Post-transcriptional Regulation of H-ferritin mRNA

IDENTIFICATION OF A PYRIMIDINE-RICH SEQUENCE IN THE 3'-UNTRANSLATED REGION ASSOCIATED WITH MESSAGE STABILITY IN HUMAN MONOCYTIC THP-1 CELLS

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We have previously demonstrated that phorbol myristate acetate (PMA) up-regulates H-ferritin gene expression in myeloid cells by stabilization of its message. In the present report, we showed that insertion of the 3'-untranslated region (3'-UTR) of H-ferritin mRNA at the 3'-end of luciferase coding sequence significantly reduced the stability of luciferase mRNA in human monocytic THP-1 cells. However, the half-life of the chimeric transcript was markedly prolonged after PMA treatment. A cytosolic protein factor from THP-1 cells was found to specifically bind to H-ferritin 3'-UTR. PMA treatment of THP-1 cells resulted in the reduction of the RNA binding activity in a time-dependent manner. Deletion analysis and RNase T1 mapping revealed a pyrimidine-rich sequence within the 3'-UTR which interacts with the protein factor. Competition experiments with homoribopolymers further demonstrated the importance of uridines for the binding activity. Point mutations in uridines of the pyrimidine-rich sequence reduced the protein binding to 3'-UTR, while increasing the stability of the chimeric luciferase transcript. Together, these results demonstrate that the pyrimidine-rich sequence in the 3'-UTR is involved in post-transcriptional regulation of H-ferritin gene expression in myeloid cells.

Ferritin is a multimeric protein with a function in controlling the iron homeostasis in cells (1, 2). In mammals, each ferritin molecule consists of 24 subunits of two types, heavy (H) and light (L), which are derived from distinct genes and share only about 50% homology in amino acid sequences. It is generally believed that H-ferritin, which contains a ferroxidase activity (3), plays a key role in the intracellular flux of iron, whereas L-ferritin is primarily responsible for the iron storage. H- and L-ferritin combine in variable ratios in different cells or tissues depending on the cellular requirement for iron as well as the differentiation or pathological states. The synthesis of H- and L-ferritin can be regulated by iron at the translational level (1, 2). When the intracellular iron concentration is low, a repressor protein would bind to a conserved iron regulatory element located in the 5'-untranslated regions of H- and L-ferritin mRNAs to inhibit the translation of both genes (4–6). Increased iron concentration will lead to the dissociation of the repressor from the iron regulatory element and result in the increase of ferritin synthesis. In addition, early studies have demonstrated that L- and H-ferritin genes are subjected to differential regulation during development, cellular differentiation, or inflammation, although the molecular mechanisms are not fully resolved (7–13). It has been shown that differentiation of human leukemia cells leads to an increase in the H/L ratio of ferritin expression (14, 15). Using the human monocytic THP-1 cell line as a model system, we have demonstrated that phorbol myristate acetate (PMA)-induced differentiation of THP-1 cells toward macrophages markedly up-regulates the expression of H-ferritin mRNA, but not L-ferritin mRNA, in a cell-type specific manner (16). Furthermore, the gene induction appears to be the result of stabilization of the H-ferritin transcript (16). Since the stability of most mRNAs has been shown to be regulated by sequences in their 3'-UTRs (17), we hypothesized that an unique cis-regulatory element within the 3'-untranslated region (3'-UTR) of H-ferritin mRNA is likely involved in regulating the stability of the H-ferritin message in PMA-treated THP-1 cells.

In the present study, we identified a pyrimidine-rich sequence within the 3'-UTR of the H-ferritin mRNA to be associated with the message turnover in THP-1 cells. Gel-mobility shift assay demonstrated the existence of a RNA-binding protein in the cytosolic fraction of THP-1 cells to interact with this sequence. PMA treatment down-regulated the binding of the protein factor to H-ferritin 3'-UTR, suggesting that the interaction between the cytosolic protein and the cis element serves as a destabilizing signal to facilitate the degradation of H-ferritin mRNA in THP-1 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Human monocytic THP-1 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum. HeLa and hepatoma PLC cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were incubated in a humidified atmosphere of 95% air, 5% CO2 at 37 °C. For PMA treatment, THP-1 cells were grown to a density of 1 × 106 cells/ml and treated with 100 ng/ml PMA for indicated times.

Plasmid Constructs—A cDNA fragment spanning the full-length 3'-UTR of the H-ferritin gene (nucleotides -1 to +150, +1 is the first nucleotide after the stop codon) was obtained by polymerase chain reaction (PCR) using the human H-ferritin cDNA as the template (18). EcoRI and HindIII restriction sites were created in sense and antisense primers, respectively. The sequences of the sense and antisense oligonucleotides are as follows: sense, 5'-GACAGTGATAAATCTCTTCA-3'; and antisense, 5'-GGCCAAGCTTCTTTATTTT-3'. The PCR fragment was subcloned into the EcoRI-HindIII site of the pGEM11Zf+ vector (Promega). To prepare the constructs containing the mutated nucleotides within the sequence located at nucleotides +72 to +88 of the 3'-UTR, the sense primers containing mutated nucleotides and the SpI restriction site at the 5'-end were used for PCR. The sequences of these oligonucleotides are as follows: M1, 5'-GACAGTGATAAATCTCTTCA-3';
expression vector, pcDNA3, to generate pcDNA3-Luc. The cDNA fragment of wild type or mutant 3'-UTR with XhoI and Apal restriction sites created at the 5' and 3' ends, respectively, was prepared by PCR and subcloned into the XhoI-Apal site of pcDNA3-Luc to generate wild type or mutant pLuc-HF-3'UTR.

**Transient Transfection and RT-PCR**—THP-1 cells (2 × 10⁴) were transfected with 2 μg of pcDNA3-Luc or pLuc-HF-3'UTR plasmid DNA using Effectene reagent (Qiagen) according to the manufacturer's instructions. After incubation in culture medium for 24 h, cells were divided into 4 dishes and each dish was treated with 10 μg/ml actinomycin D for indicated times. Total RNA was then isolated and the expression level of luciferase mRNA was quantified by RT-PCR. Briefly, 1 μg of total RNA was reverse transcribed into cDNA by incubation with 10 units of RNasin, 0.2 mM hexamers, and 0.8 mM dNTPs at 37 °C for 1 h. The reaction was terminated by heating at 95 °C for 10 min and the mixture was diluted to 500 μl with deionized H₂O. An aliquot (2.5 μl) was used as a negative control for the binding reaction. The complex formed is indicated by an arrow. BSA, bovine serum albumin.

**Preparation of Cytosolic Extracts and Gel Mobility Shift Assay**—Cells were harvested by centrifugation at 200 × g for 5 min, washed twice with ice-cold phosphate-buffered saline, and centrifuged again. Cell pellets were resuspended in ice-cold hypotonic buffer containing 25 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 0.2% Nonidet P-40. After placing on ice for 5 min, cell lysate was subjected to centrifugation at 20,000 × g for 30 min at 4 °C. The supernatant (cytosolic fraction) was removed and stored at −70 °C. The protein concentration was determined by a Bio-Rad protein assay. To prepare the radiolabeled transl-
In vitro transcription was performed with T7 polymerase in a reaction containing 0.4 mM unlabeled ATP, GTP, CTP, 0.04 mM \(^{32}\)P-UTP, and 30 \(\mu\)Ci of \(\alpha\)-\(^{32}\)P-UTP (Amersham Pharmacia Biotech, >3000 Ci/mmol). The \(^{32}\)P-labeled transcript was filtered through a TE-100 Chroma spin column (CLONTECH) to remove free \(^{32}\)P-UTP. For the RNA binding reaction, 10–40 \(\mu\)g of cytosolic proteins were incubated with \(^{32}\)P-labeled transcript (30,000–50,000 cpm) in 20 \(\mu\)l of 15 mM HEPES, pH 7.4, containing 1 mM EDTA, 50 mM KCl, 3 mM MgCl\(_2\), 10% glycerol, and 10 \(\mu\)g/ml yeast tRNA for 30 min at room temperature. Twenty units of RNase T1 were added and incubation continued for an additional 30 min, followed by addition of heparin to a final concentration of 2 mg/ml for an additional 10 min. Samples were then subjected to electrophoresis performed on a 6% native polyacrylamide gel using 0.25 \(\times\) TBE as electrophoresis buffer. Gels were dried and exposed to Kodak X-Omat AR films overnight at \(-70^\circ\)C.

**UV Cross-linking**—Following electrophoresis of the binding reaction, gel was exposed to x-ray film at 4 \(^\circ\)C overnight. The region of specific RNA-protein complex was identified and excised. The protected RNA fragment was eluted from gels by electrophoresis through a NA45 membrane and then recovered from membrane by extraction with high salt buffer. After ethanol precipitation, the recovered RNA was digested with or without RNase T1 (20 units) at 37 \(^\circ\)C for 30 min. The \(^{32}\)P-labeled 3'-UTR transcript was also subjected to RNase T1 digestion. The digested products were analyzed by a 12\% polyacrylamide, 8 M urea sequencing gel.

**RESULTS**

To explore the possibility that the 3'-UTR of H-ferritin mRNA contains a regulatory element affecting mRNA stability, a chimeric luciferase reporter gene construct carrying the entire H-ferritin 3'-UTR at the 3'-end of the luciferase coding sequence was prepared. After transient transfection into THP-1 cells, the expression of chimeric luciferase mRNA was assessed by semiquantitative RT-PCR. As shown in Fig. 1, insertion of H-ferritin 3'-UTR led to the decrease in the half-life of luciferase mRNA from 8.0 \(\pm\) 0.5 to 4.5 \(\pm\) 0.3 h as measured in the presence of actinomycin D. PMA treatment markedly prolonged the half-life of the chimeric transcript to greater than 9 h. In contrast, the half-life of the parental transcript was not significantly altered by PMA treatment. This result supports the idea that the H-ferritin 3'-UTR contains a regulatory sequence mediating the effect of PMA. When the \(^{32}\)P-labeled transcript of H-ferritin 3'-UTR was incubated with the cytosolic extracts prepared from monocytic THP-1 cells, followed by digestion with RNase T1, and analyzed by a native polyacrylamide gel, a RNase-resistant band was observed (Fig. 2). This band was abolished by the addition of proteinase K or SDS in the binding reaction, indicating that it was formed by the interaction of a protein factor with the radiolabeled RNA probe. The RNA binding activity was proportional to the increments of the cytosolic proteins (Fig. 3A). When the radiolabeled antisense RNA probe was used, there was virtually no binding activity detected with the same amounts of proteins. The sequence specificity of the RNA-protein complex was further revealed by the competition experiment showing that the complex formation was inhibited by the addition of excess amounts of unlabeled 3'-UTR (Fig. 3C). Further experiments demonstrated that the RNA binding activity was barely detectable in the cytosolic extracts prepared from HeLa or hepatoma PLC cells, indicating that the protein factor binding to the H-ferritin 3'-UTR is predominantly present in myeloid cells (Fig. 3B). The effect of PMA treatment on the RNA-protein complex formation was examined. As shown in Fig. 4A, the RNA binding activity in PMA-treated THP-1 cells was substantially less than that in control cells. Time course experiments further revealed that down-regulation in RNA binding is evident at 3 h, reaches a maximum at 12 h, and is prolonged to 36 h following PMA treatment (Fig. 4B).

To locate the sequence interacting with the cytosolic protein factor, the 3'-deletion RNA probes were prepared and used for the binding assay. As shown in Fig. 5, when the RNA probe prepared from the \(\Delta/\delta\)-digested 3'-UTR cDNA template was used for binding assay, the RNA-protein complex was still observed. This complex, however, was not detected by using shorter RNA probes prepared from the \(\delta\)-digested DNA template. This result indicates that the sequence responsible for the interaction with the protein factor is located in the region between \(\delta\)-I and \(\delta\)-III restriction sites in H-ferritin 3'-UTR. This sequence was further identified by RNase T1 mapping assay. As shown in Fig. 6, digestion of the entire
32P-labeled 3'-UTR with RNase T1, which cleaves after each G residue, results in oligonucleotides varying from 22 through 2 bases. When the protected 32P-labeled RNA fragment isolated from the RNA-protein complex was electrophoresed, it migrated as a 22-base oligonucleotide. Further digestion of the protected RNA fragment with RNase T1 did not yield smaller fragments, indicating that the protected RNA is a G-free sequence located at 72–92 nucleotides downstream of the stop codon of H-ferritin gene. It was noted that this sequence is rich in pyrimidines, particularly the uridines. To test whether the U residues are important for binding with the protein factor, a competition experiment with various homopolymers of ribonucleotides was performed. As shown in Fig. 7, the formation of the RNA-protein complex could be completely inhibited by excess amounts of poly(U) homopolymers but not by poly(C) or poly(A), supporting that the poly(U) tract is essential for the binding activity.

To confirm the role of the U-rich sequence on mRNA stability, the effects of point mutations within the poly(U) tract of the H-ferritin 3'-UTR on the RNA-protein complex formation and the stability of chimeric transcript were examined. As shown in Fig. 8B, substitution of uridines at positions +77, +79, +83, and +85 by adenines (M1) reduced the binding of the cytosolic protein factor to 3'-UTR by ~50%. Further substitution of uridines at positions +77, +79, +83, and +85 by adenines (M2) completely abolished the binding. When the chimeric luciferase constructs carrying the mutant 3'-UTRs were transfected into THP-1 cells and the stability of their transcripts was assessed, it was shown that the mutant Luc/M1 mRNA has a calculated half-life of 7.4 ± 1.4 h, and mutant Luc/M2 mRNA has a half-life of >9 h. Apparently, both mutant transcripts are more stable than the chimeric transcript carrying the wild type 3'-UTR shown in Fig. 1.

**DISCUSSION**

As revealed by the transient transfection experiment, the 3'-UTR of the human H-ferritin gene placed at the 3'-end of luciferase gene destabilized the reporter transcript by decreasing its half-life from 8.0 ± 0.5 to 4.5 ± 0.3 h in monocytic THP-1 cells. Nevertheless, PMA treatment significantly prolonged the half-life of the chimeric mRNA to greater than 9 h, indicating that the 3'-UTR contains a sequence determinant mediating the PMA-induced message stabilization in these cells. Identification and sequence analysis of this regulatory element revealed that it is pyrimidine-rich and interacts with a novel protein factor which is present in cytoplasm of THP-1 cells but not HeLa or hepatoma PLC cells. The restriction in cell-type distribution is consistent with the early finding that induction of H-ferritin gene expression by PMA is myeloid cell-specific (16). When we examined the 3'-UTRs of H-ferritin mRNAs from different species, it is clearly shown that the homologous pyrimidine-rich sequence is present in all of them (Fig. 9), indicating that this sequence is highly conserved and may have
an important role in H-ferritin gene expression. PMA treatment of THP-1 cells resulted in the decrease of the protein binding to the H-ferritin 3'-UTR, suggesting that the binding protein acts as a destabilizer. A similar phenomenon has been reported in 3T3 cells, in which the induction of ribonucleotide reductase R1 and R2 genes by PMA is associated with the decrease in the binding activities of the RNA-binding proteins to their 3'-UTRs (19, 20). Likewise, a recent study on human pleural mesothelioma cells has shown that stabilization of the urokinase receptor mRNA by PMA is correlated with the down-regulation of the formation of a urokinase receptor RNA-protein complex (21).

The competition experiment with homoribopolymers demonstrated the importance of poly(U) within the pyrimidine-rich sequence for the interaction with the protein factor. The correlation between protein binding to the U-rich sequence and message stability was further supported by the observation that mutations in some of the uridines led to the reduction in RNA binding, but increase in the stability of mutant chimeric luciferase mRNA. Recently, numerous studies have demonstrated the involvement of U-rich regions in 3'-UTRs to regulate the message stability in many mRNAs. Accumulative evidence has revealed that the AUUUA pentamer and U-rich sequence present in 3'-UTRs of unstable mRNAs encoding cytokines, lymphokines, oncogenes, and growth factors plays an important role in facilitating the degradation of their tran-

**Fig. 6.** RNase T1 mapping of the sequence on 3'-UTR of H-ferritin mRNA interacting with the protein factor. Radiolabeled 3'-UTR or protected RNA fragment eluted from the RNA-protein complex was digested with or without RNase T1 and analyzed by sequencing gel. Asterisk indicates the protected fragment. The sites of cleavage by RNase T1 on the 3'-UTR of H-ferritin mRNA are indicated by arrows. The protected 22-base sequence is underlined.

**Fig. 7.** Effects of homoribopolymers on the formation of the RNA-protein complex. Cytosolic extracts (20 μg) from THP-1 cells were incubated with radiolabeled H-ferritin 3'-UTR in the absence or presence of the indicated amounts of poly(U), poly(C), or poly(A). After RNase T1 digestion and addition of heparin, the formation of the RNA-protein complex was analyzed by gel mobility shift assay.

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scripts (17, 22–24). The identification of U-rich sequences in 3′-UTRs to control the stability of mRNAs encoding amyloid precursor and GAP-43 proteins has also been reported (25–28). Furthermore, the expression of some RNA-binding proteins with preferential binding activity to the U-rich region in RNA has been shown to be implicated in neuronal development in Drosophila, Xenopus, and mouse embryo (29–31). Whether the protein factor binding to the H-ferritin 3′-UTR would also interact with the U-rich region located in the 3′-UTRs of other genes remains to be clarified.

UV cross-linking experiments revealed that the H-ferritin 3′-UTR-binding protein has a molecular size of ~43 kDa (data not shown). Recently, a number of pyrimidine tract-binding proteins which participate in RNA splicing or belong to a family of heterogeneous nuclear ribonucleoproteins have been cloned and characterized (24, 32–36). These nuclear proteins have apparent size in the ranges of 56–70 kDa and some of them exhibit preferential binding activity to the sequence rich in U. Based on the differences in subcellular localization, molecular size, and the restriction in cell-type origin, the H-ferritin 3′-UTR-binding protein appears to be distinct from these identified pyrimidine-binding proteins. Nevertheless, a recent study has showed that a protein factor which binds to a U-rich sequence in the 3′-UTR of GAP-43 mRNA shares sequence homology with PTB, an identified pyrimidine tract-binding protein implicated in RNA splicing (28). Whether the 43-kDa protein binding to the H-ferritin 3′-UTR is a PTB-like protein is an intriguing question awaiting to be further investigated. It is apparent that disclosure of the molecular nature of this RNA-binding protein should provide insight into the mechanism underlying the differential regulation of H-ferritin gene expression at the post-transcriptional level in myeloid cells.

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