RNase-L-dependent Destabilization of Interferon-induced mRNAs

A ROLE FOR THE 2–5A SYSTEM IN ATTENUATION OF THE INTERFERON RESPONSE*

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The 2–5A system is an interferon-regulated RNA degradation pathway with antiviral, growth-inhibitory, and pro-apoptotic activities. RNase-L mediates the antiviral activity through the degradation of viral RNAs, and the anticellular effects of the 2–5A system are thought to be similarly mediated through the degradation of cellular transcripts. However, specific RNase-L-regulated cellular RNAs have not been identified. To isolate candidate RNase-L substrates, differential display was used to identify mRNAs that exhibited increased expression in RNase-L-deficient NIH-3T3 fibroblasts as compared with RNase-L-transfected cells. A novel interferon-stimulated gene encoding a 43-kDa ubiquitin-specific protease, designated ISG43, was identified in this screen. ISG43 expression is induced by interferon and negatively regulated by RNase-L. ISG43 induction is a primary response to interferon treatment and requires a functional JAK/STAT signaling pathway. The kinetics of ISG43 induction were identical in wild type and RNase-L knock-out fibroblasts; however, the decline in ISG43 mRNA following interferon treatment was markedly attenuated in RNase-L knock-out fibroblasts. The delayed shut-off kinetics of ISG43 mRNA corresponded to an increase in its half-life in RNase-L-deficient cells. ISG15 mRNA also displayed RNase-L-dependent regulation. These findings identify a novel role for the 2–5A system in the attenuation of the interferon response.

Cellular mRNAs exhibit half-lives ranging from minutes to days, and the stability of a given message can change dramatically in response to diverse stimuli (1). The control of mRNA turnover thus provides a mechanism to effect rapid changes in gene expression. Accordingly, the stringent regulation of genes functionally regulated by RNase-L. ISG43 induction is a primary response to interferon treatment and requires a functional JAK/STAT signaling pathway. The kinetics of ISG43 induction were identical in wild type and RNase-L knock-out fibroblasts; however, the decline in ISG43 mRNA following interferon treatment was markedly attenuated in RNase-L knock-out fibroblasts. The delayed shut-off kinetics of ISG43 mRNA corresponded to an increase in its half-life in RNase-L-deficient cells. ISG15 mRNA also displayed RNase-L-dependent regulation. These findings identify a novel role for the 2–5A system in the attenuation of the interferon response.

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1 The abbreviations used are: UTR, untranslated region; ARE, AU-rich element; IFN, interferon; dsRNA, double-stranded RNA; ISG, interferon-stimulated gene; UBP, ubiquitin-specific protease; KO, knock-out; WT, wild type; ORP, open reading frame; MEF, mouse embryo fibroblast; DUB, deubiquitinating; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; bp, base pair; JAK, Janus kinase; STAT, signal transducers and activators of transcription.

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nuclease, RNase-L (reviewed in Ref. 16). 2–5A synthetase is induced in IFN-treated cells and, in the presence of double-stranded RNA (dsRNA), polymerizes ATP into unique 5′-phosphorylated, 2′,5′-linked oligoadenylates (2–5A). 2–5A, in turn, binds the latent RNase-L, leading to its dimerization and activation (17); activated RNase-L catalyzes the cleavage of single-stranded RNA. The 2–5A system was first studied as a mediator of the antiviral activity of IFN, and transfection of cDNAs encoding 2–5A synthetase (18) or RNase-L (19, 20) has confirmed this role. In addition, a protein inhibitor of RNase-L has been implicated in regulating RNase-L activity in virus-infected cells (21). Inhibition of RNase-L activity using dominant-negative and targeted gene disruption strategies revealed that RNase-L also functions in IFN-mediated growth inhibition and in apoptosis (22–24). In virus-infected cells, viral RNA appears to be targeted for degradation by RNase-L, possibly through a localized activation of the 2–5A system by viral dsRNA (25, 26). However, in the absence of virus infection, the cellular RNA substrates of RNase-L are not known.

Interferon-stimulated genes (ISGs), including those of the 2–5A system, encode the proteins that mediate the effects of IFN in cells. Studies of the IFN system therefore focused initially on the identification of ISGs and more recently on the transcriptional activation of ISGs through the JAK/STAT signaling pathway (27). Interferon-regulated gene expression is transient, characterized by distinct induction and shut-off phases. Indeed, the tight regulation of ISGs is critical, as constitutive expression of ISGs is often deleterious to cells (23, 28). Furthermore, constitutive expression of ISGs is often deleterious to cells (23, 28). Several inhibitors of IFN signaling that function to limit the transcriptional induction of ISGs have recently been identified (e.g. suppressors of cytokine signaling and protein inhibitors of activated STATs; reviewed in Ref. 29). However, posttranscriptional mechanisms to eliminate existing ISG-encoded gene products have not been described. Such posttranscriptional regulation would permit a more rapid and efficient attenuation of the IFN response.

In this study, we sought to identify mRNAs regulated by RNase-L as a first step in understanding the mechanisms by which the 2–5A system elicits its antiviral effects. Toward this end, we characterized an RNase-L-deficient cell line and restored RNase-L activity in stable transfectants, providing a system in which differentially expressed mRNAs represent candidate RNase-L substrates. Differential display analysis of RNase-L-deficient and competent cell lines identified a novel ISG as a candidate RNase-L substrate. This ISG encodes a 43-kDa ubiquitin-specific protease (UBP), designated ISG43. The decline of ISG43 mRNA following IFN treatment is markedly reduced in fibroblasts derived from RNase-L knock-out (KO) as compared with wild type (WT) mice. Moreover, ISG43 mRNA exhibits an increased half-life in RNase-L KO fibroblasts, demonstrating RNase-L-dependent regulation of ISG43 mRNA stability in intact cells. The transcript encoding ISG15 is also regulated by RNase-L. These data provide evidence of a novel role for RNase-L in the posttranscriptional attenuation of the IFN response; experimental evidence consistent with a model for RNase-L functioning as an effector and an attenuator of IFN action is discussed.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections

All cells were maintained in a humidified atmosphere of 5% CO2, 95% balanced air at 37 °C. Cells were cultured in the following growth media: RNase-L−/−, +/+ MEFs (generously supplied by Robert H. Silverman, The Cleveland Clinic Foundation), and L929- Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, and antibiotic/antimycotic; N1E-115-medium 199, 10% newborn calf serum, non-essential amino acids, sodium pyruvate, vitamin mix, and antibiotic/antimycotic; 2TGH, USA, and U4A (kindly provided by George R. Stark, The Cleveland Clinic Foundation)-Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 250 μg/ml hygromycin, and antibiotic/antimycotic (all cell culture reagents from Life Technologies, Inc.). The human RNase-L cDNA in the pcDNAneo vector (Invitrogen) in the sense orientation or vector alone was transfected into N1E-115 cells by calcium phosphate precipitation (Life Technologies, Inc.). Stable transfectants were then selected in 250 μg/ml G418; N1E-RNase-L-1 and -L2 refer to independent clonal cell lines. Interferon treatment used murine α + β (Lee Biomolecular Laboratories), human IFNα (Hoffmann-La Roche), or human IFNγ (Ciba-Geigy) at the concentrations indicated.

2–5A Transfection

For 2–5A-trimer phosphorothiolate a concentration of 1 μM was transfected into cells by calcium phosphate co-precipitation for 75 min. The cells were then washed with phosphate-buffered saline, refed with growth medium, and incubated for 2.5 h; total RNA was then harvested for analysis using Trizol Reagent (Life Technologies, Inc.).

Analyses of Gene Expression

Protein—RNase-L in postmitochondrial supernatants was labeled by UV cross-linking to [α-32P]2–5A and analyzed by SDS-PAGE as described previously (30). Transfected RNase-L protein was measured by Western blot analysis using a monoclonal antibody specific for the human enzyme (kindly provided by Beihua Dong and Robert H. Silverman, The Cleveland Clinic Foundation); RNase-L was visualized by reacting blots with ECL (Amersham Pharmacia Biotech) which were used to expose X-Omat AR film (Eastman Kodak Co.).

RNA—Total and poly(A)+ RNA was analyzed on glyoxal-agarose gels by ethidium staining and Northern blot hybridization. Hybridization probes were labeled with [α-32P]dCTP (Amersham Pharmacia Biotech) by random priming (Amersham Pharmacia Biotech). The cDNA hybridization probe for ISG15 (31) was described previously. For analyses of mRNA half-life, cells were treated with 5 μg/ml actinomycin D (Sigma) for the indicated periods. Northern blots were quantified by PhosphorImager (Molecular Dynamics); these data were used in determinations of mRNA half-life (1).
sequence was submitted to the GenBank™ under accession number AF176642.

In Vitro Transcription and Translation

The plasmids containing the murine and human ISG43 cDNAs were linearized with HindIII and in vitro transcribed using T7 RNA polymerase as described by the supplier (Promega). In vitro transcripts (500 ng/reaction) were translated in rabbit reticulocyte lysate (Promega) in the presence of [α-35S]methionine (Amersham Pharmacia Biotech) and analyzed by PAGE and autoradiography.

RESULTS

Characterization of an RNase-L-deficient Cell Line—The murine neuroblastoma cell line, N1E-115, was previously determined to lack detectable RNase-L protein (33); we sought to characterize further this RNase-L deficiency and to restore RNase-L activity by stable expression of a transfected RNase-L cDNA. RNase-L mRNA was not detected in total or poly(A)+ RNA from control and IFN-treated N1E cells by Northern blot analysis (Fig. 1A) or reverse transcriptase-PCR (not shown). In contrast, RNase-L mRNA was readily detected in IFN-treated murine L929 cells (Fig. 1A). 2–5A synthetase was induced to comparable levels by IFN in both N1E and L929 cells (Fig. 1A, lower panel), demonstrating that the lack of RNase-L induction in N1E cells is not due to a defect in IFN signaling.

To restore RNase-L expression, N1E cells were stably transfected with a human RNase-L cDNA expression construct (N1E-RNase-L) or vector alone (N1E-vector; pcDNAIneo, Invitrogen). Interestingly, in contrast to many other cell types studied in which expression of transfected RNase-L induces apoptosis (23, 34), constitutive RNase-L expression did not reduce the viability of N1E cells (data not shown). RNase-L protein was measured by covalent cross-linking of [α-32P]Pcp-labeled 2–5A (30). No RNase-L protein was observed in parental N1E or vector control lysates using this highly sensitive method (Fig. 1B, 1st and 4th lanes), whereas RNase-L expression was easily detected in lysates from clonally derived transfectants (Fig. 1B, 2nd and 3rd lanes). Western blot analyses using a monoclonal antibody that is specific for the transfected human RNase-L (17) demonstrated that RNase-L expression was derived from the transgene rather than from an activation of the endogenous murine gene (Fig. 1B, lower panel).

To determine if RNase-L activity was detectable in N1E cells, untreated and IFN-treated cells were transfected with 2–5A activator, and total RNA was analyzed for rRNA cleavage. In the presence of saturating amounts of 2–5A as were used in this experiment (1 μM trimer triphosphate), RNase-L cleaves rRNA into discrete characteristic products (35). No rRNA cleavage was detected in 2–5A-transfected N1E cells (Fig. 1C, 6th and 8th lanes). In contrast, rRNA cleavage products were clearly observed following 2–5A transfection of N1E-RNase-L cells (Fig. 1C, 2nd and 4th lanes), demonstrating that the transgene-encoded RNase-L was enzymatically active. Taken together, these data demonstrate that RNase-L expression in N1E cells is either completely lacking or exceedingly low and that these cells are functionally null for RNase-L activity. N1E-derived vector control and RNase-L N1E transfectants that lack and possess functional RNase-L activity, respectively, thus provided a system to identify RNase-L-regulated mRNAs.

Identification of RNase-L-regulated mRNAs—RNA substrates of RNase-L are predicted to exhibit a relative increase in expression in RNase-L-deficient as compared with RNase-L-competent cells as a result of an increase in their mRNA stability. Differential display PCR analysis was used to identify mRNAs that were differentially expressed in N1E-vector and N1E-RNase-L cell lines. Cells were treated for 18 h with murine IFNα + β to induce 2–5A synthetase and activate RNase-L, thereby enhancing RNase-L-dependent differences in gene expression between N1E-RNase-L and N1E-vector cells. Total RNA was reverse-transcribed and PCR-amplified in the presence of [α-32P]dATP using multiple primer sets (GenHunter). PCR products that displayed a reduced signal in cDNA from N1E-RNase-L as compared with N1E-vector cells represented candidate RNase-L substrates; changes in gene expression resulting from IFN treatment were also detected by differential display. PCR products that did not change in intensity provided an internal control for gel loading and amplification (e.g. clone NC3.1 and bands labeled C in Fig. 2A). Clone NC3.1 was identified as the L27a ribosomal RNA protein and was employed as a constitutively expressed control mRNA in subsequent Northern blots. Many of the PCR products isolated exhibited apparent differential expression that was not reproduced in Northern blot analyses, as has been previously reported for the differential display technique (36). Interestingly, one PCR product, clone NA4.1, displayed both IFN- and RNase-
L-dependent regulation. In the absence of IFN, NA4.1 was undetectable; however, this PCR product was dramatically increased in IFN-treated samples. Although IFN treatment induced NA4.1 in both N1E-vector and N1E-RNase-L cells, the induced levels of the NA4.1 PCR product were markedly reduced in samples from N1E-RNase-L cells, fulfilling the criteria for a candidate RNase-L substrate (Fig. 2A). Importantly, Northern blot analysis confirmed the NA4.1 mRNA regulation observed using differential display. The 350-bp NA4.1 PCR product hybridized to a 1.7-kilobase pair IFN-induced mRNA, and the IFN-induced levels of this transcript were significantly reduced in N1E-RNase-L as compared with N1E-vector cells (Fig. 2B). These results indicated that NA4.1 mRNA was induced by IFN and negatively regulated by RNase-L. To dissect further this unique pattern of regulation, we focused our studies on the characterization and regulation of NA4.1 mRNA.

**Clone NA4.1 Encodes a Ubiquitin-specific Protease—**Clone NA4.1 was used to screen a cDNA library prepared from an IFNγ-treated murine macrophage cell line (RAW). Several positive clones were isolated, one of which contained the full-length NA4.1 sequence. Sequence analysis of this 1735-bp clone revealed an 1107-bp open reading frame (ORF) encoding a 368-amino acid, 43-kDa protein (Fig. 3B). Consistent with this prediction, in vitro transcription and translation of NA4.1 resulted in a 43-kDa product (Fig. 3C); accordingly, we have named this interferon-stimulated gene, ISG43. The amino acid sequence contained regions of strong homology to ubiquitin-specific proteases, a family of enzymes that function to cleave ubiquitin from a broad range of protein substrates. In fact, a cDNA identical to ISG43 has recently been shown to encode a functional UBP (Ref. 37 and see “Discussion”). UBP homology in ISG43 is restricted primarily to four conserved domains including the Cys box and His box motifs (Fig. 3B). The Cys box cysteine residue, Cys-61 in murine ISG43, is thought to be the active site nucleophile. The QHDAQL and LPQTLTIILMRF motifs (Fig. 3B) as well as less conserved regions following the His box (not shown) are present in all UBPs (38). Extensive divergence in the remaining residues typifies UBP family members and may reflect unique properties of individual UBPs such as substrate specificity (38).

**ISG43** exhibited homology to several human expressed sequence tags; therefore, the human ISG43 cDNA was isolated by PCR and 3′-rapid amplification of cDNA ends (Fig. 3A). The human gene encodes a 372-amino acid protein; consistent with this ORF, in vitro transcription and translation of this cDNA produced a protein of approximately 43 kDa (Fig. 3C). The human protein is highly conserved, with 70% identity to the murine gene (Fig. 3B) and 76% identity to the recently isolated porcine gene (39). The complete human coding region is comprised of 10 exons spanning 15.7 kilobase pairs of a chromosome 22q11 genomic clone (GenBank™ accession number AC008079). Consistent with the dramatic induction of ISG43 by IFN (Fig. 2), a strong IFNβ response element was identified in the putative promoter region of the human gene.2 Little sequence similarity is observed in the 3′-UTRs of the human, murine, and porcine cDNAs; however, several features are conserved. Specifically, the presence of (i) ARE elements, (ii) sequences recently determined to activate 2–5A synthetase (Ref. 40; Fig. 3A), and (iii) regions with the potential to form secondary structures (not shown) in the ISG43 3′-UTR may be important in RNase-L-mediated mRNA destabilization. Indeed, the coordinate activation of 2–5A synthetase and RNase-L has been implicated in substrate recognition by RNase-L (Refs. 25 and 40, and see “Discussion”).

**Regulation of ISG43 by IFN through the JAK/STAT Pathway—**ISG43 was isolated based on its regulation by IFN and RNase-L. To characterize the activation of ISG43 by IFN and determine if RNase-L influenced its induction, we employed mouse embryo fibroblasts (MEFs) derived from WT or RNase-L KO mice (24). The kinetics of ISG43 mRNA induction were identical in WT and KO cells, with maximal levels attained by 8 h post-IFN treatment (Fig. 4A). Treatment of cells with actinomycin D blocked ISG43 induction by IFN, demonstrating the requirement for transcription (Fig. 4A, lower panel). In contrast, ISG43 induction did not require protein synthesis (Fig. 4A, lower panel); rather, cycloheximide treatment resulted in a superinduction of ISG43 mRNA, suggesting that a labile, perhaps IFN-regulated, repressor inhibits ISG43 transcription in the absence of IFN. ISG43 induction and response to inhibitors of transcription and translation were thus identical in WT and RNase-L KO cells indicating that RNase-L did not affect this phase of ISG43 expression.

Transcriptional induction of ISGs occurs primarily through activation of the JAK/STAT signaling pathway (27). To determine if a functional JAK/STAT pathway was required for IFNα induction of ISG43, RNA was prepared from mutant human cell lines lacking either JAK1 (U4A) or STAT1 (U3A) and from parental control (2fTGH) cells following IFN treatment (27). ISG43 mRNA was clearly induced in IFNα-treated 2fTGH cells, whereas no signal was detected in RNA from U4A or U3A cells (Fig. 4B). A similar requirement for JAK/STAT components was observed for ISG15 induction by IFNα (Fig. 4B). IFNα induction of ISG43 thus occurs through JAK/STAT-mediated signal transduction.

Type 1 and type 2 IFNs employ overlapping but distinct combinations of JAK/STAT signaling components to induce

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2 X.-L. Li and B. A. Hassel, unpublished data.
transcription from ligand-specific promoter elements (16); moreover, differential gene regulation by IFNα and IFNβ has been reported (41). To determine the relative responsiveness of ISG43 to induction by IFNα, -β and -γ, RNA was isolated from 2fTGH cells treated with these cytokines. Interestingly, ISG43 mRNA was induced to the greatest extent by IFNβ and was induced to a significant but lesser degree by IFNα and γ (Fig. 4C). In addition, ISG43 was induced in response to dsRNA (data not shown), as has been reported for several ISGs (42).

RNAse-L-dependent Regulation of mRNA Stability—Regulation of gene expression by IFN is transient, characterized by rapid induction and shut-off phases. The induction of ISG43 was not altered in cells lacking RNase-L; therefore, we investigated whether RNase-L functioned in the decline in ISG43 mRNA following IFN treatment. The kinetics of ISG43 mRNA induction and shut-off were first examined in WT and RNase-L KO fibroblasts. Northern blot analysis revealed an identical induction profile for ISG43 mRNA in WT and KO cells; however, the decline of ISG43 mRNA was markedly attenuated in KO as compared with WT cells (Fig. 5A). Analysis of ISG43 mRNA levels following IFN induction revealed that the apparent half-life of ISG43 mRNA increased more than 3-fold, from 3.3 h in WT cells to 11.1 h in KO cells (Fig. 5B and Table I). To confirm that the delayed shut-off of ISG43 mRNA in KO cells reflected an increase in its mRNA stability, WT and KO cells were first treated with IFN to induce ISG43 expression and then treated with actinomycin D to inhibit further transcription. ISG43 mRNA was analyzed by Northern blot at various times after actinomycin D treatment (Fig. 6A). PhosphorImager analysis of this blot revealed a 1.8-fold increase in the half-life of ISG43 mRNA in WT as compared with KO cells (Fig. 6B and Table I). The difference in half-life values between WT and KO cells determined in the presence and absence of actinomycin D chase may reflect effects of transcriptional inhibition on RNA decay (43). Specific ISG43 mRNA degradation products were not observed in RNA from IFN-treated cells (not shown), consistent with previous findings that endonucleolytic decay products are rapidly removed by cellular exonucleases (6). The reduced stability of ISG43 mRNA in IFN-treated WT cells was not due to a widespread activation of RNase-L, as no rRNA cleavage products were detected. Moreover, the levels of NC3.1 mRNA were not affected (Figs. 5A and 6A). Thus in cells lacking RNase-L, ISG43 mRNA displays delayed shut-off kinetics in response to IFN and an increase in the half-life of the induced mRNA. Furthermore, restoration of RNase-L activity in RNase-L-deficient N1E cells reduced the half-life of IFN-
induced ISG43 mRNA by 2.8-fold (Table I). Taken together, these data demonstrate the RNase-L-dependent regulation of ISG43 mRNA in intact cells.

To determine the extent to which RNase-L may affect the expression of other ISGs, the Northern blot in Fig. 5A was stripped and rehybridized with an ISG15 cDNA probe. The decline in steady state levels of ISG15 mRNA following IFN treatment was also attenuated in KO as compared with WT cells (Fig. 7A). To determine if the delayed shut-off kinetics of ISG15 mRNA reflected an RNase-L-dependent change in mRNA half-life, the blot in Fig. 6A was rehybridized with an ISG15 cDNA probe (Fig. 7B). The half-life of ISG15 mRNA displayed a dramatic, greater than 10-fold increase in KO as compared with WT cells (Fig. 7C, Table I), indicating that ISG15 mRNA stability is also regulated by RNase-L. Further studies are required to determine if all ISG mRNAs are negatively regulated by RNase-L.

**DISCUSSION**

The control of mRNA stability is an important mechanism in the regulation of gene expression. RNase-L is one of the few well characterized ribonucleases, yet its RNA substrates in the absence of viral infection have not been identified. Our findings demonstrate for the first time the modulation of the half-lives of specific cellular mRNAs by RNase-L, providing strong evidence that they represent authentic RNase-L substrates. Furthermore, we have identified a novel ISG as a member of this subset of RNase-L-regulated transcripts. ISG15 mRNA also exhibited RNase-L-dependent regulation, suggesting a novel function for the 2–5A system in the posttranscriptional attenuation of the IFN response. The identification of cellular RNase-L substrates permits direct studies of the cis- and trans-acting factors that modulate RNase-L-substrate interactions in cells. Indeed, it remains to be determined how specific features of the enzyme, substrate, and cellular components mediate the apparent selectivity of RNase-L.

**Cellular Substrates of RNase-L—**While the full spectrum of biological activities attributable to the 2–5A system is not yet known, RNase-L is now recognized to function in the antiviral and growth inhibitory effects of IFN and in apoptosis independent of IFN (22–24). The antiviral effects of the 2–5A system appear to be mediated through the preferential degradation of viral RNAs by RNase-L (25, 26); similarly, the antiproliferative/pro-apoptotic effects of RNase-L are thought to occur through the degradation of cellular mRNAs. For example, the degradation of mRNAs encoding growth-promoting or cell death inhibitory gene products may mediate the anticellular
effects associated with RNase-L activation. However, our initial differential display screen to identify RNase-L-regulated transcripts employed 36 primer sets that should theoretically amplify 60% of the expressed mRNAs in a cell (44), yet we did not detect any known proliferation regulatory genes. Rather, we identified a novel ISG, ISG43, which displayed RNase-L dependent regulation. The differential display screen identified RNase-L-regulated mRNAs, potentially including both RNase-L substrates and mRNAs that were up-regulated as a secondary effect of RNase-L inactivation. Therefore, RNase-L-dependent regulation of mRNA half-life was used as a criterion to distinguish strong candidate substrates. The induction kinetics of ISG43 mRNA were identical in RNase-L KO and WT cells; therefore, analysis of its natural mRNA decay rate from IFN-induced levels provided an accurate measurement of half-life in the absence of potential nonspecific effects of transcriptional inhibitors on mRNA decay (43). ISG43 mRNA displayed an increased half-life in IFN-treated RNase-L KO as compared with WT cells when measured by the decline in steady state mRNA levels and by inhibition of transcription (Table I). In addition, restoration of RNase-L expression in RNase-L-deficient N1E cells reduced the half-life of ISG43 mRNA, providing further evidence of its RNase-L-dependent regulation. The RNase-L-dependent destabilization of ISG43 mRNA did not reflect a global increase in RNA turnover due to widespread RNase-L activation as demonstrated by the following: (i) the absence of detectable rRNA cleavage products in IFN-treated cells, (ii) the lack of RNase-L-dependent regulation of the L27a ribosomal protein mRNA, and (iii) the absence of RNase-L-dependent changes in the majority of differential display products.

The mechanism by which ISGs are targeted for degradation by RNase-L remains to be determined. The requisite activation of 2–5A synthetase has been implicated as a potential link to

**TABLE I**

Half-life values of ISG43 and ISG15 mRNAs in cell lines which lack or express RNase-L.

| Half-life determination | Actinomycin D chase | Post-IFN induction decay | Actinomycin D chase |
|-------------------------|---------------------|------------------------|---------------------|
| ISG43                   | t1/2 (h) -Fold change | t1/2 (h) -Fold change | t1/2 (h) -Fold change |
| RNase-L +/+             | 4.2                 | 1.8                    | 3.3                 | 3.4 |
| RNase-L −/−             | 7.7                 | ND                     | 11.1                | 55.0 |
| N1E-RNase-L             | 5.0                 | 2.8                    | ND                  | ND  |
| N1E-vector              | 13.9                | ND                     | ND                  | ND  |

**FIG. 6.** RNase-L-dependent regulation of ISG43 mRNA stability. A, total RNA (20 μg/lane) from RNase-L +/+ or −/− fibroblasts treated for 17 h with murine IFNα + β (500 units/ml) in the presence or absence of actinomycin D (5 μg/ml) as indicated was analyzed for expression of ISG43 (upper panel) and NC3.1 (middle panel) by Northern blot analysis. Lower panel shows the ethidium-stained gel. B, graph of the data from PhosphorImager analysis of ISG43 expression in the blot shown in A; open squares are data points from RNase-L +/+ cells, and closed circles are data points from RNase-L −/− cells.

**FIG. 7.** Regulation of ISG15 expression by RNase-L. A, the Northern blot described in Fig. 5A was stripped and hybridized to an ISG15 cDNA probe. B, the Northern blot described in Fig. 6A was stripped and hybridized to an ISG15 cDNA probe. C, graph of the data from PhosphorImager analysis of ISG15 expression in the blot shown in B; open squares are data points from RNase-L +/+ cells, and closed circles are data points from RNase-L −/− cells.
substrate recognition by RNase-L. Specifically, contiguous double-stranded structures on target RNAs may activate 2–5A synthetase and RNase-L in a localized manner, thus limiting the extent of mRNA degradation (45). In the case of viral RNAs, encephalomyocarditis virus replicative intermediates and the human immunodeficiency virus trans-activating response element bind and activate 2–5A synthetase (46, 47). The source of dsRNA in the absence of virus infection is not known. Modeling of secondary structure in the 3′-UTR of human, mouse, and pig ISG43 mRNAs (M-FOLD, Ref. 48) revealed hairpins potentially capable of activating 2–5A synthetase (not shown), supporting the idea that dsRNA elements may identify RNase-L substrates. A recent study using the SELEX approach identified RNA ligands of 2–5A synthetase that were more potent enzyme activators than the synthetic dsRNA activator, poly(IC) (40). Interestingly, the strongest activators of 2–5A synthetase lacked significant double-stranded structure, suggesting that specific single-stranded RNA sequences may serve as natural activators of 2–5A synthetase. In this regard, the 3′-UTR of human ISG43 contains nine copies of the two consensus motifs found in the strongest 2–5A synthetase agonist identified in the SELEX screen (Fig. 3A; Ref. 40). Analysis of 2–5A synthetase activation by ISG43 mRNA will thus constitute a direct test of the localized activation hypothesis with a physiologically relevant mRNA. Furthermore, the capacity of the ISG43 3′-UTR to render heterologous mRNAs sensitive to RNase-L-mediated degradation will directly address the role of this cis element in RNase-L substrate recognition.3

Ubiquitin and Ubiquitin-like Proteins in IFN Action—ISG43 is induced as a primary response to IFN treatment, suggesting that it functions in some aspect of IFN action. The porcine orthologue of ISG43 was recently isolated in a screen to identify genes induced in porcine reproductive and respiratory syndrome virus-infected macrophages (39), and ISG43 is directly induced in response to dsRNA (data not shown), implicating ISG43 in antiviral activity. Sequence analysis revealed that ISG43 encodes a ubiquitin-specific protease; this family of enzymes function to remove ubiquitin adducts from a broad range of substrates (38). Indeed, the ubiquitin system functions in antigen presentation and viral pathogenesis through the targeting of viral and cellular proteins for proteosomal degradation via ubiquitin conjugation (49). Both ubiquitin conjugation and removal are regulated steps. Distinct deubiquitinating enzymes (DUBs) have been identified that function in critical cellular processes including development (50), growth control (51, 52), and oncogenesis (53). The specialized roles of individual DUBs are thought to reflect a substrate-specific activity of these enzymes (39). Ubiquitin mediated degradation of STAT1 functions in the down-regulation of IFN-induced signal transduction (54), and trophoblast IFN induces ubiquitin during pregnancy (55); however, a direct link between ubiquitin and type 1 IFN action has not been described. Other ubiquitin pathway enzymes that are induced by IFN have been recently identified, but their role in IFN action is not known (56). ISG15 encodes a ubiquitin-like protein that forms conjugates with cellular proteins (57); these conjugates were initially thought to represent potential physiologic substrates for the deconjugating activity of ISG43. However, the proteases that remove ubiquitin-like proteins from their conjugates were recently identified (58), and these enzymes do not share the conserved domains found in DUBs.

The recent cloning of a cDNA identical to ISG43 in a screen to identify genes activated in AML1-ETO knock-in mice may provide some insights into its function. AML1-ETO is the transcrip- tion factor gene product of an 8;21 chromosomal translocation implicated in human leukemias (37). AML1-ETO knock-in mice exhibit defective hematopoiesis, central nervous system-associated hemorrhaging, and embryonic lethality (37); therefore, ISG43 induction in these embryos may have resulted from increased embryonic or maternal IFN associated with the knock-in phenotype. Indeed, the direct induction of ISG43 by AML1-ETO was not demonstrated; however, ISG43 was expressed in hematopoietic tissues (i.e. thymus and peritoneal macrophages) and cell lines. Furthermore, constitutive expression of ISG43 blocked differentiation of myeloid cells, suggesting a role for ISG43 in hematopoiesis (37). In contrast, type 1 IFNs typically promote hematopoietic differentiation through both growth inhibition and the induction of hematopoietic specific genes (59). Taken together, these observations suggest a role for ISG43 in the feedback inhibition of IFN-induced hematopoietic differentiation. A complete understanding of how ISG43 functions in the antiviral, growth inhibitory, or other effects of IFN will require identification of its cellular ubiquitin-conjugated substrates.

The 2–5A System in Attenuation of the IFN Response—The identification of ISG43 and ISG15 as RNase-L-regulated mRNAs suggests a novel role for the 2–5A system in the attenuation of the IFN response. The degree to which other ISG mRNAs display RNase-L-dependent regulation remains to be determined. Interestingly, an attenuated decline in ISG expression, similar to that observed in RNase-L KO cells, was seen in cells treated with IFN in the presence of protein kinase C activators (60). Protein kinase C activation is known to inhibit RNase-L activation (61); thus similar effects on ISG expression appear to result from the suppression of RNase-L activity by genetic or biochemical means.

RNase-L is an established mediator of the antiviral and antiproliferative effects of IFN, and inhibition of RNase-L expression or activity typically results in a diminished response to IFN (22, 24). However, the negative regulation of ISG mRNAs by RNase-L suggests it also functions to limit the IFN response (Fig. 8). A direct prediction of this model is that the biological activities mediated by the proteins encoded by RNase-L substrates will be enhanced in RNase-L-deficient cells. Thus in the physiological conditions in which these substrate-encoded proteins serve a critical function, RNase-L-deficient cells may exhibit an enhanced response to IFN. Consistent with this prediction, when cells were infected at a virus multiplicity of infection of 1.0 or less, IFN-induced antiencephalomyocarditis virus activity was reduced in RNase-L KO as compared with WT cells (24). However, RNase-L KO cells infected at higher multiplicity of infections showed an enhanced antiviral effect of IFN (62). This observation suggests that RNase-L mediates the antipicornavirus effects of IFN in infections involving a relatively low viral load, whereas other ISGs, including those encoded by RNase-L substrates, are responsible for the antiviral activity in conditions of acute infection. Studies of RNase-L KO mice in diverse physiological con-

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3 C. S. Judge and B. A. Hassel, manuscript in preparation.
texts may reveal a more complex phenotype reflecting this broader role for RNase-L in the IFN response.

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RNase-L-dependent Destabilization of Interferon-induced mRNAs: A ROLE FOR THE 2–5A SYSTEM IN ATTENUATION OF THE INTERFERON RESPONSE
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