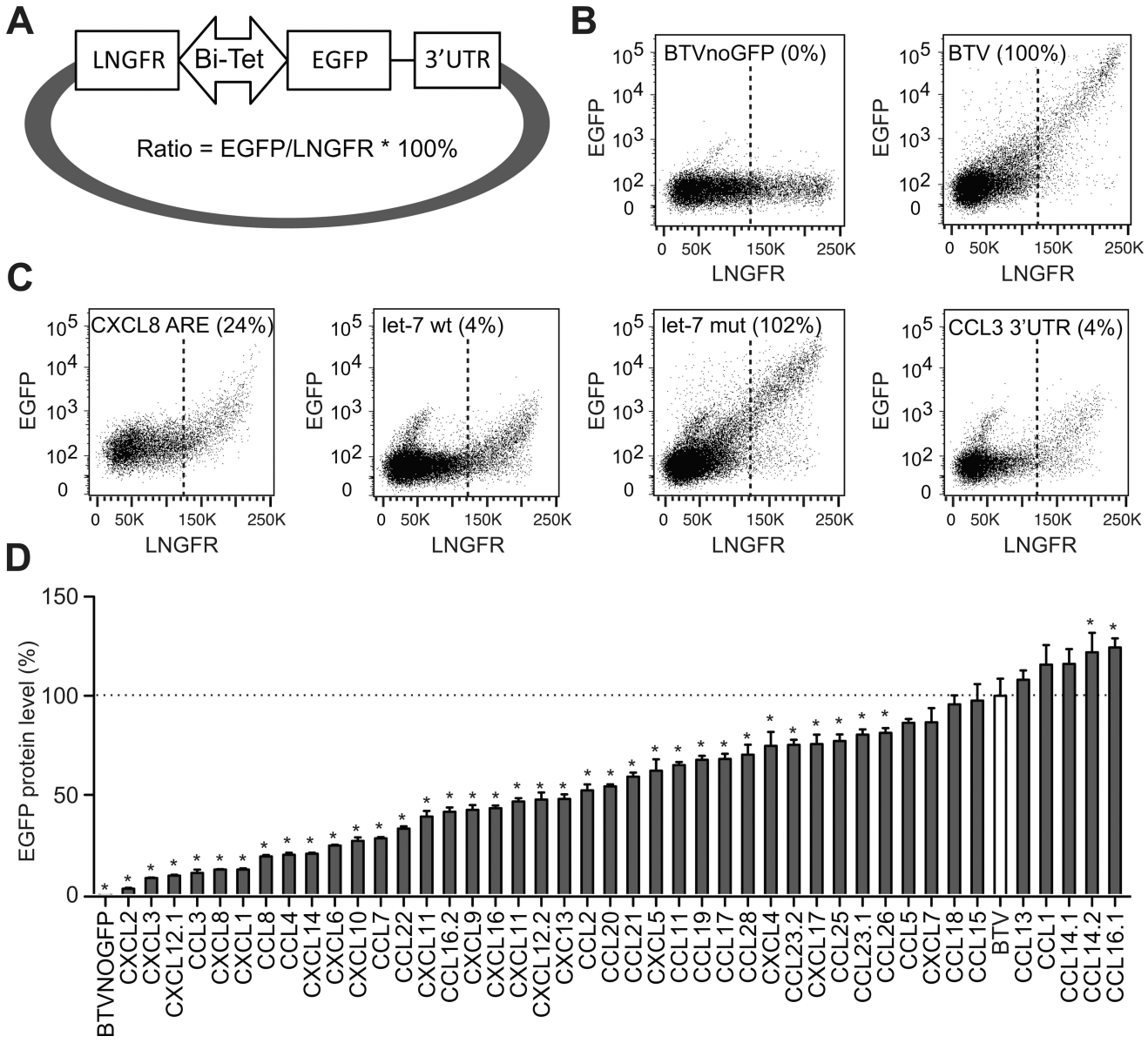

FIGURE 1.

Quantification of the effects of chemokine 3'-UTRs on *EGFP* protein production with a reporter assay. (**A**) BTV reporter. Bi-Tet: bi-directional tetracycline-regulated promoter driving both *EGFP* and *LNGFR* expression. The 3'-UTR test sequence was cloned downstream of the *EGFP* open reading frame. (**B**) Flow cytometric analysis of BEAS-2B cells transduced with a negative control without *EGFP* (BTVnoGFP) or BTV without a 3'-UTR test sequence. (**C**) Flow cytometric analysis of effects of 3'-UTR test sequences. *CXCL8* ARE: 160-nt ARE-containing sequence from *CXCL8* 3'-UTR; let-7 wt: synthetic let-7 target; let-7 mut: mutant let-7 target; *CCL3*: full length 3'-UTR of *CCL3* mRNA. The dotted lines show the threshold for gating of LNGFR positive cells used in the analysis. (**D**) Effects of 43 chemokine 3'-UTRs on *EGFP* protein expression in BEAS-2B cells. The suffixes ".1" and ".2" represent 3'-UTR variants arising from alternative polyadenylation; ".1" is the longer version. All values represent mean ± s.d.. *, $p < 0.05$.

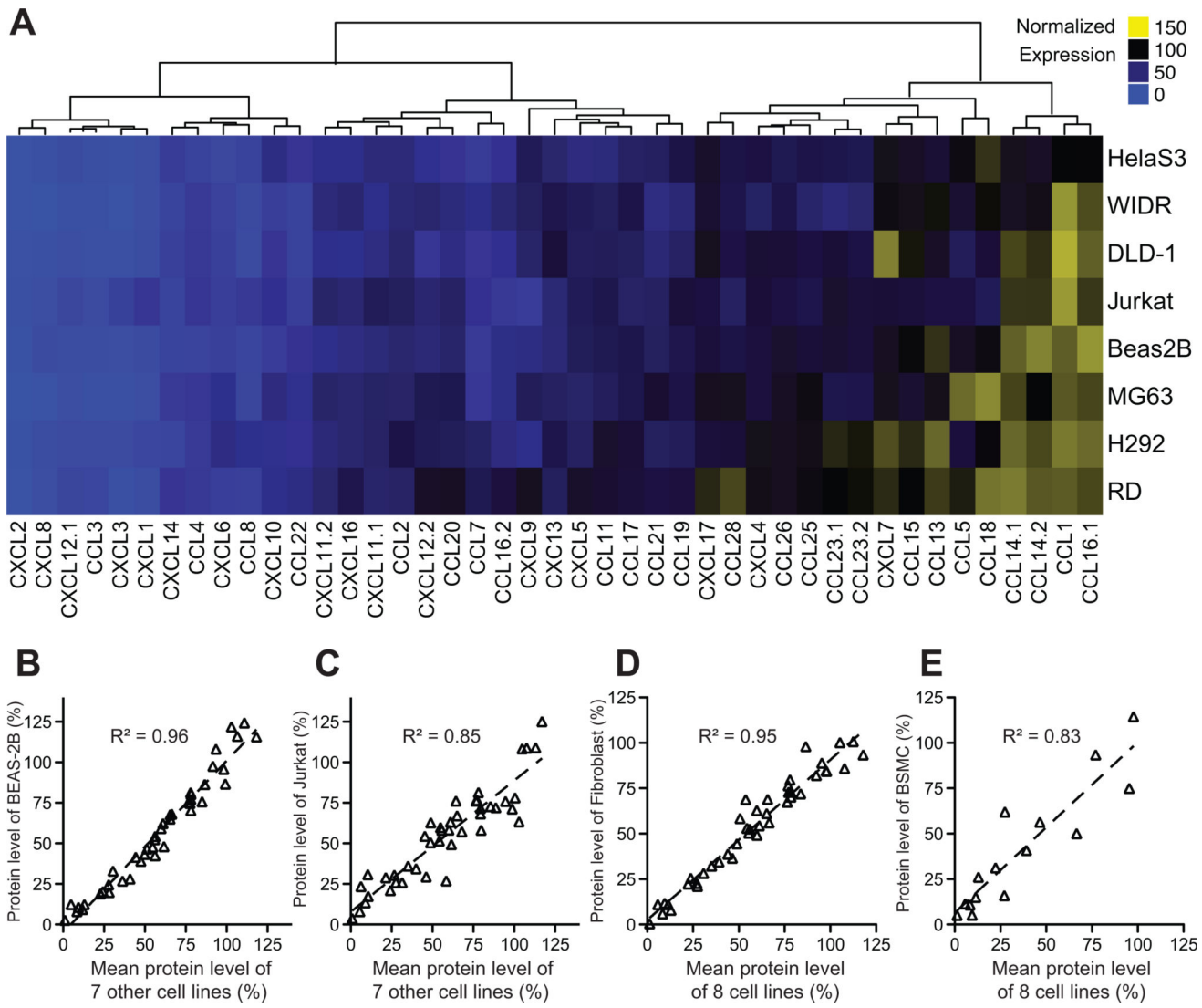

FIGURE 2.

Comparison of the effects of chemokine 3'-UTRs on *EGFP* protein in cell lines and primary cells. (**A**) Effects of chemokine 3'-UTRs in immortalized cell lines. Reporters were introduced into each of 8 cell lines: RD (rhabdomyosarcoma), MG-63 (osteosarcoma), DLD-1 (colon cancer), BEAS-2B (airway epithelial), Jurkat (T lymphoblast), WIDR (colorectal adenocarcinoma), HelaS3 (cervical carcinoma), and H292 (airway epithelial). The color scale represents reporter protein expression relative to the control reporter with no 3'-UTR test sequence (100%) in the same cell line. (**B, C**) Correlation of reporter protein levels in one cell type with mean reporter protein levels in the other seven cell lines. BEAS-2B cells (B) had the highest correlation and Jurkat cells (C) had the lowest. Each point represents one of the 43 chemokine 3'-UTRs tested. (**D, E**) Correlation of reporter protein levels in primary lung fibroblasts (D) or primary bronchial smooth muscle cells (E) with mean reporter protein levels in the eight cell lines. All 43 chemokine 3'-UTRs were analyzed in fibroblasts and a subset of 15 chemokine 3'-UTRs were analyzed in smooth muscle cells.

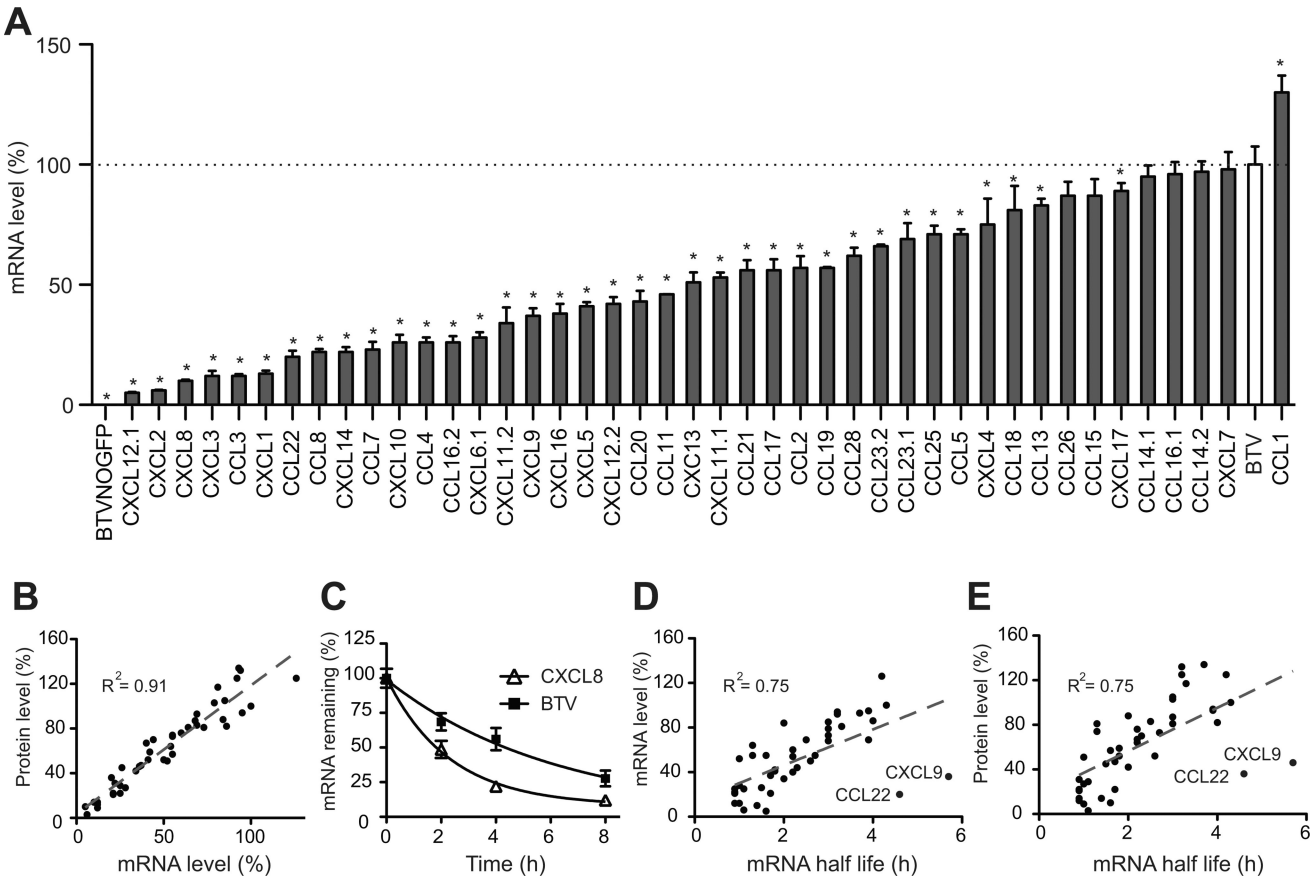

FIGURE 3.

Effects of chemokine 3'-UTRs on steady state mRNA levels and mRNA stability. (**A**) Quantification of the steady state *EGFP* mRNA in BEAS-2B cells with qRT-PCR. Results were normalized by comparing with BTV reporter with no 3'-UTR test sequence (100%). Values represent mean ± s.d.. *, $p < 0.05$. (**B**) Effects of chemokine 3'-UTRs on steady state *EGFP* reporter mRNA versus protein levels ($R^2 = 0.91$, $p < 0.0001$). (**C**) *CXCL8* 3'-UTR effects on mRNA degradation. (**D**) Effects of chemokine 3'-UTRs on reporter mRNA half-lives versus steady state mRNA levels ($R^2 = 0.75$, $p < 0.0001$). (**E**) Effects of chemokine 3'-UTRs on reporter mRNA half-lives versus protein levels ($R^2 = 0.75$, $p < 0.0001$). The dotted lines are linear regression lines. *CCL22* and *CXCL9* 3'-UTRs have lower levels of mRNA than other 3'-UTRs with similar mRNA half-lives.

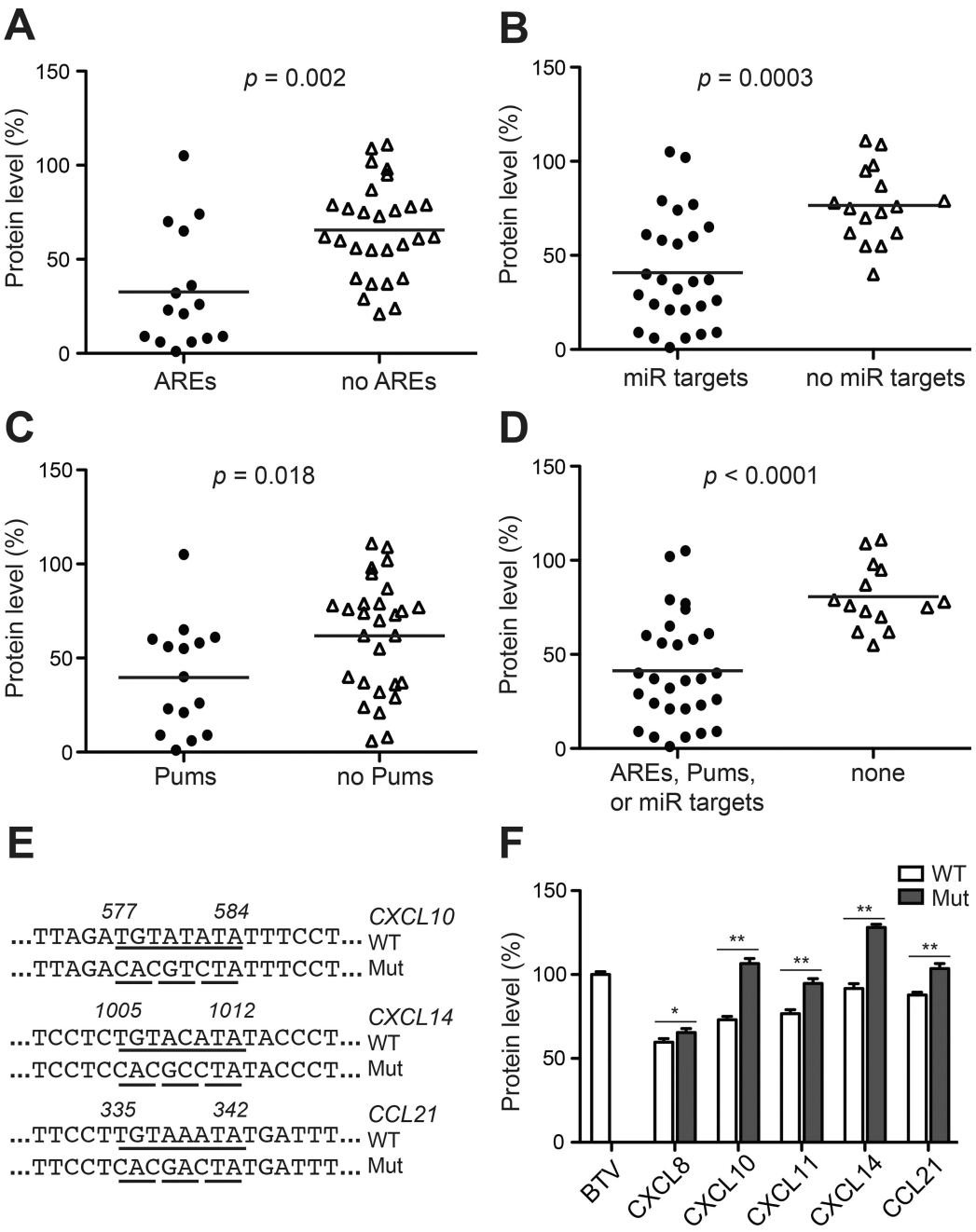

FIGURE 4.

Relationships between chemokine 3'-UTR activity and the presence of predicted cis-elements. Comparison of the effects chemokine 3'-UTRs containing or lacking AREs (**A**), miRNA targets (**B**), Pumilio binding sites (**C**) or any of these elements (**D**) on *EGFP* protein level in BEAS-2B cells. Each points represents one chemokine 3'-UTR; bars represent group medians. P values were calculated using the Mann-Whitney test. (**E**) Examples of three predicted Pumilio binding sites (WT) and mutations tested in the BTV reporter assay. Numbers denote motif positions within the 3'-UTR. (**F**) Effects of mutating predicted

Pumilio binding sites on reporter protein expression in BEAS-2B cells. *, $p < 0.05$; **, $p < 0.005$ by Student's t-test.

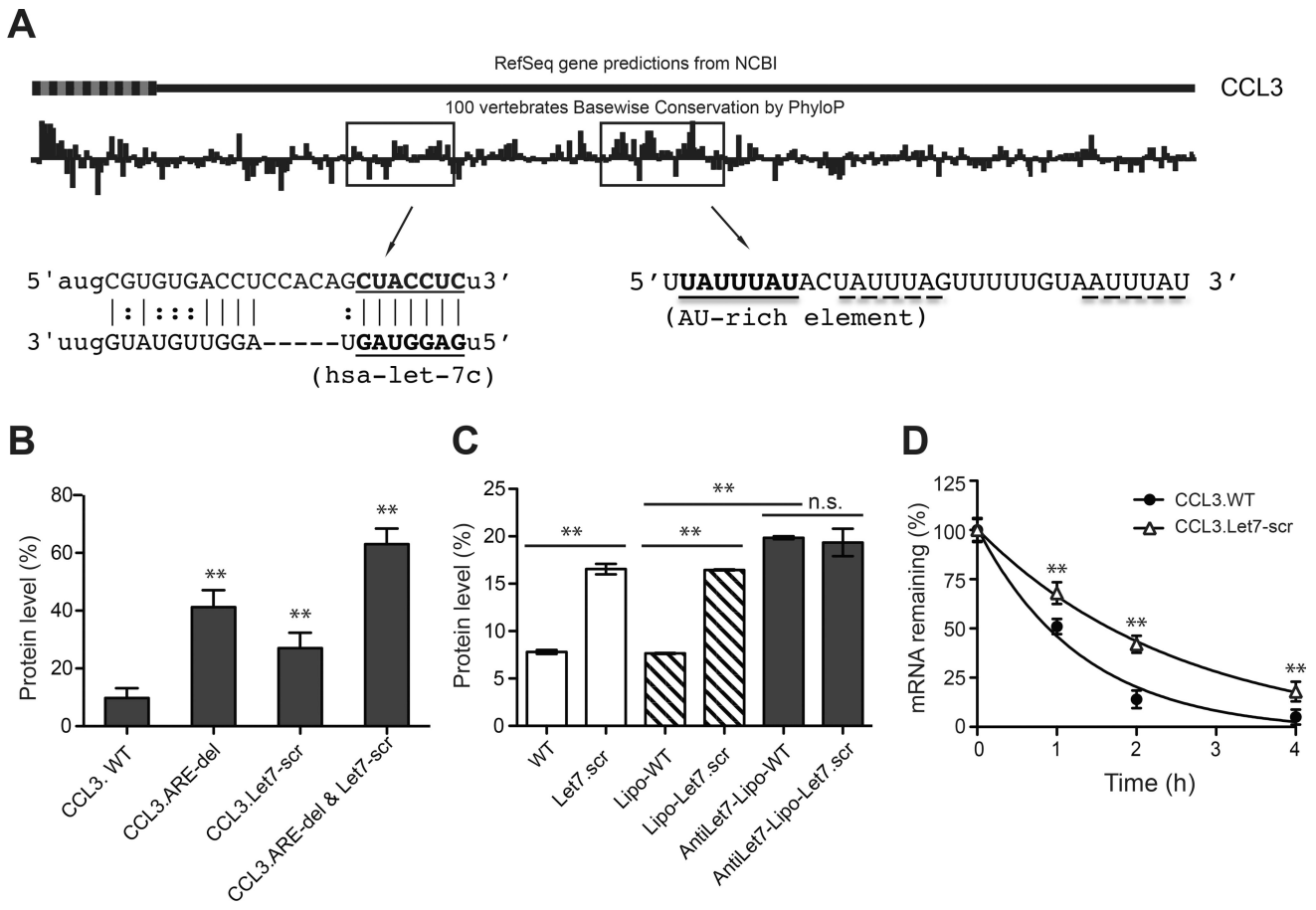

FIGURE 5.

A let-7 target and an ARE in the *CCL3* 3'-UTR co-regulate gene expression. (**A**) *CCL3* 3'-UTR contains a conserved let-7 target and an ARE. The vertical bars represent conservation scores from PhyloP. The let-7 target is predicted by TargetScan; the ARE (based on ARED) contains a typical ARE motif (solid underline) and two non-typical ARE motifs (broken underline). (**B**) Mutagenesis of the let-7 target and the ARE resulted in the reduction of inhibitory activity of *CCL3* 3'-UTR in BEAS-2B cells. WT: *CCL3* 3'-UTR; ARE-del, deletion of the ARE in *CCL3* 3'-UTR; Let7-scr, scrambled seed sequence binding site in *CCL3* 3'-UTR; ARE-del&Let7-scr: mutation of both the ARE and the let-7 target. (**C**) The let-7 inhibitor abrogated the inhibitory effect of the let-7 target in *CCL3* 3'-UTR. Lipo: negative control transfection with lipofectamine; AntiLet7, let-7 inhibitor. (**D**) Scrambling the let-7 target seed sequence in *CCL3* 3'-UTR stabilized mRNA. **, $p < 0.005$; ns, no significant difference by one-way ANOVA and Dunnett's test. All values represent mean ± s.d.