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Novel Role for Molecular Transporter Importin 9 in Posttranscriptional Regulation of IFN-ε Expression

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IFN-ε is a unique type I IFN whose constitutive expression in lung, brain, small intestine, and reproductive tissues is only partially understood. Our previous observation that posttranscriptional events participate in the regulation of IFN-ε mRNA expression led us to investigate whether the 5′- and/or 3′-untranslated regions (UTR) have regulatory functions. Surprisingly, we found that full-length IFN-ε 5′UTR markedly suppressed mRNA expression under basal conditions. Analysis of the secondary structure of this region predicted formation of two stable stem-loop structures, loops 1 and 2. Studies using luciferase constructs harboring various stretches of IFN-ε 5′UTR and mutant constructs in which the conformation of loop structures was disrupted showed that loop 1 is essential for regulation of mRNA expression. Incubation of HeLa cell extracts with agarose-bound RNAs harboring IFN-ε loop structures identified importin 9 (IPO9), a molecular transporter and chaperone, as a candidate that associates with these regions of the 5′UTR. IPO9 overexpression decreased, and IPO9 silencing increased basal IFN-ε expression. Our studies uncover a previously undescribed function for IPO9 as a specific, and negative, posttranscriptional regulator of IFN-ε expression, and they identify key roles for IFN-ε stem-loop structure 1 in this process. IPO9-mediated effects on 5′UTRs appear to extend to additional mRNAs, including hypoxia-inducible factor–1α, that can form specific loop structures. The Journal of Immunology, 2013, 191: 1907–1915.

Viral invasion of mammalian cells is followed by uncoating of the viral particles, exposure of nucleic acids, and host cell–mediated replication of the viral genome (1). Recognition of viral nucleic acids by host intracellular receptors initiates a cascade of events that culminate in the production of type I IFNs and other cytokines (2, 3). Type I IFNs are encoded by multiple, usually intronless, genes, and, in humans, most IFNs (e.g., IFN-β, -o, -κ, -ε, and 14 IFN-α species) map to chromosome 9 and are expressed in tissue-specific manners (4). IFN-ε, a recently identified member of this family, is expressed in specific tissues, including female reproductive organs such as the ovary and uterus (5). It appears to play a role in immunity and protection against viruses owing to its ability to induce a short-lived and localized mucosal immune response (6). Seminal plasma has been reported to upregulate expression of IFN-ε in cervical and vaginal tissues (7), a response that may represent an antimicrobial defense mechanism that evolved to fight infections. Importantly, a recent study demonstrated that the reproductive tracts of female mice deficient in Ifn-ε display increased susceptibility to vaginal infections (8), suggesting that IFN-ε is a cardinal type I IFN that plays key roles as a mediator of innate immunity.

We previously reported that, in human cervical cancer cells, IFN-ε mediates STAT1 phosphorylation in response to stimulation with TNF-α and presented evidence indicating that transcriptional and posttranscriptional mechanisms participate in the regulation of IFN-ε expression (9). Gene expression is regulated at the levels of replication, transcription, mRNA splicing, stability and translation, protein posttranslational modification and stability, and others (10). Posttranscriptional control of gene expression relies on specific RNA–protein interactions that stabilize mRNAs, promote targeted degradation, or prevent access of the ribosome to the translation start codon (10). Such interactions can be mediated by the 5′ or 3′ untranslated regions (UTR) of mRNAs (11, 12). Sequences located in 3′UTRs are thought to participate primarily in the regulation of mRNA stability. A number of unstable mRNAs, including those encoding cytokines, oncogenes, and transcription factors, harbor AU-rich elements (AREs) whose signature sequence (i.e., AUUUA) is located in the 3′UTR (13). The 5′UTR-mediated regulation of expression is associated with events related to the initiation of mRNA translation, although mRNA-destabilizing effects also have been noted (11). Mammalian 5′UTRs often form stable secondary structures such as stem loops positioned between the cap structure and the AUG codon, which can inhibit translation initiation; the extent of this inhibitory effect depends on the thermodynamic stability and position of the structures (14).

In this study, we investigated whether regions in the 5′ and 3′UTRs of IFN-ε mRNA regulate expression. Our motivation to conduct these analyses was based on previous studies showing that...
IFN-ε expression is regulated posttranscriptionally (9). We present novel evidence demonstrating that cis 5'UTR sequences negatively regulate constitutive IFN-ε expression. This effect is mediated by a 5'UTR stem-loop structure that, when disrupted, leads to enhanced mRNA expression. The effect is specific and involves direct or indirect interaction with importin 9 (IPO9), a molecular transporter and chaperone that belongs to the superfamily of karyopherins (15).

**FIGURE 1.** Effect of IFN-ε 3'UTR on posttranscriptional regulatory events. (A) DNA sequences immediately downstream of the stop codon UAG represent the 3'UTR of IFN-ε mRNA. Two AUUUA stretches are underlined. (B) Diagrammatic representation of Luc and Luc-3' vectors tested in (C). (C) We transfected HeLa cells with Luc and Luc-3', incubated the cells for 24 h, and assessed luciferase activity in cellular extracts. Data represent the mean ± SD from four experiments.

**FIGURE 2.** Effect of IFN-ε 5'UTR on posttranscriptional regulatory events. (A) DNA sequences immediately upstream of the start codon AUG represent the 5'UTR of IFN-ε mRNA. Two regions showing predicted stem-loop structures are underlined. (B) Diagrammatic representation of Luc, 5'-Luc-, and 5'-Luc-3' vectors tested in (C). (C) We transfected HeLa cells with Luc, 5'-Luc-, and 5'-Luc-3'; incubated the cells for 24 h; and assessed luciferase activity in cellular extracts. The results obtained with Luc are the same as those presented in Fig. 1C and are reproduced in this panel to facilitate direct comparisons. Data represent the mean ± SD from four experiments. *p < 0.001.
Materials and Methods

Cell culture and transfection

The human cervical cancer cell lines HeLa and HeLa S3 were purchased from American Type Culture Collection and were maintained in a 5% CO₂ atmosphere at 37°C in DMEM (Life Technologies) supplemented with 10% FBS (ThermoFisher Scientific). Transient transfection was accomplished by plating HeLa cells at a density of 1.5 x 10⁵ cells/well of 12-well culture plates. After 18-20 h, we transfected 0.5 μg plasmid DNA using Lipofectamine LTX (Life Technologies), following the manufacturer’s recommendations. We incubated the cells for 24 h, and then determined luciferase activity and/or mRNA levels in cellular extracts. RNA interference was performed by transfection of HeLa cells with nonsilencing (control) small interfering RNA (siRNA; Life Technologies) or with siRNAs against IPO9 (Life Technologies 442703) using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s protocol.

IFN-ε 5’UTR cloning

We cloned the 5’UTR of human IFN-ε using a 5’ RACE kit (Life Technologies). The template consisted of 1 μg total RNA from HeLa cells previously transfected with polynosinic-polycytidylic acid (polyIC; 100 ng, 6 h), a treatment previously shown to increase IFN-ε mRNA expression. We generated cDNA using SuperScript II reverse transcriptase (Life Technologies) combined with gene-specific primer 1 (5’-GAGGACACACAGCCTCTCTTTGATTC-3’), and then cloned into the XbaI forward, 3’-CTGAACACCATGA-3’. The product was purified, digested with XbaI, and then cloned into the pcDNA3.1/Zeo + cloning vector. We generated cDNA using Reverse Transcription (Life Technologies) combined with gene-specific primer 1 (5’-GAGGACACACAGCCTCTCTTTGATTC-3’), and then cloned into the XbaI forward, 3’-CTGAACACCATGA-3’. The amplified products were cloned into the XbaI forward and 3’-CTGAACACCATGA-3’ sites of the mammalian expression vector, p3XFLAG-CMV7.1 (Sigma-Aldrich), according to the manufacturer’s protocol.

Plasmid construction

Luciferase reporter constructs were generated in the mammalian expression vector pcDNA3.1/Zeo™ modified to express freely luciferase cDNA (generated from pGL3-Basic (Promega) by digestion with HindIII and XbaI, as described by Dixon et al. (16)). We used the 5’ RACE PCR product as template to generate constructs harboring stem loops 1 and 2 (el + 2), posterior stem loop 2 (el2), and no stem loops (ε), using Platinum PfX DNA Polymerase (Life Technologies). The reverse primer harbored a 5’-GGGAACCTGAAAATCatAA-3’ tail at the 5’ end using TdT. Initial amplification was accomplished by PCR (94°C, 30 s; 65°C, 30 s; 72°C, 3 min; 25 cycles followed by 10 min at 72°C) using gene-specific primer 2 (5’-TCTTTCCCAACTCTACCAGAAAGAAA-3’). A second round of PCR (94°C, 30 s; 55°C, 30 s; 72°C, 3 min; 30 cycles followed by 10 min at 72°C) was performed using 2.5 μl of a 50× dilution of the initial PCR product combined with a nested gene-specific primer 3 (5’-GCCAGCAGCACCAACACAGT-3’). The forward primer (AUAP: 5’-GGGCCACCGTGACTGAC-3’) was provided by Life Technologies. The generated 5’ RACE PCR product was gel purified, cloned, and sequenced at the University of Utah DNA Sequencing Core Facility.

Quantitative RT-PCR

Total RNA was extracted using a RNeasy total RNA isolation kit with on-column DNase I digestion (Qiagen). We used 1 μg total RNA as template for first-strand cDNA synthesis, combined with oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (Life Technologies). An Opticon 2 Real-Time PCR System (Bio-Rad) was used for quantitative analyses of luciferase and zeocin expression. The sequences of the primers were as follows: IFN-ε forward, 5’-AGGAGACCACCTGCTCCAGTTTTG-3’; IFN-ε reverse, 5’-TGTCTCTATGCTGTCACAAG-3’; Luciferase forward, 5’-ACGGATTACCCAGAGTTTTCAT-3’; Luciferase reverse, 5’-AGGGCTCTTCTAAGACCTTTTCT-3’. Amplifications were performed using iQ SYBR Green Supermix (Bio-Rad), according to the manufacturer’s specifications. Cycling conditions were as follows: 50°C, 2 min; 95°C, 5 min; 40 cycles of 95°C (15 s) plus 58°C (30 s) plus 72°C (30 s). A melting curve was generated by acquiring fluorescence measurements while slowly heating to 95°C at a rate of 0.1°C/s. Melting curves and quantitative analysis of the data were performed using an Opticon monitor, version 3.1, as previously reported (17).

Reporter assays

Twenty-four hours after transfection with reporter constructs, we harvested cells using reporter lysis buffer and determined luciferase activity using a commercially available assay system (Promega). When indicated, the cells
were cotransfected with a β-galactosidase cDNA (pSV-β-Gal). For normalization purposes, we assessed either total protein concentration in the cell lysates or β-galactosidase activity. The latter was determined using a chemiluminescence-based reporter assay (Roche Applied Science). The data presented are representative of at least three independent experiments performed in duplicate, and they are reported as the mean ± SD.

Identification of 5'-UTR-binding proteins

HeLa S3 cells were grown in suspension in DMEM containing 10% FBS. A cytoplasmic extract was obtained by harvesting 5 × 10^7 cells in buffer A (10 mM HEPES-KOH [pH 7.5], 3 mM MgCl2, 14 mM KCl, 5% glycerol, 1 mM DTT, and 0.5% protease inhibitor mixture [Sigma-Aldrich]). Following brief incubation (15 min on ice), the lysate was homogenized with 20 strokes of a Dounce homogenizer and then subjected to centrifugation (5000 × g, 15 min). The supernatant was stored at −80°C in small aliquots.

We immobilized RNA on agarose beads essentially as described by Caputi et al. (18). Briefly, the vectors diagrammatically shown in Fig. 5A were linearized using NcoI and then were in vitro transcribed using T7 RiboMAX (Promega), following the manufacturer’s recommendations. RNAs (500 pmol) were placed in 400 μl reaction mixtures containing 100 mM sodium acetate (pH 5.0) and 5 mM sodium m-periodate, and were incubated for 1 h in the dark at room temperature. RNAs were precipitated using ethanol, and then were resuspended in 400 μl 100 mM sodium acetate (pH 5.0). The 200-μl aliquots of adipic acid dihydrazide agarose beads (Sigma-Aldrich) were washed four times with 10 ml 0.1 M sodium acetate (pH 5.0). After the final wash, the beads were resuspended in 400 μL 0.1 M sodium acetate (pH 5.0), mixed with periodate-treated RNAs, and then rotated for 16 h at 4°C. Next, we washed the beads with 3 × 1 ml 2 M NaCl and then 3 × 1 ml buffer D (20 mM HEPES-KOH [pH 7.6], 5% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT). We then mixed agarose-bound RNAs with HeLa S3 cytoplasmic extracts and rotated the mixtures for 2 h at 4°C. Beads were decanted (5000 rpm, 1 min) and washed four times with 1 ml buffer D. After the final centrifugation, agarose-bound proteins were eluted with 2× SDS sample buffer, subjected to electrophoresis on 10% SDS-polyacrylamide gels, and visualized with Coomassie Blue. We excised several bands of interest from the stained gel, and performed in-gel trypic digestion, as described (19). The resulting tryptic peptides were dissolved in 20 μl 0.1% trifluoroacetic acid and then desalted using C18 ZipTip (Millpore, Bedford, MA), according to the manufacturer’s recommendations. Next, the peptides were dissolved in 0.1% formic acid and subjected to nanoflow-liquid chromatography-tandem mass spectrometry analyses and identification, according to Ozaki et al. (20).

Results

The 3’ end of IFN-ε mRNA harbors AUUUA pentamers that are not required for constitutive expression

Our first goal was to investigate whether, in addition to transcriptional regulation (9), posttranscriptional mechanisms regulate expression of the IFN-ε gene. We first hypothesized that one or more AREs might regulate the stability of IFN-ε mRNA under basal conditions. AREs are usually located in the 3’UTR of mRNAs (21), and sequence analyses revealed the presence of two AUUUA elements in the 3’UTR of IFN-ε mRNA (Fig. 1A). We cloned this region using RACE and then generated a mammalian expression vector in which the 3’UTR was cloned downstream of the firefly luciferase cDNA (Fig. 1B). Transfection of HeLa cells with this construct resulted in luciferase expression at levels similar to those achieved by a construct lacking the 3’UTR (Fig. 1C). These results suggest that the 3’UTR is not required for constitutive expression of IFN-ε.
Effect of the 5'UTR of IFN-ε mRNA on firefly luciferase production under basal conditions

We next focused our attention on the 5’UTR of IFN-ε mRNA. We cloned this region using RACE and found that it harbors 265 nt upstream of the human IFN-ε translation start site (Fig. 2A). We then generated a mammalian expression construct in which this region was inserted at the 5’ end of the firefly luciferase cDNA (5’-Luc; Fig. 2B). In addition, we created a construct that harbored both the 5’ and 3’UTRs (5’-Luc-3’) to assess potential synergistic effects (Fig. 2B). HeLa cells transfected with 5’-Luc expressed significantly lower levels of luciferase activity relative to total protein, compared with cells transfected with a construct that lacked UTRs (Fig. 2C). These results point at 5’UTR-mediated negative regulation of constitutive IFN-ε expression. Inclusion of IFN-ε 3’UTR did not affect 5’UTR-mediated effects (5’-Luc-3’; Fig. 2C), thus confirming lack of participation of the 3’UTR in posttranscriptional regulatory events.

The 5’UTR of IFN-ε mRNA does not affect mRNA stability

Regulation of mRNA stability is a common posttranscriptional mechanism to control protein levels in response to cellular needs. In previous work, we provided evidence suggesting that HeLa cells regulate IFN-ε expression by modulating mRNA stability (9). Thus, we hypothesized that the ability of the 5’UTR of IFN-ε to decrease luciferase mRNA levels (Fig. 2C) might involve destabilization of the mRNA. To test this, we compared the stability of IFN-ε mRNA in the presence and absence of the 5’UTR, under experimental conditions that precluded transcription, as previously described (9). We observed no 5’UTR-mediated effects on mRNA 1/2, suggesting that the 5’UTR is not involved in mRNA-destabilizing effects (data not shown).

The 5’UTR of IFN-ε mRNA is predicted to form stem-loop structures that affect constitutive mRNA expression

RNA secondary structures located between the cap structure and the initiation codon can inhibit translation initiation, and the extent of this effect depends on the thermodynamic stability and position of the structure (10). Based on findings presented in Fig. 2C, we hypothesized that defined regions in the 5’UTR of IFN-ε mRNA might adopt secondary structures that impact posttranscriptional events. We conducted computer-aided analyses of the 5’UTR of human IFN-ε mRNA using CentroidFold (www.ncrna.org/centroidfold), a web-based approach frequently employed as a tool to predict RNA secondary structures (22). This analysis identified two sequences potentially capable of forming stem-loop structures, as follows: loop 1 spanning bases 23–49, and loop 2 spanning bases 110–142 (Fig. 3). The formation of stem-loop structures was consistently predicted by multiple additional algorithms, including KineFold (http://kinefold.curie.fr/; Supplemental Fig. 1A), RNAfold (http://rna.bi.univie.ac.at; Supplemental Fig. 1B, 1C), and Mobyle (http://mobyle.pasteur.fr; data not shown). Moreover, stem-loop formation at the 5’UTR of IFN-ε is predicted for a variety of primates (Fig. 4) (23), suggesting evolutionarily conserved functions for these regions. We used this information to generate firefly luciferase expression constructs in which IFN-ε mRNA 5’UTR sequences harboring 0-1-2 loops were cloned between the CMV promoter and luciferase cDNA (Fig. 5A), and then used these constructs to assess the impact of loop structures on posttranscriptional events. We found that the presence of loop 2 (ε2) did not affect luciferase mRNA expression compared with that resulting from a construct that lacked stem-loop structures (compare ε0 with ε2; Fig. 5B), suggesting that this structure does...
not regulate IFN-ε mRNA expression. In contrast, inclusion of loops 1 and 2 robustly decreased relative luciferase mRNA levels (compare ε0 with ε1 + 2; Fig. 5B). These combined observations suggest that loop 1, or the combined presence of loops 1 and 2, suppresses IFN-ε mRNA expression.

Effect of loop structures within 5' UTR of IFN-ε on constitutive mRNA expression

Our next goal was to investigate whether proper conformation of stem-loop structures is involved in the regulation of IFN-ε mRNA expression. To accomplish this, we generated a series of 5'UTR mutant constructs in which each of the predicted secondary structures was individually disrupted (Supplemental Fig. 2A, 2B). We found that disruption of loop 1 rescued luciferase mRNA expression to a level comparable to that of ε2, which lacks loop 1 (Fig. 6). In contrast, disruption of loop 2 had no effect compared with the control (ε1 + 2). These data are consistent with studies shown in Fig. 5, and they suggest that proper folding of loop 1 is essential for constitutive suppression of IFN-ε mRNA. Our results also suggest that loop 1, and not the combined presence of loops 1 and 2, is responsible for negative regulation of IFN-ε expression.

Identification of IPO9 as a protein that binds to IFN-ε 5' UTR stem-loop structures

We next sought to identify proteins that specifically interact with IFN-ε 5' UTR stem loops, and determine whether the identified candidates play a role in posttranscriptional regulation of IFN-ε expression. To accomplish this, we generated RNAs corresponding to IFN-ε 5' UTR harboring both loops (ε1 + 2) or no loops (ε0). We attached individual RNAs to agarose beads, incubated them with HeLa cell extracts, and then subjected bound proteins to

FIGURE 7. RNA harboring stem-loop structures 1 and 2 bind 68-, 108-, and 118-kDa HeLa cell proteins. We generated IFN-ε RNAs harboring stem loops 1 and 2 (ε1 + 2) or no stem-loop structures (ε0) from constructs shown in Fig. 5A. The RNAs were covalently attached to agarose beads, and the products then were incubated with HeLa cell extracts. Proteins adsorbed to control and RNA-treated beads were subjected to SDS-PAGE and visualized by staining with Coomassie Blue.

FIGURE 8. Knockdown of IPO9 enhances IFN-ε mRNA expression. (A) We transfected HeLa cells with IPO9 siRNA and then incubated the cells for 48 h. Knockdown efficiency of IPO9 was determined by Western blot analyses, as described in Materials and Methods. (B–F) We extracted total RNA from IPO9-silenced and determined mRNA levels of IFN-ε (B), HIF-1α (C), GAPDH (D), CCL5 (E), and IL-6 (F), using quantitative RT-PCR. The 18S rRNA levels served as normalization controls. Data represent the mean ± SD of three independent experiments. **p < 0.01.
We found that removal of loops 1 and 2 robustly decreased association with three proteins of apparent molecular masses 68, 108, and 118 kDa (Fig. 7). We subjected the 100- to 130-kDa region of the SDS-PAGE gel shown in Fig. 7 to mass spectrometric analyses, and identified IPO9 (predicted molecular mass = 110 kDa), a member of the importin-β superfamily of nuclear transport receptors, as a candidate involved in posttranscriptional regulation of IFN-ε expression. We were unable to identify the 68-kDa protein owing to technical difficulties.

**IPO9 silencing enhances constitutive expression of IFN-ε mRNA**

To investigate whether IPO9 functionally affects IFN-ε expression, we silenced its expression using siRNA (Fig. 8A) and found that decreased IPO9 expression enhanced constitutive IFN-ε mRNA expression (Fig. 8B). Stimulation with TNF-α modestly, but significantly, increased the level of IFN-ε mRNA, in agreement with our previous findings (9). However, IPO9 silencing restored IFN-ε mRNA to an extent equal to that observed in unstimulated cells (Fig. 8B). These results indicate that IPO9 suppresses constitutive, but not stimulated, IFN-ε mRNA expression. We next investigated whether IPO9 suppressed expression of other mRNAs and found that constitutive expression of HIF-1α, predicted to form a large secondary structure within its 5’UTR (Supplemental Fig. 3), was enhanced by IPO9 silencing, in a manner similar to that observed for IFN-ε (Fig. 8C). In contrast, the levels of constitutively expressed (housekeeping) gene GAPDH (24) and those of the chemokine CCL5 and the cytokine IL-6 were not affected by IPO9 silencing (Fig. 8D–F). Although the 5’UTRs of these mRNAs also are predicted to adopt secondary structures (data not shown), the stem loops formed by IFN-ε and HIF-1α appear to be unique.

**Loop 1 is required for IPO9-mediated suppression of IFN-ε expression**

To investigate whether the regulatory effects of IPO9 and loop 1 on IFN-ε expression are functionally related, we assessed the impact of IPO9 on inhibition of IFN-ε expression mediated by our 5’UTR constructs. We used a two-pronged approach whereby IPO9 was either overexpressed (Fig. 9A) or silenced (Fig. 9B). These studies showed that IPO9 regulates IFN-ε mRNA levels in a loop-dependent fashion. Increased IPO9 led to marked reductions in the expression of a construct harboring loops 1 and 2 (ε1 + 2), but had no effect when loops 1 and 2 were deleted (Fig. 9A). Conversely, silencing IPO9 enhanced expression of ε1 + 2, but not that of constructs lacking loop 1 (ε0 and ε2; Fig. 9B). These results demonstrate that IPO9 regulates constitutive expression of IFN-ε mRNA through loop 1 within 5’UTR of IFN-ε. Loop 1 in IFN-ε and a stem loop in HIF-1α (candidate loop structure; Supplemental Fig. 3) share unique features; the stems are composed of 6–10 bp, and the loops harbor 5–7 nt. Whereas this requirement remains to be confirmed experimentally, it appears that the suppressive effect of IPO9 on mRNA expression is dependent on the formation of peculiar secondary structures within 5’UTRs.

**IPO9 does not regulate IFN-ε expression at the promoter level**

Our final goal was to investigate whether IPO9 transcriptionally regulates IFN-ε expression. To test this, we transfected IPO9-silenced and control HeLa cells with a previously described IFN-ε promoter construct, and then determined promoter activity, as before (9). We found that IPO9 silencing did not activate the IFN-ε promoter, in contrast to transfection with polyI:C, which was used as a positive control (Fig. 10) (9). We conclude that IPO9 does not regulate IFN-ε expression at the promoter level.
Discussion

Previous studies have shown that IFN-ε expression is induced upon infection with Semliki Forest (5) and vaccinia (14) viruses, and in response to exposure of cervicovaginal tissues to seminal fluid (7). In addition, RNA interference–mediated silencing of IFN-ε significantly inhibits activation of STAT1 (9), a key transcriptional activator of antiviral, immune, and antitumorigenic responses (25). These observations point at the importance of IFN-ε in host immune responses such as those required to establish antimicrobial, antiviral, and antitumorigenic states in specific organs. The mechanisms that control IFN-ε levels in various tissues, including those of the female reproductive tract, are only partially understood. Previous work rightly focused on transcriptional initiation events shown to participate in the regulation of IFN-ε expression (5, 9). However, this is not the only mechanism whereby the levels of IFN-ε are regulated, as early evidence suggested that posttranscriptional mechanisms also are involved (9). In the current study, we investigated whether, and how, IFN-ε expression is regulated at the posttranscriptional level because constitutive expression of IFN-ε was recently shown to play essential roles in the protection of female reproductive organs from sexually-transmitted infections (8). We found evidence supporting a role for the 5′ UTR of IFN-ε mRNA in regulation of expression under basal conditions.

The 5′ UTRs often include cis elements that survey individual mRNAs and affect their stability. Examples include regions in the 5′ UTRs of mRNAs encoding IL-2 and growth-related oncogenes (26, 27). Several RNA secondary structure prediction algorithms identified two potential stem-loop structures in IFN-ε 5′ UTR, and the studies presented in this work show evidence supporting a role for loop 1 as a negative regulator of IFN-ε mRNA expression. This inhibitory effect does not appear to involve changes in mRNA stability, and, whereas the precise mechanism involved remains to be elucidated, thermodynamically stable stem loops have been shown to stall ribosomes and initiate endonucleolytic cleavage events (28).

The inhibitory effect exerted by IFN-ε 5′ UTR on mRNA expression may be specific for certain cell types and most likely depends on expression of proteins that recognize defined structures or sequences located in the 5′ UTR. Our studies identified IPO9 as a protein that binds primarily to stem-loop structure 1 in IFN-ε 5′ UTR and negatively impacts mRNA expression. To our knowledge, this constitutes the first report identifying IPO9 as a regulator of IFN-ε mRNA expression, a function suggesting roles additional to those previously reported. Importins, including IPO9, are known for their ability to mediate active transport through nuclear pore complexes (29) and effectively suppress the aggregation of their basic import cargoes in polyamoronic environments (15). Importins exert their functions through protein–protein interactions; they recognize nuclear localization signals and prevent protein aggregation by shielding basic patches such as those found in a variety of ribosomal proteins (15). No studies to date have reported direct interactions between importins and nucleic acids, leading us to speculate that, in our studies, IPO9–mediated posttranscriptional effects on IFN-ε mRNA expression involve intermediates, such as unidentified RNA-binding protein(s). Our observation that IPO9 inhibited expression of IFN-ε and HIF-1α, but not that of other mRNAs, points at specific interactions limited to a subset of mRNAs whose 5′ UTR fold into defined secondary structures such as IFN-ε loop 1.

In conclusion, we have identified a novel mechanism for posttranscriptional regulation of IFN-ε expression. A distinct stem-loop structure predicted to form in the 5′ UTR directly or indirectly interacts with IPO9, a member of the importin family, negatively regulating IFN-ε mRNA expression under basal conditions. These observations provide a novel mechanism for regulation of IFN-ε, and describe a previously unidentified function for IPO9 in posttranslational, gene-specific regulation of expression. This regulatory mechanism also may be used to regulate the expression of other mRNAs, including HIF-1α.

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Disclosures

The authors have no financial conflicts of interest.

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