



**HAL**  
open science

## The 2–5A/RNase L/RNase L Inhibitor (RNI) Pathway Regulates Mitochondrial mRNAs Stability in Interferon $\alpha$ -treated H9 Cells

Florence Le Roy, Catherine Bisbal, Michelle Silhol, Camille Martinand,  
Bernard Lebleu, Tamim Salehzada

► **To cite this version:**

Florence Le Roy, Catherine Bisbal, Michelle Silhol, Camille Martinand, Bernard Lebleu, et al.. The 2–5A/RNase L/RNase L Inhibitor (RNI) Pathway Regulates Mitochondrial mRNAs Stability in Interferon  $\alpha$ -treated H9 Cells. *Journal of Biological Chemistry*, 2001, 276 (51), pp.48473 - 48482. 10.1074/jbc.M107482200 . hal-01878511

**HAL Id: hal-01878511**

**<https://hal.umontpellier.fr/hal-01878511>**

Submitted on 21 Sep 2018

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

---

**RNA: STRUCTURE METABOLISM AND  
CATALYSIS:**

**The 2–5A/RNase L/RNase L Inhibitor  
(RNI) Pathway Regulates Mitochondrial  
mRNAs Stability in Interferon  $\alpha$ -treated  
H9 Cells**

Florence Le Roy, Catherine Bisbal, Michelle  
Silhol, Camille Martinand, Bernard Lebleu  
and Tamim Salehzada

*J. Biol. Chem.* 2001, 276:48473-48482.

doi: 10.1074/jbc.M107482200 originally published online October 3, 2001

---

Access the most updated version of this article at doi: [10.1074/jbc.M107482200](https://doi.org/10.1074/jbc.M107482200)

Find articles, minireviews, Reflections and Classics on similar topics on the [JBC Affinity Sites](#).

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 64 references, 23 of which can be accessed free at  
<http://www.jbc.org/content/276/51/48473.full.html#ref-list-1>

## The 2–5A/RNase L/RNase L Inhibitor (RLI) Pathway Regulates Mitochondrial mRNAs Stability in Interferon $\alpha$ -treated H9 Cells\*

Received for publication, August 5, 2001, and in revised form, October 1, 2001  
Published, JBC Papers in Press, October 3, 2001, DOI 10.1074/jbc.M107482200

Florence Le Roy‡, Catherine Bisbal§¶, Michelle Silhol||, Camille Martinand, Bernard Lebleu, and Tamim Salehzada§

From the EP2030 CNRS, Institut de Génétique Moléculaire, 1919 route de Mende, 34293 Montpellier, France

Interferon  $\alpha$  (IFN $\alpha$ ) belongs to a cytokine family that exhibits antiviral properties, immuno-modulating effects, and antiproliferative activity on normal and neoplastic cells *in vitro* and *in vivo*. IFN $\alpha$  exerts antitumor action by inducing direct cytotoxicity against tumor cells. This toxicity is at least partly due to induction of apoptosis. Although the molecular basis of the inhibition of cell growth by IFN $\alpha$  is only partially understood, there is a direct correlation between the sensitivity of cells to the antiproliferative action of IFN $\alpha$  and the down-regulation of their mitochondrial mRNAs. Here, we studied the role of the 2–5A/RNase L system and its inhibitor RLI in this regulation of the mitochondrial mRNAs by IFN $\alpha$ . We found that a fraction of cellular RNase L and RLI is localized in the mitochondria. Thus, we down-regulated RNase L activity in human H9 cells by stably transfecting (i) RNase L antisense cDNA or (ii) RLI sense cDNA constructions. In contrast to control cells, no post-transcriptional down-regulation of mitochondrial mRNAs and no cell growth inhibition were observed after IFN $\alpha$  treatment in these transfectants. These results demonstrate that IFN $\alpha$  exerts its antiproliferative effect on H9 cells at least in part via the degradation of mitochondrial mRNAs by RNase L.

Interferons (IFNs)<sup>1</sup> belong to a family of cytokines produced and secreted by mammalian cells in response to various stimuli. Although originally discovered due to their participation in cellular defense against viral infection (1), IFNs are also known to modulate immune responses as well as control various cellular functions by altering the transcription of a large family of genes involved in cell proliferation and differentiation (2, 3).

\* This study was supported by grants from the Association pour la Recherche contre le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a pre-doctoral fellowship from Association pour la Recherche contre le Cancer. Present address: University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Dept. of Molecular Genetics and Microbiology, 675 Hoes Lane, Piscataway, NJ 08854.

§ Present address: UPR CNRS 1142, Institut de Génétique Humaine, 141 rue de la Cardonille, 34396 Montpellier, France.

¶ Present address: Laboratoire de Plasticité Cérébrale, Unité Mixte de Recherche CNRS 5102, Place Eugène Bataillon, 34095 Montpellier, France.

|| To whom correspondence should be addressed. Tel.: 4 99 61 99 73; Fax: 4 99 61 99 01; E-mail: Catherine.Bisbal@igh.cnrs.fr.

<sup>1</sup> The abbreviations used are: IFN, interferon; RLI, RNase L inhibitor; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphodehydrogenase; TIM, translocase complex in the inner membrane; TOM, translocase complex in the outer membrane.

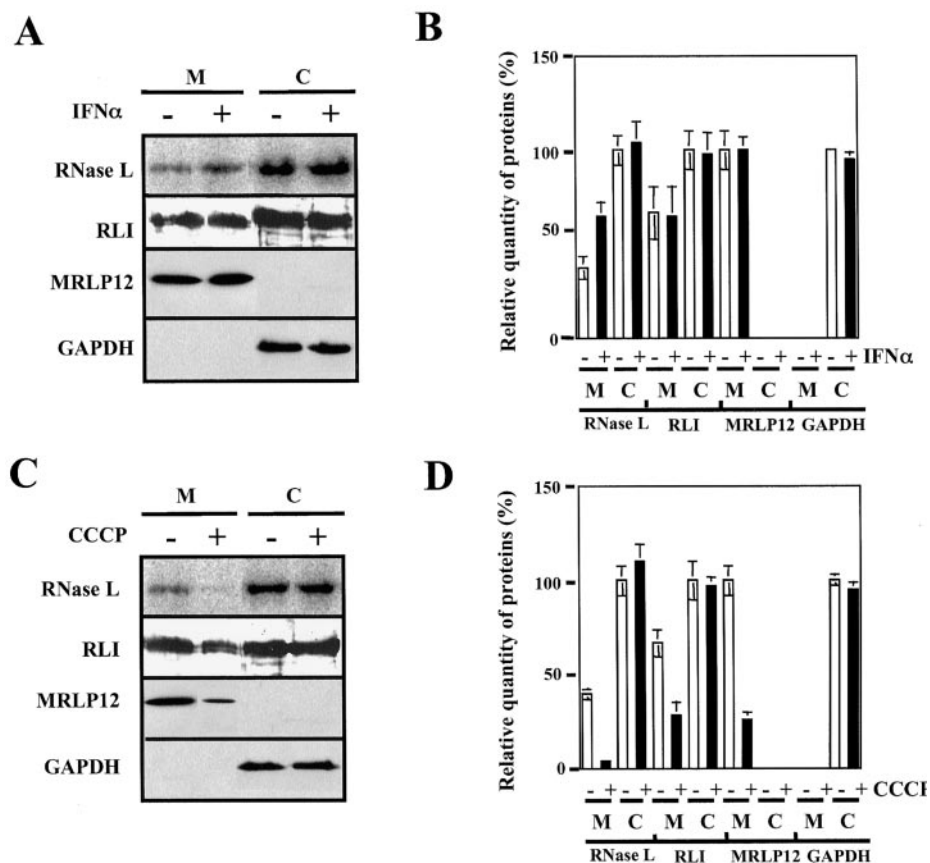
IFNs are negative regulators of cell proliferation through induction of cell cycle arrest and apoptosis, which has also been suggested to be of central importance in IFN antitumor action. IFN $\alpha$  (type I family) has emerged as a tumor suppressor protein. A number of clinical trials were thus carried out in cancer therapy with IFN $\alpha$  alone or in combination with other negative regulators of cell proliferation (4, 5). It has been shown to be effective against hematological malignancies including hairy cell leukemia, chronic myelogenous leukemia, and cutaneous T-cell lymphoma (6). IFN $\alpha$  has been used successfully in cancer therapy, but development of resistance to IFN $\alpha$  therapy has often been observed in patients. Although the molecular basis of the inhibition of cell growth by IFN $\alpha$  is partially understood, there is a correlation between the sensitivity of cells to the antiproliferative action of IFN $\alpha$  and the down-regulation of their mitochondrial mRNAs and the impairment of mitochondrial function upon IFN $\alpha$  treatment (7, 8).

In this work, we have studied the role of one of the enzymatic pathways induced by IFN $\alpha$ , the 2–5A/RNase L pathway, in the regulation of mitochondrial mRNAs. The 2–5A/RNase L pathway is an RNA degradation pathway (9). IFN $\alpha$  induces the expression of 2–5A synthetase(s) which, upon activation by double-stranded RNA, convert ATP into an unusual series of oligomers known as 2–5A (10, 11). 2–5A activates RNase L, a latent endoribonuclease, which inhibits protein synthesis by cleaving mRNAs 3' of UpNp (uracyl nucleotide, the nucleotide sequence of mRNA recognized by RNase L) sequences (12, 13). RNase L activity is also regulated by RNase L inhibitor (RLI), which inhibits 2–5A binding to RNase L, thereby switching off the 2–5A/RNase L system (14).

We postulated that RNase L may participate in the antiproliferative effect of IFN $\alpha$  by playing an important role in the IFN $\alpha$ -induced down-regulation of mitochondrial mRNAs, which leads to a decrease in cellular ATP levels and suppression of mitochondrial function (7, 8). In this report, we demonstrate the presence of RNase L and RLI in mitochondria. Furthermore we show that in IFN $\alpha$ -treated H9 cells, the level of mitochondrial mRNAs is a function of RNase L activity in the mitochondria. Inhibition of RNase L can prolong the stability of mitochondrial mRNAs.

### EXPERIMENTAL PROCEDURES

*Cells, IFN, and Carbonyl Cyanide *m*-Chlorophenylhydrazone (CCCP)*—Human H9 cells (lymphoma, cutaneous T lymphocyte; American Type Culture Collection (ATCC): HTB-176), human HeLa cells (adenocarcinoma, cervix; ATCC: CCL-2), and human Daudi cells (Burkitt's lymphoma, B lymphoblast; ATCC: CCL-213) were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal calf serum. Cells were incubated at 37 °C in a 5% CO<sub>2</sub>, 95% air atmosphere. Cell growth was determined by counting viable cells as determined by trypan blue exclusion. Human IFN $\alpha$  (IFN $\alpha$ 2) was used at 500 units/ml. The CCCP was added to culture media at 20  $\mu$ M for the same period of time as IFN $\alpha$  treatment. The experiments were done in triplicate, and the S.D. is indicated on the plots.



**FIG. 1. RNase L and RLI are present in mitochondria.** *A*, H9 cells were treated (+) or not (-) with IFN $\alpha$  (500 units/ml) for 24 h. Mitochondria were isolated as described under "Material and Methods." Mitochondrial extract (*M*, 100  $\mu$ g of protein) and cytoplasmic extract (*C*; 100  $\mu$ g of protein) were analyzed by the radiochemical affinity-labeling assay (2-5ApCp) for RNase L 2-5A binding activity and by Western blot with the appropriate polyclonal antibodies for RLI, MRLP12, and GAPDH protein content. *B*, densitometric analysis of the gels shown in panel *A* treated by IFN $\alpha$  (500 units/ml) (■) or not (□). For RNase L 2-5A binding activity of RLI and GAPDH proteins, a value of 100% corresponds to the amount of these proteins in cytoplasmic extract at time 0, before IFN $\alpha$  treatment. For MRLP12, a value of 100% corresponds to the amount of this protein in mitochondrial extract at time 0, before IFN $\alpha$  treatment. *Error bars* refer to the S.D. obtained in three independent experiments. *C*, H9 cells were treated (+) or not (-) with CCCP (20  $\mu$ M) for 24 h. Mitochondria were isolated as described under "Material and Methods," and proteins were analyzed as described above. *D*, densitometric analysis of the gels shown in panel *C* treated by CCCP (20  $\mu$ M) (■) or not (□). For RNase L 2-5A binding activity of RLI and GAPDH proteins, a value of 100% corresponds to the amount of these proteins in cytoplasmic extract at time 0, before CCCP treatment. For MRLP12, a value of 100% corresponds to the amount of this protein in mitochondrial extract at time 0, before CCCP treatment. *Error bars* refer to the S.D. obtained in three independent experiments.

**Cell Extracts and Isolation of H9 Mitochondria**—H9 cells were washed twice in ice-cold phosphate-buffered saline (PBS: 140 mM NaCl, 2 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4), pelleted, resuspended in 2 volumes of hypotonic buffer (0.5% (v/v) Nonidet P-40 (NP-40), 20 mM Hepes pH 7.5, 10 mM potassium acetate, 15 mM magnesium acetate, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml aprotinin, 150  $\mu$ g/ml leupeptin), incubated 10 min on ice, disrupted with a Dounce homogenizer, and centrifuged 10 min at 10,000  $\times$  *g*. The obtained supernatant was designated post-nuclear extract. For mitochondria isolation, H9 cells (2.0  $\times$  10<sup>8</sup> cells) were washed twice in ice-cold PBS, pelleted, and resuspended in 5 ml of a 10 mM Tris-HCl, pH 6.7, 10 mM KCl, 0.15 mM MgCl<sub>2</sub> solution. After incubation for 10 min on ice, cells were disrupted with a Dounce homogenizer and, after the addition of sucrose (0.25 M final concentration), centrifuged at 1,000  $\times$  *g* for 5 min to remove the nuclei. The supernatant was complemented with K-EDTA (1 mM final concentration) and centrifuged at 10,000  $\times$  *g* for 10 min at 4  $^{\circ}$ C. The mitochondrial pellet was resuspended in cold buffer A (0.25 M sucrose, 10 mM Tris acetate, pH 7.0). The supernatant was named the cytoplasmic extract (without mitochondria). The mitochondria were purified on a 30% (v/v) Percoll (Amersham Pharmacia Biotech) cushion in buffer A supplemented with 1 mM K-EDTA. The mitochondria were collected from the cushion and centrifuged to eliminate Percoll. Mitochondria were lysed by sonication, and the total mitochondrial extract was used for further experiments. The protein concentration was determined by Bradford reaction (15).

**Expression Vectors and Transfections**—The coding sequences of human RLI cDNA (14) and human RNase L cDNA (13) were subcloned in pcDNA3neo (Invitrogen) by standard procedures (16). RLI sense/pcDNA3neo (7  $\mu$ g), RNase L antisense/pcDNA3neo (7  $\mu$ g), and

the empty vector (7  $\mu$ g) were transfected in H9 cells by electroporation as previously described (17). Transfected H9 cell clones were isolated by dilution of 1 cell/slot in 96-slot plates in the presence of 1 mg/ml Geneticin (Life Technologies, Inc.). Two clones were selected for each construction and named RLI S1 and RLI S2 for clones expressing sense RLI cDNA and RNase L AS1 and RNase L AS2 for clones expressing RNase L cDNA in the antisense orientation. An empty pcDNA3 vector-transfected clone was used as a control.

**Isolation of Mitochondrial DNA and Mitochondrial cDNA Synthesis**—Mitochondrial DNA was isolated from human Daudi cells by lysis in a 2% (w/v) SDS solution, two phenol dichloromethane extractions, and ethanol precipitation. Polymerase chain reaction was performed directly on mitochondrial DNA to amplify cytochrome *b*, ATPase 6, and cytochrome oxidase subunit II cDNA. Primer 1 (5'-GGGAATTCACAA-GAACACCAATGACCCCAA-3') and primer 2 (5'-GGGAATTCACAA-GACAGGCCCATTTGAGT-3') were used to amplify cytochrome *b* cDNA. Primer 3 (5'-GGGAATTCACAAATGAACGAAAATCTGTTC-3') and primer 4 (5'-GGGAATTCCTGGTCAATTATGTGTGTCTGTCG-3') were used to amplify ATPase6 cDNA. Primer 5 (5'-GGGAATTCAT-CTTAATGGCACATGCAGCGC-3') and primer 6 (5'-GGGAATTCCTT-ACAGTGGGCTCTAGAGGG-3') were used to amplify cytochrome oxidase subunit II cDNA.

**RNA Analysis**—RLIS1, RLIS2, RNase L AS1, RNase L AS2, and control cells were plated at 2.0  $\times$  10<sup>5</sup> cells/ml, treated for 24 or 48 h by IFN $\alpha$  (500 units/ml), and collected for RNA extraction using the guanidine thiocyanate-lithium chloride procedure (18). Total RNA (20  $\mu$ g) were analyzed by Northern blot (16). After transfer onto nylon membranes (Appligen), rRNA were revealed with 0.5 M (sodium acetate) pH 5, 0.04% (w/v) methylene blue. The nylon membranes were then incu-

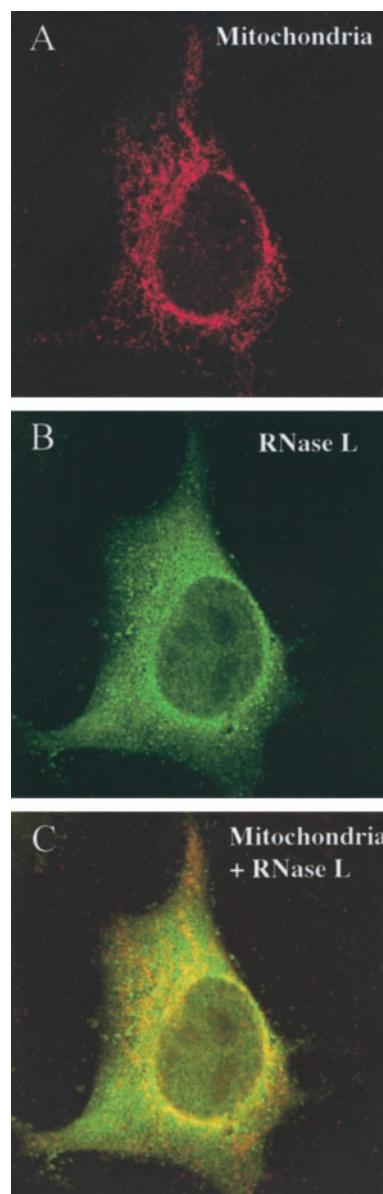
bated with the appropriate [<sup>32</sup>P]cDNA probes (as indicated in the figure legends) synthesized using the Multiprime radiolabeling kit (Life Technologies, Inc.). After autoradiography on a PhosphorImager 445-SI (Molecular Dynamics), the mRNA were quantified by image analysis with the Image Quant program (Molecular Dynamics). The experiments were done in triplicate, and the S.D. is indicated on the plots.

**Western Blot**—Proteins (100 μg of post-nuclear extract, 100 μg of cytoplasmic extract, 100 or 200 μg of mitochondrial extract) were fractionated by SDS/polyacrylamide gel electrophoresis and transferred electrophoretically onto a nitrocellulose membrane (Schleicher and Schuell) according to Towbin *et al.* (19). For detection with antibody, the nitrocellulose membranes were incubated in PBS supplemented with 10% (w/v) skim milk powder for 30 min and then soaked overnight at 4 °C in the same buffer with the appropriate antibodies: rabbit polyclonal anti-glyceraldehyde-3-phosphodehydrogenase (anti-GAPDH) at 1:3000 dilution, rabbit polyclonal anti-RLI at 1:500 dilution (20), rabbit polyclonal anti-RNase L at 1:1000 dilution (20), rabbit polyclonal anti-MRLP12 at 1:5000 dilution. The membranes were washed with PBS supplemented with 0.05% (v/v) Tween 20 and incubated for 1 h at room temperature with donkey anti-rabbit IgG antibody or with sheep anti-mouse IgG antibody conjugated to horseradish peroxidase at 1:2000 dilution (Amersham Pharmacia Biotech). Chemiluminescence was generated by reaction with the PerkinElmer Life Sciences enhanced chemiluminescence kit. The autoradiographies were scanned, and protein bands were quantified by image analysis with the Intelligent Quantifier program (BioImage Systems Corp.). The experiments were done in triplicate, and the S.D. is indicated on the plots.

**Radiocovalent Affinity Labeling of RNase L**—The radiolabeled 2–5A<sub>4</sub>-3'-[<sup>32</sup>P]pCp probe was oxidized at its 3' end (21). This modified probe can be covalently linked to amine groups on RNase L and allows analysis by denaturing gel electrophoresis as initially described by Wreschner *et al.* (22) with our modifications (23). Post-nuclear extracts (100 μg), cytoplasmic extracts (100 μg), or mitochondrial extracts (100 or 200 μg) were incubated with the oxidized 2–5A<sub>4</sub>-3'-[<sup>32</sup>P]pCp probe for 30 min in ice and for a further 20 min at room temperature with sodium cyanoborohydride (20 mM final concentration). Proteins were analyzed by 10% (v/v) SDS/polyacrylamide gel electrophoresis (24). Labeled proteins were visualized after autoradiography, and protein bands were quantified by image analysis with the Intelligent Quantifier program (BioImage Systems Corp.). The experiments were done in triplicate, and the S.D. is indicated on the plots.

**Immunofluorescence**—Human HeLa cells were cultivated at a concentration of 10<sup>5</sup> cells/ml in RPMI 1640 supplemented with 10% (v/v) fetal calf serum for 24 h. The cell culture medium was then changed to RPMI 1640 supplemented with 10% (v/v) fetal calf serum and 100 nM MitoTracker dye (red CM-H2XRos, Molecular Probes), incubated at 37 °C for 30 min, washed at 37 °C with PBS, and incubated for 5 min at room temperature in PBS, 3.7% (v/v) formalin. After several washes with PBS, cells were incubated in PBS, 0.1% (v/v) Triton X-100, 10% (w/v) bovine serum albumin for 20 min, then in PBS, 10% (w/v) bovine serum albumin for 1 h. Cells were further incubated for 1 h at room temperature with a rabbit anti-RNase L polyclonal antibody at 1:200 dilution, washed with PBS, incubated for 1 h at room temperature with fluorescein isothiocyanate isomer-conjugated goat anti-rabbit IgG at 1:500 dilution, washed with PBS, fixed with MOWIOL (2.4 g of MOWIOL 4–88 in 6 g of glycerol, 6 ml of water, 12 ml of 0.2 M Tris-HCl, pH 8.5), and analyzed by confocal microscopy on a Bio-Rad MRC 1024. The experiments were done in triplicate.

**In Organello Pulse Chase**—Immediately after purification, aliquots of the mitochondrial fraction (1 mg of mitochondrial protein) were resuspended in 0.5 ml of an incubation buffer containing 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM K<sub>2</sub>HPO<sub>4</sub>, 0.05 mM EDTA, 5 mM MgCl<sub>2</sub>, 1 mM ADP, 10 mM glutamate, 2.5 mM malate, 10 mM Tris/HCl pH 7.4, 1 mg/ml bovine serum albumin, and 20 μCi of [<sup>32</sup>P]UTP (800 Ci/mmol) (25). Incubation was carried out at 37 °C for 60 min, and the mitochondrial samples were pelleted at 13,000 × g for 1 min to remove non-incorporated [<sup>32</sup>P]UTP. The mitochondria were resuspended in the incubation buffer described above in the presence of a 200-fold excess of unlabeled UTP and incubated for increasing periods of time as indicated in the figure legends. Mitochondrial RNAs were extracted twice with an equal volume of a phenol/dichloromethane/isoamyl alcohol mixture (25:25:1; v/v) at room temperature. After ethanol precipitation of the aqueous phase and centrifugation, the nucleic acids pellet was dissolved in sterile water. The labeled transcripts from the different mitochondrial fractions were hybridized at 42 °C with mitochondrial cDNA spotted (5 μg) on a nitrocellulose membrane already prehybridized for 48 h in 50% (v/v) formamide, 50 mM Na<sub>2</sub>HPO<sub>4</sub> pH 6.5, 0.8 mM NaCl, 1 mM EDTA, 2.5× Denhardt's, 250 μg/ml salmon sperm DNA,



**FIG. 2. Mitochondrial localization of RNase L by confocal analysis.** HeLa cells were incubated with MitoTracker dye (red fluorescence, A) then with a rabbit anti-RNase L polyclonal antibody and fluorescein-conjugated goat anti-rabbit IgG (green fluorescence, B). Confocal microscopy analysis was performed using appropriate filters for visualization of red (A), green (B), or combined (C) fluorescence resulting from fluorescein linked to the secondary antibody against the rabbit anti-RNase L polyclonal antibody and MitoTracker molecules.

and 500 μg/ml baker yeast tRNA. After 48 h of hybridization, the membranes were washed for 10 min at 20 °C with 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% (w/v) SDS, 30 min at 65 °C with 0.2× SSC, 15 min at 37 °C with 2× SSC, RNase A (10 μg/ml), and 2 times for 30 min at 37 °C with 2× SSC (26). After autoradiography on a PhosphorImager 445-SI (Molecular Dynamics), the mRNAs were quantified by image analysis with the Image Quant program (Molecular Dynamics). The experiments were done in triplicate, and the S.D. is indicated on the plots.

**In Organello Mitochondrial mRNA Stability in Presence of 2–5A**—Transcription of mitochondrial RNA was done on isolated mitochondria in the presence of 20 μCi of [<sup>32</sup>P]UTP (800 Ci/mmol) as described in the previous paragraph (25). The mitochondria were then resuspended in the incubation buffer described above in the presence of a 200-fold excess of unlabeled UTP and incubated for increasing periods of time as indicated in the figure legends in the presence or absence of 2–5A<sub>4</sub> (100 μM). Mitochondrial RNA was extracted, spotted, and analyzed as described above.

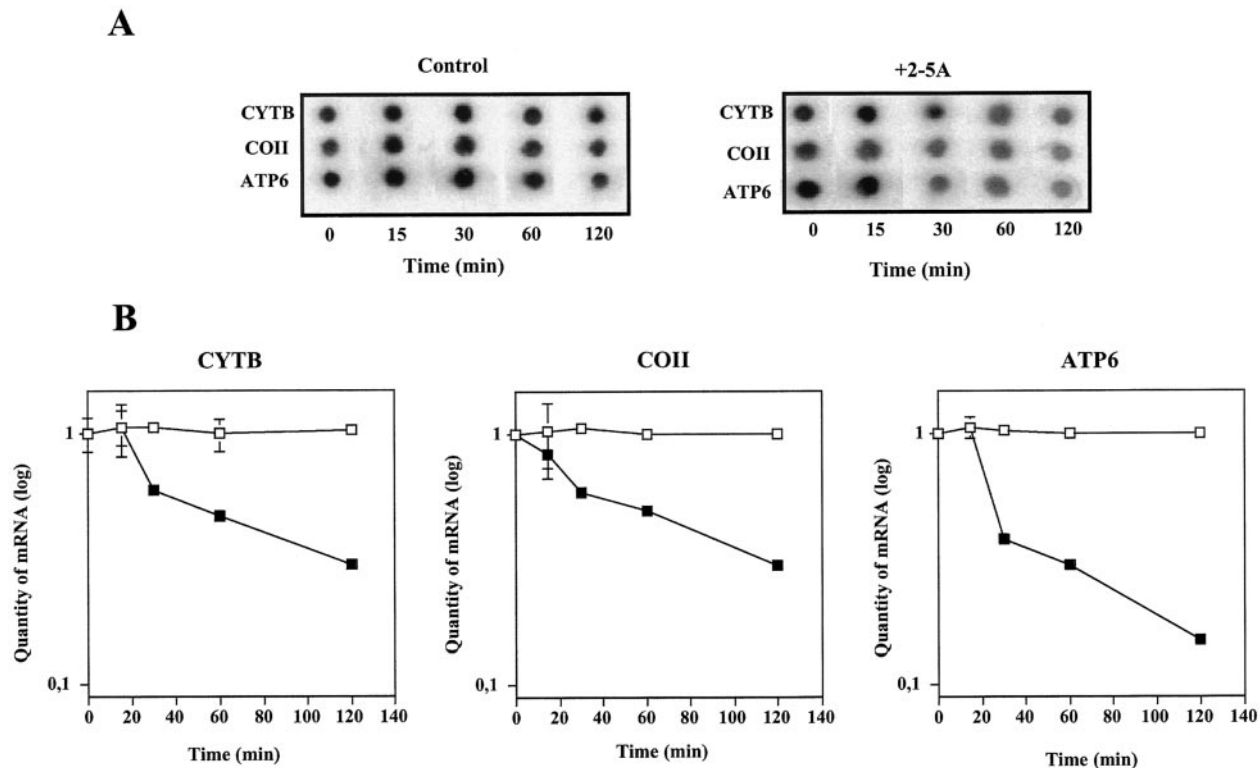


FIG. 3. **Activation of RNase L by 2-5A destabilizes mitochondrial mRNAs in isolated mitochondria.** A, mitochondria were isolated from H9 cells, and radioactive transcription was performed with [ $\alpha$ - $^{32}$ P]UTP for 1 h at 37 °C followed by a chase with unlabeled UTP in the presence or not of 2-5A<sub>4</sub> (100  $\mu$ M). At the indicated times, mitochondrial RNAs were extracted and hybridized with spotted mitochondrial cDNA. *CYTB*, cytochrome *b*; *ATP6*, ATPase 6; *COII*, cytochrome oxidase subunit II. B, densitometric analysis of the gels shown in panel A. Data are presented as the log of RNA remaining versus time. A value of 1 corresponds to the log of the amount of RNA at time 0, before incubation with unlabeled UTP and 2-5A<sub>4</sub> (■) or UTP alone (□). The S.D. obtained from three independent experiments are indicated by error bars.

## RESULTS

**RNase L and RLI Are Present in the Mitochondria**—As mentioned in the introduction, our working hypothesis is that down-regulation of mitochondrial mRNAs in IFN $\alpha$ -treated cells could be due to RNase L activation. For RNase L and RLI to act as regulators of mitochondrial mRNA stability they must, first, be present in mitochondria and, second, RNase L must be able to degrade mitochondrial mRNAs. In keeping with this hypothesis we provide the first evidence for the presence of RNase L and RLI in isolated mitochondria (Fig. 1, panels A and B). RNase L was detected by radiocovalent affinity labeling, because the level of RNase L present in the mitochondria is lower than the detection threshold level on Western blot, using our polyclonal antibody against RNase L. Twenty-four hours of IFN $\alpha$  treatment increased mitochondrial quantities of RNase L but did not affect RLI level (panels A and B). The absence of GAPDH in the mitochondrial extract (panels A and C) indicated that there was no cytoplasmic contamination during mitochondrial fractionation. As a positive control of mitochondria isolation and purity, we probed the Western blot for MRLP12 protein, which is the mitochondrial mammalian homologue to chloroplastic and bacterial L12 ribosomal proteins (27).

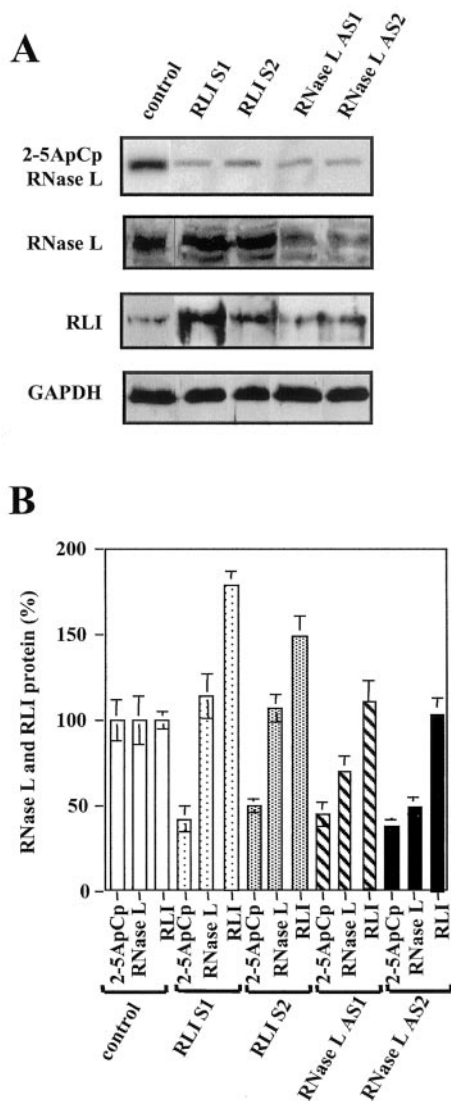
To prove that RNase L and RLI are imported into mitochondria from the cytoplasm and that their detection in mitochondria is not due to nonspecific adsorption of these proteins on the outer mitochondrial membrane, CCCP was used to abolish the membrane potential required for polypeptide import through the inner mitochondrial membrane (28, 29). CCCP treatment of H9 cells decreased MRLP12 protein levels 4-fold, RNase L protein levels 8-fold, and RLI protein levels 2-fold in mitochondria, indicating that these proteins are imported from the cytoplasm into the mitochondria (Fig. 1, panels C and D). No changes were observed in the cytoplasmic amounts of RNase L,

RLI, or GAPDH after CCCP treatment.

The presence of RNase L in mitochondria was also established by immunofluorescence and confocal microscopy. A fraction of the cellular RNase L population clearly co-localized with MitoTracker, a mitochondria-specific marker (30–32). The yellow coloration (Fig. 2, panel C) resulting from the combined fluorescence of MitoTracker (red fluorescence, Fig. 2, panel A) and fluorescein linked to a secondary antibody against the rabbit anti-RNase L polyclonal antibody (green fluorescence, Fig. 2, panel B) clearly showed this co-localization.

**Mitochondrial mRNAs Are Targets of RNase L**—Because RNase L was present in the mitochondria, we proceeded with pulse-chase experiments on intact mitochondria isolated from H9 cells to confirm that mitochondrial mRNAs could be destabilized by mitochondrial RNase L. We followed the stability of radiolabeled mitochondrial mRNAs in the presence or not of 2-5A, the specific activator of RNase L (10, 13). As illustrated by the data in Fig. 3, the addition of 2-5A to isolated intact mitochondria destabilizes mitochondrial mRNAs including cytochrome *b*, ATPase 6, and cytochrome oxidase subunit II. This result clearly shows that mitochondrial RNase L, once activated directly by 2-5A, can destabilize mitochondrial mRNAs.

**Decreased RNase L Activity Inhibits IFN $\alpha$ -induced Down-regulation of Mitochondrial mRNAs**—To study the potential role of RNase L and RLI in the down-regulation of mitochondrial mRNAs by IFN $\alpha$ , we reduced endogenous RNase L activity in human H9 T lymphocytes, chosen for their sensitivity to the antiproliferative effect of IFN $\alpha$ , by stably transfecting an RNase L cDNA antisense construct or an RLI sense cDNA construct. Two clones from each stable transfection were selected based on their decreased 2-5A binding capacity, as an indicator of diminished cellular RNase L activity and their RLI and RNase L protein content (Fig. 4). The two RLI sense cDNA



**FIG. 4. Characterization of the H9 transfectants.** *A*, post-nuclear extracts (100  $\mu$ g of proteins) from H9 cells transfected with the empty vector (control), the RLI sense cDNA (RLI S1 and RLI S2), or the RNase L cDNA in the antisense orientation (RNase L AS1 and RNase L AS2) were analyzed by the radiocovalent affinity labeling assay (2-5ApCp) for RNase L 2-5A binding activity or by Western blot with the appropriate polyclonal antibodies for RNase L, RLI, and GAPDH proteins. *B*, densitometric analysis of the gels shown in *panel A*. A value of 100% corresponds to the amount of RNase L 2-5A binding activity (2-5ApCp) or RNase L and RLI protein in control cells. Error bars refer to the S.D. obtained in three independent experiments.

transfectant clones (RLI S1 and RLI S2) expressed the same level of RNase L compared with the control cells but exhibited a 2-fold decrease in 2-5A binding activity due to RLI overexpression (Fig. 4, *panels A and B*). In these two clones, there was a strong correlation between RLI overexpression and down-regulation of RNase L 2-5A binding activity. In the two RNase L antisense cDNA transfectant clones (RNase L AS1 and RNase L AS2), decreased 2-5A binding activity correlated with lower expression levels of RNase L and no change in RLI expression levels. The level of GAPDH protein was constant in control and in transfected cells.

These clones were treated with IFN $\alpha$  (500 units/ml) for 24 and 48 h, and their mitochondrial mRNAs levels were analyzed by Northern blot (Fig. 5). In the control H9 cells, IFN $\alpha$  treatment down-regulated several mitochondrial mRNAs such as cytochrome *b*, ATPase 6, and cytochrome oxidase subunit II, as previously observed in other cell types (7, 8, 33). In contrast, in

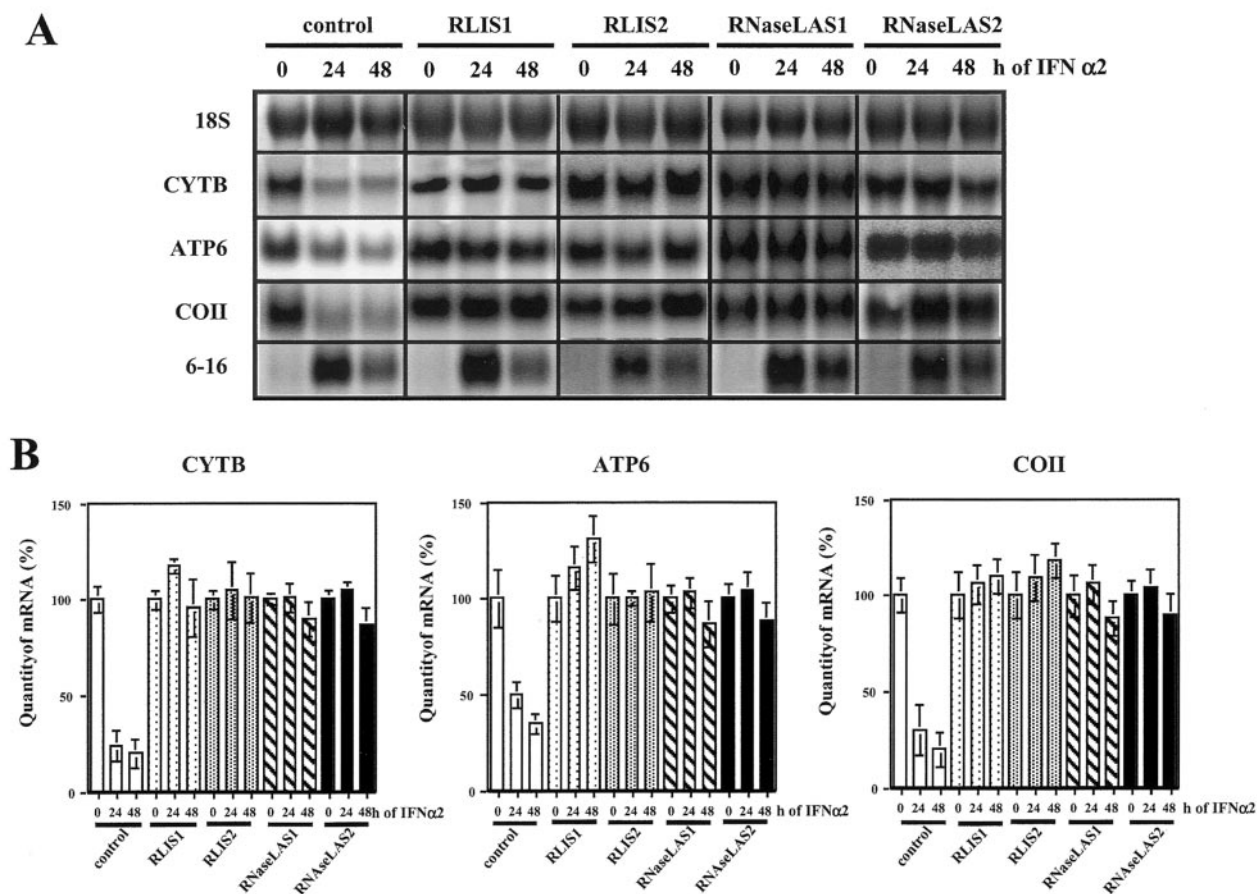
the RLI S and RNase L AS transfectants, no decrease in these mitochondrial mRNAs was observed after IFN $\alpha$  treatment. The 6-16 mRNA, a known IFN $\alpha$ -induced gene, was correctly induced by IFN $\alpha$  treatment in all transfectants as in control cells and as previously described (34), indicating that these cells responded to IFN $\alpha$  treatment. The level of 18 S rRNA was constant in control and in transfected cells. These experiments thus support a role of RNase L in the accelerated degradation of mitochondrial mRNAs in IFN $\alpha$ -treated cells.

**Inhibition of the Antiproliferative Effect of IFN $\alpha$  in H9 Transfectants**—IFN $\alpha$  exerts an antiproliferative effect on various cell lines, and the down-regulation of mitochondrial mRNAs is only observed in cells sensitive to the antiproliferative effect of IFN $\alpha$ .

There is a correlation between the antiproliferative response and the mitochondrial response (7, 8, 33). This correlation and the fact that a decrease in mitochondrial mRNAs leads to a decrease in mitochondrial proteins and to a decrease in cellular ATP levels (7, 8) suggests that the down-regulation of mitochondrial mRNAs could participate in the growth inhibition induced by IFN $\alpha$ . Therefore, we charted the growth rate of H9 cells and H9 transfectants for 5 days after IFN $\alpha$  (500 units/ml) treatment. IFN $\alpha$  inhibited cell proliferation in the control H9 cells, transfected with an empty vector (Fig. 6, *panel A*). On the contrary, the H9 transfectants with no IFN $\alpha$ -induced down-regulation of mitochondrial mRNAs (Fig. 5) were resistant to the growth inhibitory effects of IFN $\alpha$  (Fig. 6, *panels B-E*).

**No Mitochondrial mRNAs Destabilization Is Observed after IFN $\alpha$  Treatment when Mitochondrial RNase L Activity Is Low**—To determine whether there is a correlation between the down-regulation of mitochondrial mRNAs and RNase L activity in the mitochondria, we performed a radiocovalent affinity labeling assay on purified mitochondrial extracts from the H9 transfectants. Less 2-5A binding activity of RNase L was detected in the mitochondria of the H9 transfectants than in the control H9 cells (Fig. 7, *panels A and B*). In the RLI S1 and RLI S2 clones, the decreased RNase L 2-5A binding activity correlated with an increased level of RLI in the mitochondria. In the RNase L AS1 and RNase L AS2 clones, the quantity of RLI found in mitochondria was the same as that in the control cells, indicating that the decrease of RNase L 2-5A binding activity is probably because of low levels of RNase L, as shown for total cell extract (Fig. 4). The absence of GAPDH confirms that there was no cytoplasmic contamination in the mitochondrial extract, and MRLP12 levels indicate that an equal amount of mitochondrial proteins was loaded for each transfectant.

Because our data demonstrated that the 2-5A/RNase L/RLI pathway is involved in the down-regulation of mitochondrial mRNAs by IFN $\alpha$  (Fig. 5), that RNase L was present in the mitochondria, and that mitochondrial mRNAs can be destabilized by mitochondrial RNase L after its activation by 2-5A (Figs. 1-3), we postulated that mitochondrial RNase L may regulate mitochondrial mRNA stability in response to IFN $\alpha$  treatment. To verify this hypothesis, we compared the stability of radiolabeled mitochondrial mRNAs by pulse-chase analysis of intact mitochondria isolated from control H9 cells and from RLI S- and RNase L AS-transfected H9 cells treated or not with IFN $\alpha$ . As shown in Fig. 8, IFN $\alpha$  treatment of control H9 cells resulted in the destabilization of mitochondrial mRNAs cytochrome *b*, ATPase 6, and cytochrome oxidase subunit II. It is worth noting that the steady state level of radiolabeled mitochondrial mRNAs obtained in control IFN $\alpha$ -treated cells was decreased by 30% (compare times 0 on autoradiographies in Fig. 8). Because these radiolabeled mitochondrial mRNAs are obtained by transcription in mitochondria isolated from control IFN $\alpha$ -treated cells, this could indicate that IFN $\alpha$  treatment



**FIG. 5. Northern blot analysis of mitochondrial mRNAs in IFN $\alpha$ -treated H9 cells.** A, H9 transfected cells with the empty vector (*control*), RLI sense cDNA (*RLI S1* and *RLI S2*), or RNase L cDNA in the antisense orientation (*RNase L AS1* and *RNase L AS2*) were treated by IFN $\alpha$  (500 units/ml) for 24 h and 48 h, and RNA was extracted. Total RNAs (20  $\mu$ g) were analyzed by Northern blot with the different probes, cytochrome *b* (*CYTB*), ATPase 6 (*ATP6*), cytochrome oxidase subunit II (*COII*) for mitochondrial mRNAs and 6–16 probe for a cytoplasmic IFN $\alpha$ -induced mRNA. 18 S rRNAs are revealed by methylene blue coloration of the nylon sheet. B, densitometric analysis of the gels shown in A. A value of 100% corresponds to the amount of RNA in control cells at time 0, before IFN $\alpha$  treatment. Error bars refer to the S.D. obtained in three independent experiments.

also decreased mitochondrial mRNAs transcription in control cells, as suggested by Lou *et al.* (7). This can also be explained either as a destabilization of the mitochondrial mRNA by the activated RNase L in the IFN $\alpha$ -treated cells simultaneous with transcription or as a combination of a decreased transcription and degradation of mitochondrial mRNA. Our results, showing a destabilization of mitochondrial mRNAs in control IFN $\alpha$ -treated cells, are in favor of the second and third hypotheses. In the RLI S1 and RNase L AS2 H9 transfectants, with low RNase L activity, the mitochondrial mRNAs remained stable upon IFN $\alpha$  treatment (Fig. 9; panels A and B).

#### DISCUSSION

We were able to detect RNase L and its inhibitor, RLI, in isolated mitochondria (Figs. 1, 2, and 7) and showed that mitochondrial RNase L could destabilize mitochondrial mRNAs after its activation by its specific activator, 2–5A, in intact mitochondria (Fig. 3). These results presented the possibility that RNase L may degrade mitochondrial mRNAs after IFN $\alpha$  treatment. Furthermore, previous reports describe the presence of the p69 isoform of 2–5A synthetase in mitochondria (35, 36). IFN $\alpha$  treatment increases the RNase L level in mitochondria (Fig. 1) as it was shown for 2–5A synthetase (35). It was therefore conceivable that IFN $\alpha$  treatment could activate the 2–5A/RNase L pathway in mitochondria and promote the destabilization of mitochondrial mRNAs, as described here. RNase L and RLI are imported from the cytoplasm into the

mitochondria because suppression of the mitochondrial membrane potential by CCCP diminished RNase L and RLI mitochondrial level (Fig. 1). The import of proteins into mitochondria is a complex mechanism that is not completely understood. In fact not all the mitochondrial-targeted proteins obey the same rule. The majority of mitochondrial proteins are nuclear-encoded and synthesized in the cytosol as precursors proteins. These proteins are imported into mitochondria by a complex process that involves cytosolic chaperones, a hetero-oligomeric translocase of the outer mitochondrial membrane (TOM) complex, a translocase complex in the inner membrane (TIM), an ATP-driven import motor associated with TIM and soluble matrix proteins mediating proteolytic maturation and folding of the imported proteins (37–39). Although most mitochondrial precursors contain an amino-terminal-targeting presequence, many proteins are imported into the mitochondria without processing, including rhodanese and 3-oxoacyl-CoA thiolase (40), the  $\beta$ -subunit of human electron-transferring flavoprotein (41), chaperonin 10 (42), and p53 (43). For these proteins the targeting information is present in the mature protein. Although the majority of the proteins imported into mitochondria possess an amino-terminal targeting sequence, this sequence is not a prerequisite for import because p53, heme lyases, and apocytochrome *c* lack this signal. Two internal segments of apocytochrome *c* are reported to be important for its mitochondria targeting (44). The mechanism by which such proteins are



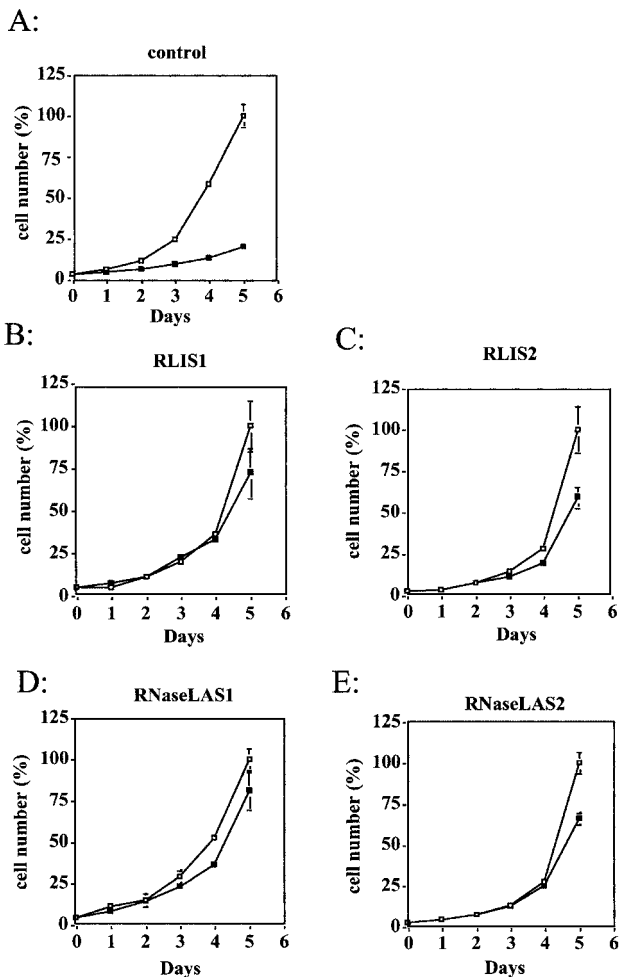


FIG. 6. Antiproliferative activity of IFN $\alpha$  on H9-transfected cells. H9-transfected cells were treated by IFN $\alpha$  (500 units/ml) (■) or not (□), and cell growth was monitored each day by counting viable cells as determined by trypan blue exclusion. A, H9 cells transfected with the empty vector (*control*). B and C, H9 cells transfected with the RLI sense cDNA (*RLI S1* and *RLI S2* respectively). D and E, H9 cells transfected with the RNase L antisense cDNA orientation (*RNase LAS1* and *RNase LAS2*, respectively). A value of 100% corresponds to the number of control or transfected cells at day 5 and untreated by IFN $\alpha$ . Error bars refer to the S.D. obtained in three independent experiments.

internalized into the mitochondria are only partially understood.

Mitochondrial N-terminal sequences do not share any primary sequence identity. Sequence analysis of a variety of processed and unprocessed mitochondrial proteins shows that these proteins have an amino-terminal sequence rich in positive charges that also share an ability to form an amphiphilic  $\alpha$ -helix (45). These observations have been confirmed experimentally (40, 46, 47).

Secondary structure analysis of RLI with PSIPred, a protein structure prediction program (48), reveals that the first 28 residues of the amino-terminal sequence form an  $\alpha$ -helix. Helical wheel analysis of this  $\alpha$ -helix structure shows its amphiphilic property. Moreover, use of MitoProt, a program that permits theoretical evaluation of mitochondrial-targeting sequences (49, 50), predicts a potential mitochondrial-targeting sequence in the N-terminal region of RLI with the probability of import into mitochondria of 39%. These computer analyses do not detect the presence of a cleavage site in RLI protein sequence, which is in agreement with our experimental results demonstrating that RLI is not processed. Both the mitochondrial and the cytoplasmic RLI have the same apparent molecular weight by SDS/

polyacrylamide gel electrophoresis, indicating that it is not processed after import into the mitochondria (Figs. 1 and 7). RLI possesses an uncleavable mitochondrial N-terminal-targeting sequence (an amphiphilic  $\alpha$ -helix), and the mitochondrial membrane potential is required for its import because CCCP treatment of H9 cells decreased the level of RLI in mitochondria (Fig. 1). One hypothesis is that RLI is imported into the mitochondria via the TOM-TIM pathway (38, 39), but this remains to be demonstrated experimentally.

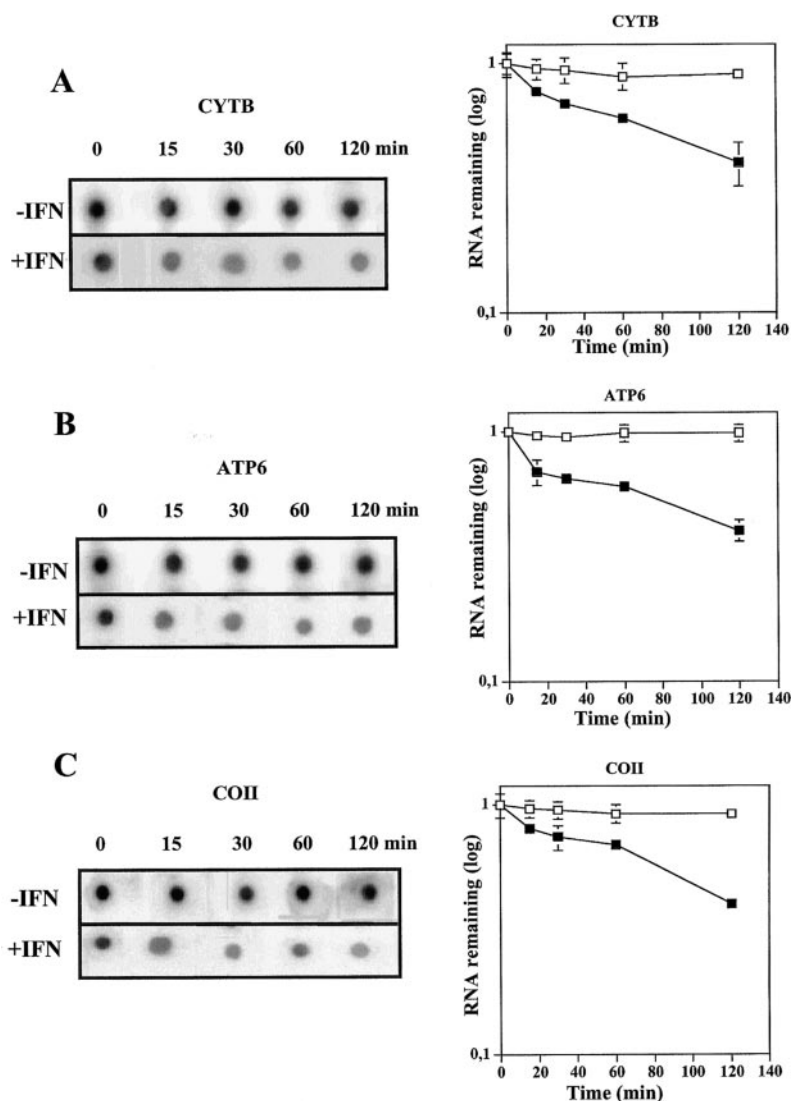
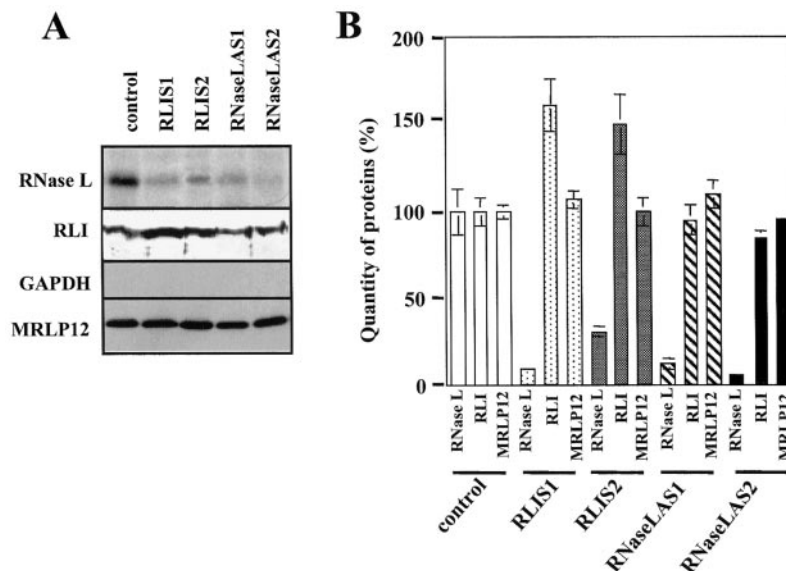
Because secondary structure analysis does not predict the presence of an amphiphilic  $\alpha$ -helix at the amino terminus of RNase L, we analyzed the RNase L sequence using PSORT II, a program used to predict protein localization (51–53). This program is based on data obtained experimentally (54). Such analysis of RNase L amino acid sequence gives the highest score (39%) for a mitochondrial localization of RNase L. Our experimental results and computer analysis together indicate that RNase L has a mitochondrial localization but does not possess an amino-terminal-targeting sequence. These results indicate that the mitochondrial-targeting sequence information of RNase L is internal, as has been described for other proteins as apocytochrome *c* or p53. The mechanisms of recognition and transport of such proteins are unknown or only partially understood (38, 43).

To study the role of the 2–5A/RNase L pathway and its inhibitor, RLI, in the IFN $\alpha$ -induced down-regulation of mitochondrial mRNAs, we down-regulated RNase L activity in human H9 T lymphocytes by stably transfecting (i) RNase L antisense (RNase L AS) cDNA, which inhibits RNase L expression and consequently RNase L activity, or (ii) RLI sense (RLI S) cDNA (Figs. 4 and 7). In contrast to mock-transfected control cells, in the transfectants with decreased RNase L activity, we did not observe a down-regulation of mitochondrial mRNAs by IFN $\alpha$  (Fig. 5). These results revealed a direct correlation between mitochondrial mRNAs decrease and the level of RNase L activity in the mitochondria (Figs. 5 and 7).

We followed the stability of radiolabeled mitochondrial mRNAs using pulse-chase analysis in intact mitochondria isolated from H9 transfectants treated or not with IFN $\alpha$ . IFN $\alpha$  treatment clearly induced a diminution of the stability of mitochondrial mRNAs in the control H9 cells (Fig. 8). These mitochondrial mRNAs remained stable in the H9 transfectants with decreased mitochondrial RNase L activity (Fig. 9). This result indicates that mitochondrial RNase L participates in mitochondrial mRNAs regulation in IFN $\alpha$ -treated cells (Figs. 5, 7, 8, and 9). Interestingly, the growth inhibition activity of IFN $\alpha$  is abolished in parallel with the stabilization of mitochondrial mRNAs (Figs. 5 and 6). This result confirms and extends the previous observations that the down-regulation of mitochondrial mRNAs by IFN $\alpha$  was observed only in cells sensitive to the antiproliferative effect of IFN (7, 8).

rRNA and viral RNAs have been the first described targets of RNase L. Recently, cellular mRNAs have been identified as regulated by RNase L. ISG43 and ISG15 mRNAs were shown to be destabilized by RNase L after IFN treatment (55), and we identified MyoD mRNA as a target of RNase L during C2 myoblast differentiation (56). Here, we demonstrate that mitochondrial mRNAs decrease during IFN $\alpha$  treatment because of post-transcriptional events in which RNase L plays a central role. These data provide new and direct evidence for a more general role of RNase L in the regulation of cellular mRNAs. The demonstration that mitochondrial mRNAs are targets for RNase L may explain how RNase L and RLI could regulate the antiproliferative effect of IFN. Moreover, these results could explain how RNase L plays a role in apoptosis. Indeed, mitochondria play a central role in apoptosis by releasing cyto-

**FIG. 7. RNase L and RLI analysis in mitochondria of H9 transfectants.** *A*, mitochondrial extracts (200  $\mu$ g of protein) from H9 cells transfected with the empty vector (*control*), with the RLI sense construction (*RLI S1* and *RLI S2*), and with the RNase L antisense construction (*RNase L AS1* and *RNase L AS2*) were analyzed by the radiocovalent affinity-labeling assay (2–5ApCp) for RNase L 2–5A binding activity and by Western blot with the appropriate polyclonal antibodies for RLI, MRLP12, and GAPDH protein contents. *B*, densitometric analysis of the gels shown in *panel A*. A value of 100% corresponds to the amount of proteins in mitochondrial extracts of control cells. *Error bars* refer to the S.D. obtained in three independent experiments.

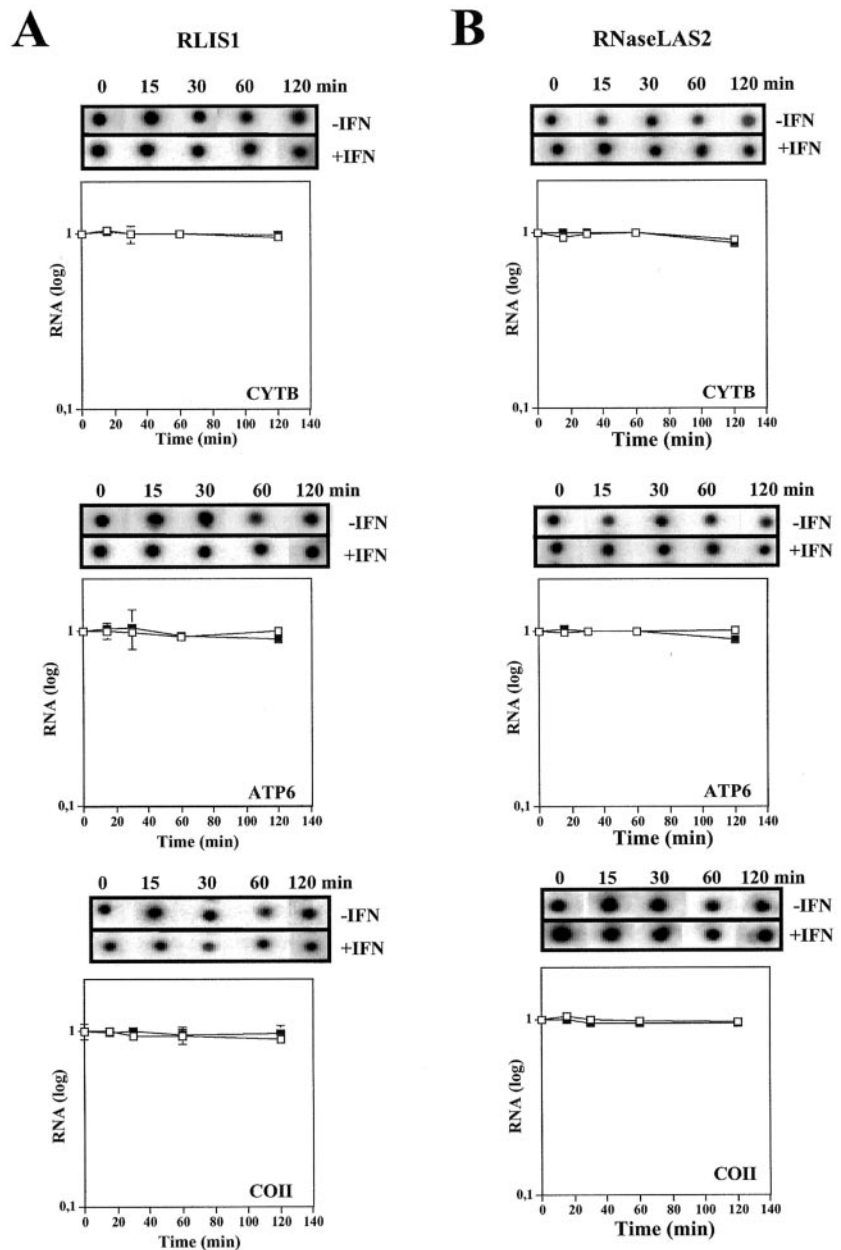


**FIG. 8. Mitochondrial mRNAs are destabilized in control H9 cells treated by IFN $\alpha$ .** Control H9 cells transfected with the empty vector were treated by IFN $\alpha$  (+) or not (–) for 24 h. Mitochondria were isolated from these cells, and radioactive transcription was performed with [ $\alpha$ - $^{32}$ P]UTP for 1 h at 37  $^{\circ}$ C followed by a chase with unlabeled UTP. At the indicated times, mitochondrial RNAs were extracted and hybridized with spotted mitochondrial cytochrome *b* cDNA (*CYTB*) (*A*), ATPase 6 cDNA (*ATP6*) (*B*), cytochrome oxidase II cDNA (*CO*) (*C*). A densitometric analysis of the gels is shown at the right side of each autoradiography. Data are presented as the log of RNA remaining *versus* time. A value of 1 corresponds to the log of the amount of RNA at time 0, before incubation with unlabeled UTP in control H9 cells treated (■) or not (□) by IFN $\alpha$ . The S.D. obtained from three independent experiments are indicated by error bars.

chrome *c*, which leads to the activation of caspase-3 (57–60), and several studies implicate RNase L in apoptosis (61–64). Degradation of mitochondrial mRNAs by RNase L will obviously lead to inhibition of mitochondrial protein synthesis, as

Lewis et al. (8) show that decrease in mitochondrial mRNA levels leads to a decrease in mitochondrial protein levels. The consequence of such an inhibition is a loss of mitochondrial membrane potential, which results in osmotic swelling and

**FIG. 9. Mitochondrial mRNAs are stable in RLI S1 and RNase L AS2 H9 transfectants treated by IFN $\alpha$ .** H9 cells transfected with the RLI sense construction (*RLI S1*) or with the RNase L antisense construction (*RNase L AS2*) were treated (+) or not (-) by IFN $\alpha$  for 24 h. Pulse-chase experiments were performed on mitochondria isolated from RLI S1 (A) and RNase L AS2 (B) H9 transfectants and analyzed as described in the legend of Fig. 8. A densitometric analysis of the gels is shown under each autoradiography. Data are presented as the log of RNA remaining *versus* time. A value of 1 corresponds to the log of the amount of RNA at time 0, before incubation with unlabeled UTP in each transfected cells treated (■) or not (□) by IFN $\alpha$ . The S.D. obtained from three independent experiments are indicated by *error bars*. *CYTB*, cytochrome *b*; *ATP6*, ATPase 6; *COII*, cytochrome oxidase subunit II.



cytochrome *c* release (58). In the same way, Yanase *et al.* (65) have recently shown that during IFN $\alpha$ -induced apoptosis there is a release of cytochrome *c* from mitochondria, a decline in mitochondrial membrane potential, and caspase-3 activation (65). On the other hand, activation of RNase L causes caspase-dependent apoptosis accompanied by cytochrome *c* release (66), (67). This induction of cell death by RNase L is inhibited by Bcl-2 (66). These results and our results show that the regulation of mitochondrial mRNAs by RNase L and its inhibitor RLI is at the center of the pathway leading from IFN $\alpha$  to apoptosis.

**Acknowledgments**—We are grateful to G. Uzé (EP2030 CNRS, Montpellier, France) for the gift of IFN $\alpha$ 2, L. Marty (Institut de Génétique Moléculaire de Montpellier (IGMM), Montpellier, France) for the gift of antibody against MRLP12, G. Cathala (IGMM) for the gift of antibody against GAPDH, N. Mechti (INSERM U475, Montpellier, France) for the gift of 6–16 cDNA, and Nicole Lautredou (Centre Régional d’Imagerie Cellulaire, INSERM, Montpellier, France) for confocal microscopy analysis. We thank Dr. Patric Turowsky (IGH, Montpellier, France) for help in computer analysis of RNase L and RLI proteins. We thank Dr. A. Shatkin (Center for Advanced Biotechnology and Medicine, Piscataway, NJ) and Dr. J. A. Langer (University of Medicine and

Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ) for helpful suggestions on the manuscript and F. Kim and Dr. L. Milligan (IGMM) for critical reading of the manuscript.

#### REFERENCES

- Isaacs, A., and Lindenmann, J. (1957) *Proc. R. Soc. Lond. B. Biol. Sci.* **147**, 258–267
- Sen, G. C., and Lengyel, P. (1992) *J. Biol. Chem.* **267**, 5017–5020
- Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) *Annu. Rev. Biochem.* **67**, 227–264
- Pelicano, L., and Chelbi, A. M. (1998) *Bull. Cancer (Paris)* **85**, 313–318
- Lindner, D. J., Hofmann, E. R., Karra, S., and Kalvakolanu, D. V. (2000) *Biochim. Biophys. Acta* **1496**, 196–206
- Gutterman, J. U. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 1198–1205
- Lou, J., Anderson, S. L., Xing, L., and Rubin, B. Y. (1994) *J. Interferon Res.* **14**, 33–40
- Lewis, J. A., Huq, A., and Najjarro, P. (1996) *J. Biol. Chem.* **271**, 13184–13190
- Silverman, R. H. (1997) in *Ribonucleases: Structure and Functions* (D’Alessio, G., and Riordan, J. F., eds) pp. 515–551, Academic Press, Inc., New York
- Kerr, I. M., and Brown, R. E. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 256–260
- Rebouillat, D., and Hovanessian, A. G. (1999) *J. Interferon Cytokine Res.* **19**, 295–308
- Floyd, S. G., Slattery, E., and Lengyel, P. (1981) *Science* **212**, 1030–1032
- Zhou, A., Hassel, B. A., and Silverman, R. H. (1993) *Cell* **72**, 753–765
- Bisbal, C., Martinand, C., Silhol, M., Lebleu, B., and Salehzada, T. (1995) *J. Biol. Chem.* **270**, 13308–13317
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–251

16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, pp. 3–87, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Martinand, C., Montavon, C., Salehzada, T., Silhol, M., Lebleu, B., and Bisbal, C. (1999) *J. Virol.* **73**, 290–296
18. Cathala, G., Savouret, J. F., Mendez, B., West, B. L., Karin, M., Martial, J. A., and Baxter, J. D. (1983) *DNA (N. Y.)* **2**, 329–335
19. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354
20. Martinand, C., Salehzada, T., Silhol, M., Lebleu, B., and Bisbal, C. (1998) *Eur. J. Biochem.* **254**, 238–247
21. Bayard, B., Bisbal, C., Silhol, M., Cnockaert, J., Huez, G., and Lebleu, B. (1984) *Eur. J. Biochem.* **142**, 291–298
22. Wreschner, D. H., Silverman, R. H., James, T. C., Gilbert, C. S., and Kerr, I. M. (1982) *Eur. J. Biochem.* **124**, 261–268
23. Bisbal, C., Salehzada, T., Lebleu, B., and Bayard, B. (1989) *Eur. J. Biochem.* **179**, 595–602
24. Laemmli, U. K. (1970) *Nature* **227**, 680–685
25. Enriquez, J. A., Perez, M. A., Lopez, P. M., and Montoya, J. (1996) *Methods Enzymol.* **264**, 50–57
26. Bonnieu, A., Rech, J., Jeanteur, P., and Fort, P. (1989) *Oncogene* **4**, 881–888
27. Marty, L., and Fort, P. (1996) *J. Biol. Chem.* **271**, 11468–11476
28. Nelson, N., and Schatz, G. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4365–4369
29. Reid, G. A., and Schatz, G. (1982) *J. Biol. Chem.* **257**, 13056–13061
30. Gonzalez, G. M., Perez, B. R., Ding, L., Duan, L., Boise, L. H., Thompson, C. B., and Nunez, G. (1994) *Development* **120**, 3033–3042
31. Wang, H. G., Rapp, U. R., and Reed, J. C. (1996) *Cell* **87**, 629–638
32. Nechushtan, A., Smith, C. L., Hsu, Y. T., and Youle, R. J. (1999) *EMBO J.* **18**, 2330–2341
33. Shan, B., Vazquez, E., and Lewis, J. A. (1990) *EMBO J.* **9**, 4307–4314
34. Kelly, J. M., Porter, A. C., Chernajovsky, Y., Gilbert, C. S., Stark, G. R., and Kerr, I. M. (1986) *EMBO J.* **5**, 1601–1606
35. Hovanessian, A. G., Laurent, A. G., Chebath, J., Galabru, J., Robert, N., and Svab, J. (1987) *EMBO J.* **6**, 1273–1280
36. Besse, S., Rebouillat, D., Marie, I., Puvion, D. F., and Hovanessian, A. G. (1998) *Exp. Cell Res.* **239**, 379–392
37. Leuenberger, D., Bally, N. A., Schatz, G., and Koehler, C. M. (1999) *EMBO J.* **18**, 4816–4822
38. Koehler, C. M. (2000) *FEBS Lett.* **476**, 27–31
39. Lithgow, T. (2000) *FEBS Lett.* **476**, 22–26
40. Hammen, P. K., Gorenstein, D. G., and Weiner, H. (1994) *Biochemistry* **33**, 8610–8617
41. Finocchiaro, G., Colombo, I., Garavaglia, B., Gellera, C., Valdameri, G., Garbuglio, N., and Didonato, S. (1993) *Eur. J. Biochem.* **213**, 1003–1008
42. Rospert, S., Junne, T., Glick, B. S., and Schatz, G. (1993) *FEBS Lett.* **335**, 358–360
43. Marchenko, N. D., Zaika, A., and Moll, U. M. (2000) *J. Biol. Chem.* **275**, 16202–16212
44. Mayer, A., Neupert, W., and Lill, R. (1995) *J. Biol. Chem.* **270**, 12390–12397
45. von, H. G., Steppuhn, J., and Herrmann, R. G. (1989) *Eur. J. Biochem.* **180**, 535–545
46. Jarvis, J. A., Ryan, M. T., Hoogenraad, N. J., Craik, D. J., and Hoj, P. B. (1995) *J. Biol. Chem.* **270**, 1323–1331
47. Ni, L., Heard, T. S., and Weiner, H. (1999) *J. Biol. Chem.* **274**, 12685–12691
48. Jones, D. T. (1999) *J. Mol. Biol.* **292**, 195–202
49. Claros, M. G. (1995) *Comput. Appl. Biosci.* **11**, 441–447
50. Claros, M. G., and Vincens, P. (1996) *Eur. J. Biochem.* **241**, 779–786
51. Horton, P., and Nakai, K. (1996) *Proc. Int. Conf. Intell. Syst. Mol. Biol.* **4**, 109–115
52. Horton, P., and Nakai, K. (1997) *Proc. Int. Conf. Intell. Syst. Mol. Biol.* **5**, 147–152
53. Nakai, K., and Horton, P. (1999) *Trends Biochem. Sci.* **24**, 34–36
54. Nakai, K., and Kanehisa, M. (1992) *Genomics* **14**, 897–911
55. Li, X. L., Blackford, J. A., Judge, C. S., Liu, M., Xiao, W., Kalvakolanu, D. V., and Hassel, B. A. (2000) *J. Biol. Chem.* **275**, 8880–8888
56. Bisbal, C., Silhol, M., Laubenthal, H., Kaluza, T., Carnac, G., Milligan, L., Le Roy, F., and Salehzada, T. (2000) *Mol. Cell. Biol.* **20**, 4959–4969
57. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) *Cell* **86**, 147–157
58. Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V., and Di, L. F. (1999) *Eur. J. Biochem.* **264**, 687–701
59. Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999) *Annu. Rev. Cell Dev. Biol.* **15**, 269–290
60. Waterhouse, N. J., and Green, D. R. (1999) *J. Clin. Immunol.* **19**, 378–387
61. Castelli, J. C., Hassel, B. A., Wood, K. A., Li, X. L., Amemiya, K., Dalakas, M. C., Torrence, P. F., and Youle, R. J. (1997) *J. Exp. Med.* **186**, 967–972
62. Zhou, A., Paranjape, J., Brown, T. L., Nie, H., Naik, S., Dong, B., Chang, A., Trapp, B., Fairchild, R., Colmenares, C., and Silverman, R. H. (1997) *EMBO J.* **16**, 6355–6363
63. Castelli, J. C., Hassel, B. A., Maran, A., Paranjape, J., Hewitt, J. A., Li, X. L., Hsu, Y. T., Silverman, R. H., and Youle, R. J. (1998) *Cell Death Differ.* **5**, 313–320
64. Zhou, A., Paranjape, J. M., Hassel, B. A., Nie, H., Shah, S., Galinski, B., and Silverman, R. H. (1998) *J. Interferon Cytokine Res.* **18**, 953–961
65. Yanase, N., Ohshima, K., Ikegami, H., and Mizuguchi, J. (2000) *J. Interferon Cytokine Res.* **20**, 1121–1129
66. Diaz, G. M., Rivas, C., and Esteban, M. (1997) *Virology* **236**, 354–363
67. Rusch, L., Zhou, A., and Silverman, R. H. (2000) *J. Interferon Cytokine Res.* **20**, 1091–1100

## Additions and Corrections

Vol. 277 (2002) 478–485

### Control of prostaglandin stereochemistry at the 15-carbon by cyclooxygenases-1 and -2. A critical role for serine 530 and valine 349.

Claus Schneider, William E. Boeglin, Jeffery J. Prusakiewicz, Scott W. Rowlinson, Lawrence J. Marnett, Nigulas Samel, and Alan R. Brash

**Page 481, Table I:** Several columns in this table were misaligned. The correctly aligned table is shown following.

TABLE I

*Stereochemical analysis of PGE<sub>2</sub>, 11-HETE, and 15-HETE formed by wild-type and Ser-530 mutant COX-2*

Incubations of wild-type and mutant COX-2 with 50 or 100  $\mu$ M [1-<sup>14</sup>C]arachidonic acid were conducted for 10 min at room temperature, extracted, and analyzed as described under "Experimental Procedures."

	PGE <sub>2</sub>		11-HETE		15-HETE	
	15S	15R	11S	11R	15S	15R
	%		%		%	
Human COX-2						
Wild-type	>99			>99	86	14
S530T	4	96	— <sup>a</sup>		13	87
S530M	13	87	—			>99
S530V	18	82		>99	1	99
Mouse COX-2						
Wild-type	>99			>99	72	28
S530M	6	94	—		4	96
S530A	95	5		>99	61	39

<sup>a</sup> —, not detected.

Vol. 276 (2001) 48473–48482

### The 2-5A/RNase L/RNase L inhibitor (RLI) pathway regulates mitochondrial mRNAs stability in interferon $\alpha$ -treated H9 cells.

Florence Le Roy, Catherine Bisbal, Michelle Silhol, Camille Martinand, Bernard Lebleu, and Tamim Salehzada

The abbreviation within the title was printed incorrectly. The correct title is shown above.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.