Number and Location of AUUUA Motifs: Role in Regulating Transiently Expressed RNAs

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Many RNAs coding for either cytokines or oncogenes are unstable and have a short half-life ($t_{1/2}$). The AUUUA motif is a highly conserved sequence and is repeated three or more times in the 3' untranslated region (3'UTR) of RNAs encoding many of these short-lived cytokines and oncogenes. These sequences can confer instability. In this study, we investigated the role of number and location of AUUUA motifs in stabilization of RNA. We introduced 1xATTATA, 2xATTATA, ATTATTATA (second adenosine of 2xATTATA was substituted with a thymidine), 3xATTATA, 5xATTATA, 7xATTATA (AT-rich sequence from granulocyte-macrophage colony-stimulating factor (GM-CSF) gene (AT-62)), and GC-62 (GC sequences were substituted for ATTTA sequences in the 3'UTR) into the 3'UTR of rabbit $\beta$-globin (R$\beta$G) gene. This construct also contained the neomycin-resistance gene. These expression vectors were transfected into human lung fibroblasts (W138), which constitutively expressed low levels of GM-CSF mRNA. Stable transfectants were selected by growth in G418. Northern blot analysis of actinomycin D-treated, stably transfected cells demonstrated that the number of AUUUA sequences correlated with rapidity of turnover of the chimeric R$\beta$G mRNA. The rank order of stability was GC-62 = 1xATTTA = 2xATTTA (no RNA decay at 4 hours) > 3xATTTA = 5xATTTA ($t_{1/2}$, 4 hours) > 7xATTTA ($t_{1/2}$, 2 hours). Stability of mRNA of R$\beta$G also was reduced ($t_{1/2}$, 2 to 4 hours) when AT-62 was introduced into the second exon of R$\beta$G gene. In these same cells, the $t_{1/2}$ of GM-CSF RNA was approximately 10 to 15 minutes, suggesting that the AUUUA motifs cannot alone account for the rapid degradation of this cytokine mRNA. Phorbol diesters, including 12-0-tetradecanoyl phorbol 13-acetate (TPA), stabilize a variety of transiently expressed RNAs, including GM-CSF RNA. We found that TPA markedly increased (>30-fold) the accumulation of GM-CSF RNA. In contrast, TPA was unable to stimulate the levels of the chimeric R$\beta$G when either 1x, 2x, 3x, or 5xATTATA motifs were fused to 3'UTR, or when either AT-62 or GC-62 control sequences were fused to the second exon. The chimeric $\beta$-globin construct with either AT-62 or ATTATTATA in the 3'UTR had only an approximately twofold to threefold increase in accumulation. Taken together, these findings show (1) the number of AUUUA cassettes in the 3'UTR may be one of the major determinants in the rate of turnover of GM-CSF mRNA; (2) the AU-rich region appears to act independent of adjacent sequences, and the AUUUA sequences when placed in an exon can still modulate stability of RNAs; and (3) although the AUUUA cassettes have a role in mRNA degradation, they cannot alone be the cis-sequences that mediate GM-CSF RNA accumulation induced by TPA. Additional sequences of GM-CSF RNA may be important in modulating accumulation of GM-CSF transcripts in TPA-stimulated cells.

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E XPRESSION of cytokines and oncogenes is finely controlled; they are usually rapidly expressed and degraded. In addition to transcriptional and translational regulation, modulation of mRNA turnover is an important determinant of gene expression by altering the amount of translatable mRNA.

Myeloid hematopoietic cells are short-lived and require continuous self-renewal. Proliferation of hematopoietic cells depends on the presence of growth factors. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a 23-kD glycoprotein that regulates the growth and differentiation of a wide range of myeloid progenitor cells. Transcriptional regulation of GM-CSF gene is important; this has been clearly shown in T lymphocytes. Previously, we have found that human normal fibroblasts constitutively transcribe GM-CSF; however, because of the short-life of this RNA, it does not accumulate in the cells. A number of inducers of GM-CSF accumulation in fibroblasts, including tumor necrosis factor (TNF), are stimulators of protein kinase C. These inducers have little effect on rates of transcription of GM-CSF, but have a major influence on stability of these transcripts. The half-life ($t_{1/2}$) of the mRNA of GM-CSF is shorter than 30 minutes in fibroblasts, and both TNF and 12-O-tetradecanoyl phorbol 13-acetate (TPA; a potent stimulator of protein kinase C) markedly increase the $t_{1/2}$ of GM-CSF RNA in these cells.

The motif AUUUA is a highly conserved sequence that is often multiply repeated in the 3' untranslated regions (3'UTRs) of RNAs encoding short-lived cytokines and proto-oncogenes. These AUUUA sequences are thought to confer instability to mRNA. The UTR of GM-CSF RNA has eight AUUUA motifs. When the AU-rich region of GM-CSF was placed in the 3'UTR of the rabbit $\beta$-globin (R$\beta$G), it resulted in rapid degradation of this chimeric transcript.

In this study, we asked three questions: (1) How many repeats of AUUUA sequences are required for destabilization of RNA? (2) Do these sequences have to be located in 3'UTR? and (3) Do the number of AUUUA motifs in a transcript influence the accumulation of the transcript after the cells are exposed to TPA? To answer these questions, expression vectors were constructed that had various numbers of ATTATA motifs either in the 3'UTR, second exon, or second intron of the R$\beta$G gene. These vectors also contained the neo gene under control of a separate promoter. They
were stably transfected into human lung fibroblasts. Stability of RNAs coding for the chimeric RβG, as well as GM-CSF, was determined, and the effect of TPA on accumulation of these RNAs was analyzed.

MATERIALS AND METHODS

Cells and cell culture. Normal human embryonic lung fibroblasts (WI38) were obtained from American Type Tissue Culture Collection, Rockville, MD. Cells were cultured in alpha medium (Cosmo, Tokyo, Japan) supplemented with 7% fetal calf serum (FCS; Mitsubishi Kasei, Tokyo, Japan) in a humidified atmosphere containing 5% CO₂. Cells were washed with phosphate-buffered saline (PBS) without either calcium or magnesium and harvested by treatment with 0.05% trypsin, 0.02% EDTA (wt/vol, Gibco Laboratories, Grand Island, NY).

Plasmid constructions. This series of constructs was synthesized using complementary synthetic oligonucleotides. AT1x, AT2x, AT3x, or AT5x have one, two, three, or five repeats of ATTATA sequences, respectively. AT62 has seven repeats of ATTATA sequences from the AT-rich region of human GM-CSF. In GC62, G and C deoxynucleotides were substituted with A and T deoxynucleotides to disrupt the seven ATTATA motifs of AT62. In the case of the second exon of genomic RβG gene. These reporter genes were inserted into SV2NEO DNA. Coding regions (B); intervening sequences (IVS; □); flanking sequences (■ in front of globin gene, and SV40 early promoter (SV40EP) in front of neo gene). Polyoma virus early promoter (PyEP) in front of globin gene, and SV40 early protein (SV40EP) in front of neo gene are shown with transcriptional start sites indicated by arrows.

annealed oligonucleotides were ligated with gel-purified, BamHI-linearized, RβG-containing plasmid DNA (Fig 1B). In the case of intron 2 (IVS2) series, the annealed oligonucleotides were treated with Klenow and dNTPs to fill in the 5' GATC protruding termini. The blunted oligonucleotides were then ligated with RβG-containing plasmid DNA, linearized with ScaI, and gel-purified (Fig 1B). Oligonucleotides, 20 mer, spanning the junction sites were used as probes in colony hybridization assays to identify plasmids with correct oligonucleotide insertions and orientations. The β-globin promotor was replaced by polyoma virus early promotor. These constructs were subcloned into pSV2NEO as previously described (Fig 1B). DNA transfection and selection of transformed cells. For each experiment, 10 μg of plasmid was introduced into 5 × 10⁵ WI38 fibroblasts by electroporation (Gene Pulser, 700V, 25 μF, Bio Rad, Hercules, CA). Twenty-four hours after exposure to DNA, the culture medium was changed, and G418 (0.5 mg/mL; Gibco) was added. This concentration prevented growth of WI38 cells that were not expressing the neo gene. Multiple, resistant colonies were trypsinized, respread, and cultured with G418. Flasks containing confluent cells were used for RNA extraction.

DNA probes. RβG DNA (0.495 kb, Xhol-BamHI) was from a RβG construct. Human GM-CSF (0.9 kb, EcoRI-BamHI) was derived from plasmid pcSF-1, a generous gift of S. Clark, Genetics Institute, Cambridge, MA. For detection of SV2NEO transcripts, fragment from pSV2NEO (BglII-SohI, 0.7 kb) was used. These probes were [32P]-labeled by random priming. The specific activities were 5 to 8 × 10⁶ cpm/μg.

RNA preparation and analysis. Total cytoplasmic RNA was prepared by the method of Clemens. WI38 cells were suspended in hypotonic buffer containing 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L KCl, and 3 mmol/L MgCl₂ and were lysed with 0.3% nonidet P-40. Cytoplasmic RNA was extracted by phenol/chloroform. After denaturation at 65°C, RNA was electrophoresed in a agarose-formaldehyde gel (1%) and transferred to a nylon-membrane filter (Hybond). Hybridization with labeled probe was for 16 to 24 hours at 42°C in 50% formamide, 2X SSC (1 × SSC = 150 mmol/L NaCl, 15 mmol/L sodium citrate), 5X Denhardt’s, 0.1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 100 μg/mL salmon sperm DNA. Filters were washed to a stringency of 0.1 × SSC, 65°C, and exposed to Kodak XAR film (Rochester, NY). Autoradiograms were developed at different exposures.

To stop the ongoing transcription, the stably transfected cells were treated for 0.5 to 4 hours with 5 μg/mL of actinomycin D. Cells were sequentially harvested and examined for levels of RNAs. Moderation in levels of RβG mRNA were quantified by initial standardization to the amount of NE0 transcripts. The relative density of neo and RβG transcripts in the different lanes was determined by laser densitometry (LKB Ultra Scan XL, Pharmacia, Piscataway, NJ) using multiple exposures of the blot, and the ratio of globin to neo in the zero time was assigned to be 100%.

Each experiment had several internal controls: (1) ethidium bromide gels confirmed that an equal amount of RNA was applied to each lane; (2) Northern blots were hybridized with 32P-probe for neo to confirm that each stable transfected contain an equivalent amount of plasmid; (3) Northern blots were hybridized with 32P-probe for GM-CSF to confirm that cells expressed GM-CSF RNA, that actinomin D was effective, and that TPA enhanced accumulation of GM-CSF RNA.

RESULTS

Stability of chimeric RβG RNA containing different numbers of AUUUA sequences in 3' UTR. GM-CSF RNA has eight repeats of AUUUA sequences. How many repeats of AUUUA sequences can destabilize a transcript? We compared in transfected fibroblasts, the stability of chimeric RβG
RNAs containing different numbers of AUUUA motifs in the 3'UTR (Fig 2A). No significant decrease of stability of the RβG RNA having either one (AT1x) or two (AT2x) AUUUA repeats was observed, when compared with the globin construct containing the GC motif (GC62) (Fig 2B). In contrast, three or more repeats of AUUUA motif, or the AUUUUUAUUUA (AT2xt) cassette, reduced stability of these RNAs (t1/2, ~4 hours). These chimeric RNAs (AT3X, AT5X) were more stable than the chimeric transcripts containing the AT62 (seven AUUUA motifs; t1/2, ~2 hours, data not shown, but reported previously in another study).10 The t1/2 of GM-CSF RNA in each transfectant was approximately 30 minutes (Fig 2A and B).

Effect of location of AUUUA sequences on stability of RβG RNA. The AU-rich sequences are located in 3'UTR of transiently expressed RNAs coding for many cytokines and oncogenes.7 We placed either the AT62 or GC62 motif in the second exon (EX2.AT62; EX2.GC62) of RβG expression vector and transfected the chimeric gene into fibroblasts (Fig 3A and B). In the EX2.AT62, the stability of RβG RNA was reduced (t1/2, ~4 hours) as compared with RβG RNA containing GC62 (no loss in stability even at 4 hours). The stability of RβG RNA was also decreased when the AU-rich region of GM-CSF RNA was placed in the second intron of RβG (1VS2.AT2; Fig 3B). The t1/2 of GM-CSF RNA in each transfectant was approximately 30 minutes.

Effect of TPA on accumulation of GM-CSF RNA and chimeric RβG RNA containing different numbers and different locations of AUUUA sequences. We previously showed that TPA, a potent diester phorbol, markedly increased the accumulation of GM-CSF RNA and that this occurred predominantly by an increased stabilization of these transcripts.5 Additional studies showed that TPA had little effect on the rate of transcription of either GM-CSF or chimeric RβG AT62 genes.1,6 Our stably transfected fibroblasts were exposed to TPA (50 nmol/L) for 2 hours (maximal GM-CSF accumulation, data not shown); samples were harvested and examined for levels of chimeric RβG and GM-CSF RNAs (Fig 4). Levels of GM-CSF transcripts markedly increased (>30-fold) after exposure of each stable transfectant to TPA. In contrast, levels of chimeric globin RNA increased only about twofold to threefold in cells having either AT62 or the AUUUUUAUUUA sequences, and did not increase at all in fibroblasts containing any of the other constructs (Fig 4).
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Fig 3. Effect of location of AUUUA sequences on stability of RβG RNA. (A) AT62 or GC62 motifs were inserted in the second exon of RβG and the chimeric genes were stably transfected into fibroblasts and Northern analysis performed as described in Fig 2 and the Methods. (B) Stability of steady-state of RβG RNA was determined as described in Fig 2 and Materials and Methods.

DISCUSSION

Mesenchymal cells, including fibroblasts and endothelial and smooth muscle cells, constitutively transcribe RNAs coding for hematopoietic growth factors, including GM-CSF; these RNAs, as well as oncogene RNAs, usually have a short $t_{1/2}$, allowing rapid modulation of levels of their protein. Although expression of these genes is controlled at both transcriptional and posttranscriptional levels, modulation of mRNA turnover is also an important mechanism in mesenchymal cells to influence production of cytokines by altering the amount of translatable mRNA.2 Production of CSFs by monocytes16-18 and lymphocytes8,19 also is reliant on regulation of stability of the transcripts.

The labile mRNAs of many transiently expressed cytokines and oncogenes possess several repeats of the pentamer, adenosine-uridine-uridine-uridine-adenosine (-AUUUA-), in 3'UTR.24 Addition of these sequences can reduce stability of the otherwise stable rabbit globin RNA8,10, removal of these sequences can markedly increase stability of ephemeral RNAs.10-23 Thus, stability of RNAs is in part conferred in cis by AUUUA sequences. Gel retardation studies suggest that several proteins can bind RNA having these sequences.24,25 Further studies have implicated the importance of either ribosomal binding or ongoing translation for selective degradation of AUUUA-containing transcripts.20-24 In this study, we investigated the role of the number and location of AUUUA sequences on stability of RNA. Transcripts from chimeric RβG gene having seven repeats of AUUUA sequences (AT62) had a short $t_{1/2}$ (2 hours, data not shown, similar data reported by us previously19) as compared with the extremely stable transcripts from the RβG chimeric gene that had GC-rich sequences (GC62, no decrease in transcripts at 4 hours). These results are consistent with our previous data, suggesting that the AUUUA sequences are focal points for destabilization of GM-CSF RNA.8,10 In this study, we have shown an inverse correlation between the number of AUUUA motifs in the chimeric RNA and the stability of the transcripts. The rank order of the ability to shorten the $t_{1/2}$ of the chimeric transcripts was $7 > 5 > 3 > 2 = 1$ cassettes of AUUUA = GC-62 (AUs mutated to GCs). Furthermore, insertion of AUUUA sequences (AT-62) in the second exon of RβG gene also destabilized its transcripts ($t_{1/2}$, 4 hours).

Our experiments suggest that destabilization of the RNA is catalyzed in cells by a pathway that can recognize specific sequences containing either three or more repeats of the AUUUA motif. Consistent with our functional studies, recent investigations by others, as well as by ourselves, have identified cellular protein(s) that can bind and form a complex with a RNA having three or more repeats of AUUUA, but cannot bind a transcript having a single AUUUA motif.24,25,27,29,30 In addition, our study suggests that the AUUU-rich region alone does not account for the short $t_{1/2}$ of GM-CSF RNA. The $t_{1/2}$ of this transcript is approximately 20 to 25 minutes, but in the same cells, RβG-AT62 RNA containing the AUUUA-rich region of GM-CSF has a $t_{1/2}$ of approximately 2 hours.

We cannot rule out that the $t_{1/2}$ of EX2AT62 and EX2GC62 RNA may have been influenced by a change in reading frame, which could effect translation of the RNA. Prior studies have shown that selective degradation of RNA containing the AUUUA motif is coupled to either ribosomal binding or ongoing translation of the mRNA.20 Nevertheless, our experiments were internally controlled and the EX2AT62 RNA was more rapidly degraded than the EX2GC62 RNA.

We also explored whether mutation of the middle adenosine of AUUUAUUAU to a uridine (AUUUAUUUA) would alter the stability of the chimeric RNA. This substitution reduced the stability of the RNA so that it behaved similar to RβG-AT5X and -AT3X ($t_{1/2}$, 4 hours) as compared with the chimeric RNA containing two repeats of AUUUA sequence, which had no decline in the level of transcripts at 4 hours. Preliminary cross-linking studies by us have shown
that both a 41- and a 39-kD nuclear and cytoplasmic protein binds to AUUUUUUUAA as avidly as RNA transcribed from AT5X and AT62. Recently, a gene coding for a protein of approximately 37 kD (AUFI) has been cloned; this protein appears to bind to the AU motif of transiently expressed genes. However, the mechanism(s) by which this or additional proteins might influence degradation of AU-rich mRNA turnover requires further study. Our studies with AT2Xt suggest the importance of a long stretch of Us for mediating AU-rich-directed mRNA degradation.

The role of AUUUA sequences in accumulation of RNA in cells exposure to TPA is unclear. Previously, we found that stimulators of protein kinase C, including TPA, markedly stabilized GM-CSF RNA in mesenchymal cells. Besides GM-CSF, many other studies showed that posttranscriptional stabilization is an important mechanism for accumulation of labile RNAs in TPA-treated cells, for example, interleukin-3 mRNA in a mast cell line, c-fos mRNA in a monocyte cell line, and interleukin-6 mRNA in fibroblasts. Factor(s) that bind AUUUA sequences have been identified in cells after they are activated with agents such as either TPA or a calcium-ionophore. Therefore, TPA might potentially activate a factor to bind and perhaps stabilize the RNAs having numerous AUUUUA cassettes or a long sequence of uridines in 3'UTR. This study showed that even though TPA markedly increased accumulation of GM-CSF RNA (>30-fold), it only slightly increased the levels of the chimeric transcripts of R/βG, which contain seven repeats of AUUUUA sequences within the 3'UTR. This study showed that even though TPA markedly increased accumulation of GM-CSF RNA (>30-fold), it only slightly increased the levels of the chimeric transcripts of R/βG, which contain seven repeats of AUUUUA sequences within the 3'UTR. This study showed that even though TPA markedly increased accumulation of GM-CSF RNA (>30-fold), it only slightly increased the levels of the chimeric transcripts of R/βG, which contain seven repeats of AUUUUA sequences within the 3'UTR. This study showed that even though TPA markedly increased accumulation of GM-CSF RNA (>30-fold), it only slightly increased the levels of the chimeric transcripts of R/βG, which contain seven repeats of AUUUUA sequences within the 3'UTR. This study showed that even though TPA markedly increased accumulation of GM-CSF RNA (>30-fold), it only slightly increased the levels of the chimeric transcripts of R/βG, which contain seven repeats of AUUUUA sequences within the 3'UTR. This study showed that even though TPA markedly increased accumulation of GM-CSF RNA (>30-fold), it only slightly increased the levels of the chimeric transcripts of R/βG, which contain seven repeats of AUUUUA sequences within the 3'UTR.

Other experiments using stable transfection in transformed cells, transient assays, and RNA-gel retardation studies suggested that the AU sequences may not be the target of stabilization of GM-CSF RNA that occurs when cells are exposed to TPA. In addition, we have shown that TNF profoundly stabilized GM-CSF RNA, but had no effect on the stability of R/βG-AT62. The data suggest that although the AT62 region of GM-CSF with its seven AUUUUA motifs is important for controlling mRNA degradation, these sequences are not a sufficient target for modulating the accumulation of GM-CSF transcripts in activated cells. Further studies are required to understand what regions of GM-CSF RNA and what binding proteins are important in stabilization and accumulation of these transcripts. Our data suggest that the AUUUUA sequences alone do not play a major role in this stabilization.

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