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A NEW COMPONENT OF THE INTERFERON-REGULATED 2–5A PATHWAY*

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The 2–5A/RNase L system is considered as a central pathway of interferon (IFN) action and could possibly play a more general physiological role as for instance in the regulation of RNA stability in mammalian cells.

We describe here the expression cloning and initial characterization of RLI (for RNase L inhibitor), a new type of endoribonuclease inhibitor.

RLI cDNA codes for a 68-kDa polypeptide whose expression is not regulated by IFN. Its expression in reticulocyte extracts antagonizes the 2–5A binding ability and the nuclease activity of endogenous RNase L or the cloned 2DR polypeptide. The inhibition requires the association of RLI with the nuclease and is dependent on the ratio between the two proteins. Likewise RLI is coimmunoprecipitated with the RNase L complex by a nuclease-specific antibody. RLI does not lead to 2–5A degradation or to irreversible modification of RNase L. The overexpression of RLI in stably transfected HeLa cells inhibits the antiviral activity of IFN on encephalomyocarditis virus but not on vesicular stomatitis virus.

RLI therefore appears as the first described and potentially important mediator of the 2–5A/RNase L pathway.

Interferons (IFNs)[1] are produced and secreted by mammalian cells in response to various inducers, such as double-stranded RNAs (1) or viral infection (2). IFNs induce the transcription of a large family of genes (3) involved in the defense against viral infections, the control of cell proliferation and differentiation, and the modulation of immune responses (4).

The 2–5A system is one of the major pathways induced by IFN; it has been implicated in some of the antiviral activities of the IFNs and might play an essential role in regulating RNA turnover and stability in cells (5, 6).

It has been described as composed of three enzymatic activities, *e.g.* 2–5A-synthetases, 2–5A-phosphodiesterase, and RNase L. 2–5A-synthetases are a family of four IFN-inducible enzymes which, upon activation by double-stranded RNA, convert ATP into the unusual series of oligomers known as 2–5A (7–9). The 2–5A-phosphodiesterase might be involved in the catabolism of 2–5A from its 2′,3′ end (10). The steady state level of 2–5A, which is very unstable, could also be controlled by dephosphorylation by phosphatases (11). The 2–5A-dependent endoribonuclease L or RNase L is the effector enzyme of this system. Its activation by subnanomolar levels of 2–5A leads to the inhibition of protein synthesis by cleavage of mRNAs at the 3′ side of UpNp sequences (12).

Variations in intracellular 2–5A and 2–5A-synthetase(s) levels have been observed during virus infection, cell growth, or cell differentiation even in the absence of exogenous IFN treatment (13). Published data are more contradictory concerning RNase L; some suggest that IFN treatment or cell growth status increase RNase L activity whereas others report no alteration in RNase L activity in these circumstances (see Ref. 13 for a review). These apparent contradictions might be explained by differences in the sensitivity of the methods used to detect RNase L. RNase L can indeed be detected by its binding to a radioactive 2–5A-3′-[^{32}P]pCp probe (14, 15), by its ability to degrade mRNAs (16), or by Western blotting with polyclonal (17) or monoclonal antibodies (18).

The correlations between variations of 2–5A/RNase L and the control of cell growth and differentiation, however, plead for a more general physiological role of this system. It is interesting to note here that RNase L is preferentially associated to polyribosomes (17).

RNase L was first described as a 200-kDa protein (19). Its molecular weight varies with the conditions of analysis, with the protein concentrations, or with the origin of cell extract (20, 21). Zhou *et al.* (1993) have cloned a 80-kDa polypeptide (2DR) which binds 2–5A and cleaves poly(uridylic acid) in a reticulocyte extract but not in a wheat-germ extract (22). More recently the properties of the 2DR were studied after expression of its cDNA in insect cells and purification to homogeneity (23).

We have recently characterized a monoclonal antibody (mAb3) which neutralizes RNase L activity; it recognizes a RNA-binding protein of 80 kDa associated (but distinct) with the previously known 2–5A binding 80-kDa protein and with 2DR. We have therefore proposed that the high molecular mass protein complex (around 200 kDa) first characterized as RNase L contains at least two distinct polypeptides *e.g.* a 2–5A-binding protein (2–5ABP or 2DR) and a mAb3 recognized polypeptide which has been called RNABP (18). Its rôle in the functioning and regulation of RNase L in intact cells has not yet been elucidated.

The control of mRNA turnover rate is now recognized as a critical element of gene expression regulation. However, the mechanisms responsible for mRNA degradation in mammalian cells are poorly understood (24). The 2–5A system appears as a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X74987 and X76388.

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[1] The abbreviations used are: IFN, interferon; RLI, RNase L inhibitor; mAb, monoclonal antibody; bp, base pair(s); PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; VSV, vesicular stomatitis virus; EMCV, encephalomyocarditis virus; kb, kilobase(s); RNABP, RNA-binding protein.

well characterized system of RNA degradation which might be involved in the control of RNA metabolism.

We describe here the cloning of a polypeptide inhibitor of the 2–5A pathway; it will be referred to as RLI for RNase L inhibitor. This protein was isolated from an expression library by binding to 2–5ApCp. *In vitro* translation of this cDNA gives rise to a protein of 68 kDa which associates specifically with 2–5ABP and RNABP as shown by immunoprecipitation with the mAb3 monoclonal antibody. This *in vitro* translated 68-kDa protein can also form a complex with the 2DR protein.

Evidence is prevailed that RLI is a regulatory protein whose co-expression inhibits the binding of 2–5A by the endogenous RNase L or by 2DR, and as a consequence the 2–5A-dependent activation of RNase L. RLI does not promote 2–5A degradation.

Moreover the overexpression of the RLI cDNA in HeLa cells results in the inhibition of the IFN-activated 2–5A pathway.

Although not regulated by IFN treatment RLI might be a key component of the IFN system and of the regulation of RNA stability in mammalian cells.

EXPERIMENTAL PROCEDURES

Cells and Cell Extracts—Daudi cells were grown in suspension culture and maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 60 IU/ml penicillin, and 50 μg/ml streptomycin. HeLa cells were grown in the same medium with 2 mM glutamine. Cells were treated with Huα/β IFN, as indicated; IFN was a generous gift of Dr. Ara Hovanessian (Institut Pasteur, Paris, France).

Cell extracts were prepared as described previously (18); briefly, the cells were resuspended in 2 volumes of hypotonic buffer, disrupted in a Dounce homogenizer, and centrifuged at 10,000 × *g*.

Radiobinding Assay for RNase L and Radiocovalent Affinity Labeling of RNase L—The radiobinding assay (25) and the covalent labeling procedure (26) were performed with S10 cell extracts, rabbit-reticulocyte lysates, or wheat-germ extracts (with or without the different cloned proteins translated) as a source of RNase L. 2–5A_4-3′-[^{32}P]pCp (3000 Ci/mmol) (27) was used as probe, as indicated in the legends of the figures. The radiobinding and radiocovalent labeling procedures were utilized with the modifications previously described (21).

Isolation of cDNA Clones—The λ-Zap Daudi cell cDNA library (10^6 plaques) was a generous gift from Dr. G. Uzé (IGMM, Montpellier, France). It was screened with 2–5A_4-3′-[^{32}P]pCp (3000 Ci/mmol). The nitrocellulose filters were soaked in 10 mM isopropyl-1-thio-β-D-galactopyranoside, dried, and overlaid onto phage plates for 3 h at 37 °C. The filters were rinsed in buffer A (10 mM HEPES, pH 7.5, 80 mM KCl, 5 mM $Mg(OAc)_2$, 1 mM EDTA, 5% (v/v) glycerol, 20 mM β-mercaptoethanol) (15) saturated with 5% (v/v) skimmed milk in buffer A, and rinsed again in buffer A without milk. They were then incubated overnight at 4 °C in buffer A with 2–5A_4-3′-[^{32}P]pCp (5 × 10^5 disintegrations/min/filter). After washing in buffer A, the filters were autoradiographed on x-ray films. This procedure has led to the isolation of a 2861-bp clone named H2ABP.

An internal primer determined from the sequence of H2APB was used to isolate by anchored PCR a full-length cDNA of 3568 bp coding for RLI (see Fig. 1A). Briefly, poly(dT) cDNA was first tailed with dG nucleotides with terminal transferase. This cDNA (0.5 μg) was amplified by PCR (30 cycles) in a final volume of 50 μl using a 5′ poly(dC) primer with *Pst*I and *Bam*HI sites (5′-TTTCTGCAGGATCCCCCCCCCCCC-3′) and an internal primer (5′-CACTTAGATCATGTTCCACCACAAT-3′) as described in Fig. 1*A*. This cDNA fragment was cloned in the *Bgl*II site of H2ABP to give the full-length cDNA of 3568 bp.

The 2DR cDNA was obtained by PCR with primers determined from the published sequence (22).

Nucleotide Sequence Analysis—H2ABP in pSK (pBluescript II SK, Stratagene) was sequenced by the Sanger dideoxy sequencing method (T7 sequencing kit, Pharmacia) after nested 3′ deletions. These deletions were generated with exonuclease III-S1 nuclease digestion followed by filling in with Klenow DNA polymerase (Pharmacia). The full-length cDNA was sequenced with the Sanger dideoxy method and internal primers.

RNA Analysis—Total cellular RNA was prepared using the guanidine thiocyanate-lithium chloride procedure (28). Northern blot hybridizations were performed by standard techniques (29). Probes were synthesized by the multiprime procedure (Ready-to-go kit, Pharmacia). After autoradiography, mRNAs were quantified by image analysis with the Bioimage program on a Millipore Sun Station. Each lane was normalized with the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe.

In Vitro Synthesis of RNA Coding for RLI and 2DR—Transcription with T3 polymerase in the presence of m^7G(5′)ppp(5′)G was performed as described by the manufacturer (Promega). RNA was analyzed and quantified by electrophoresis in 1.2% (w/v) agarose gels in TBE buffer (50 mM Tris, pH 8, 50 mM boric acid, 1 mM EDTA). RNA was extracted by phenol-dichloromethan (v/v) and precipitated with ethanol, 250 mM NaCl.

In vitro translation in rabbit reticulocyte lysates (with or without canine pancreatic microsomes), or in wheat-germ extracts were performed according to the manufacturer's instructions (Promega). The quantity of endogenous RNase L was estimated by the amount of bound 2–5ApCp, *e.g.* 9 × 10^3 disintegrations/min. Since the 2–5ApCp had a specific activity of 3 × 10^3 Ci/mmol and since it is considered that RNase L binds 2–5ApCp in a 1:1 ratio (30), the amount of RNase L in the reticulocyte lysate assay is 1.3 × 10^{-15} mol. The translation of 2DR led to a 2-fold increase in the binding of 2–5ApCp; the amount of RNase L in the reticulocyte extract is therefore 2.6 × 10^{-15} mol. The concentration of the translated proteins was also quantified by [^{35}S]methionine incorporation (specific activity 10^3 Ci/mmol). This later technique gave the same result for 2DR and 0.8 × 10^{-15} mol for the RLI.

Reticulocyte translation incubations were used as a source of RLI, 2DR, or endogenous RNase L. Translation usually took place for 60 min at 30 °C. Aliquots of each were mixed to generate various ratios of these proteins in 2–5A binding or RNA cleavage assays.

Ribosomal RNA Cleavage by RNase L—Specific hydrolysis of rRNAs by 2–5A-activated RNase L was monitored in reticulocyte extracts with some modifications previously described (18). RLI and 2DR were produced in rabbit reticulocyte extracts. The control extract was incubated in the same conditions as the extracts used for cell-free expression of 2DR or RLI cDNA. The different translation incubations were complemented or not with 100 nM 2–5A_4 and further incubated for 30 min at 30 °C. RNAs were extracted with phenol-dichloromethan and precipitated with 2 volumes of ethanol, 300 mM sodium acetate. The pellet was dried and resuspended in an electrophoresis loading buffer containing 50% (v/v) glycerol, TBE buffer, and 0.25% (w/v) bromphenol blue. Samples were analyzed on a 1.2% (w/v) agarose gel in TBE buffer. rRNAs and their degradation products were quantified by image analysis with the Bioimage program on a Millipore Sun Station.

Western Blot Analysis and Immunoprecipitation—Cell extracts or *in vitro* translated polypeptides were analyzed by Western blot (31). Proteins were fractionated on SDS-PAGE and transferred electrophoretically to a nitrocellulose sheet. The nitrocellulose membrane was hybridized with 2–5ApCp (15) or with mAb3 monoclonal antibody (18).

For immunoprecipitation, cell extracts or translation incubations were incubated 3 h at room temperature with a 1/1,000 dilution of mAb3 or control antibody. RNase L-antibody complexes were incubated overnight at 4 °C with protein A-Sepharose (Pharmacia) and recovered by centrifugation. The beads were washed several times with 10 mM Tris, pH 7.4, 0.5% (v/v) aprotinin, 1% (v/v) Nonidet P-40, 2 mM EDTA, 150 mM NaCl, and resuspended in one volume of 300 mM Tris, pH 8.9, 5% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 20% (v/v) glycerol buffer. The protein A-Sepharose was heated for 5 min at 95 °C, and the immunoprecipitated proteins were analyzed by 10% (w/v) SDS-PAGE. The gel was dried and subjected to autoradiography.

Stability of 2–5ApCp—Reticulocyte extracts were complemented or not with RLI or 2DR mRNA and incubated 1 h at 30 °C in translation conditions. 2–5ApCp was then added, and the extracts were incubated at 4 °C for 15 min (as for radiobinding) or at 37 °C for increasing periods of time as indicated in the legend of Fig. 7. The loading buffer containing 50% (v/v) formamide, 1% (v/v) bromphenol blue, and 1% (v/;V) xylene cyanol was added. The samples were boiled 3 min and centrifuged at 10,000 × *g*. The supernatants were loaded onto 20% (w/v) polyacrylamide gels containing 7 M urea. The samples were analyzed by electrophoresis at 1,100–1,600 V until the bromphenol blue dye reached 15 cm from the bottom of the gel. The wet gel was exposed to a Kodak XAR5 x-ray film (32).

Expression Vectors and Transfections—The human H2ABP cDNA which encoded a truncated active form of RLI was directionally subcloned in pcDNAIneo (Invitrogen) by standard methods (29). H2ABP/pcDNAIneo or plasmid pcDNAneoI (7 μg each) were transfected in HeLa cells by calcium phosphate coprecipitation (29). Stable transfectants were selected by culturing the cells in the presence of 1 mg/ml G418 (Life Technologies, Inc.). Individual clones were isolated for analysis of expression of the transfected cDNA. The clone which expressed the cDNA at the highest level was selected for further studies.

Antiviral Activity of Interferons—pcDNAneo or H2ABP/pcDNAIneo

B

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                                                                ccggtcctgagacacgctgtgtggctgaaaagtgaaggcaagagc   -73
tcatttggcctctgtgctcccctccgcaagggatcgtttctccagaagagctggatattctttcgcccagtt    -1

ATG GCA GAC AAG TTA ACG AGA ATT GCT ATT GTC AAC CAT GAC AAA TGT AAA CCT     54
Met Ala Asp Lys Leu Thr Arg Ile Ala Ile Val Asn His Asp Lys Cys Lys Pro     18

AAG AAA TGT CGA CAG GAA TGC AAA AAG AGT TGT CCT GTA GTT CGA ATG GGA AAA    108
Lys Lys Cys Arg Gln Glu Cys Lys Lys Ser Cys Pro Val Val Arg Met Gly Lys     36

TTA TGC ATA GAG GTT ACA CCC CAG AGC AAA ATA GCA TGG ATT TCC GAA ACT CTT    162
Leu Cys Ile Glu Val Thr Pro Gln Ser Lys Ile Ala Trp Ile Ser Glu Thr Leu     54

TGT ATT GGT TGT GGT ATC TGT ATT AAG AAA TGC CCC TTT GGC GCC TTA TCA ATT    216
Cys Ile Gly Cys Gly Ile Cys Ile Lys Lys Cys Pro Phe Gly Ala Leu Ser Ile     72

GTC AAT CTA CCA AGC AAC TTG GAA AAA GAA ACC ACA CAT CGA TAT TGT GCC AAT    270
Val Asn Leu Pro Ser Asn Leu Glu Lys Glu Thr Thr His Arg Tyr Cys Ala Asn     90

GCC TTC AAA CTT CAC AGG TTG CCT ATC CCT CGT CCA GGT GAA GTT TTG GGA TTA    324
Ala Phe Lys Leu His Arg Leu Pro Ile Pro Arg Pro Gly Glu Val Leu Gly Leu    108

GTT GGA ACT AAT GGT ATT GGA AAG TCA GCT GCT TTA AAA ATT TTA GCA GGA AAA    378
Val Gly Thr Asn Gly Ile Gly Lys Ser Ala Ala Leu Lys Ile Leu Ala Gly Lys    126

CAA AAG CCA AAC CTT GGA AAG TAC GAT GAT CCT CCT GAC TGG CAG GAG ATT TTG    432
Gln Lys Pro Asn Leu Gly Lys Tyr Asp Asp Pro Pro Asp Trp Gln Glu Ile Leu    144

ACT TAT TTC CGT GGA TCT GAA TTA CAA AAT TAC TTT ACA AAG ATT CTA GAA GAT    486
Thr Tyr Phe Arg Gly Ser Glu Leu Gln Asn Tyr Phe Thr Lys Ile Leu Glu Asp    162

GAC CTA AAA GCC ATC ATC AAA CCT CAA TAT GTA GCC AGA TTC CTA AGG CTG GCA    540
Asp Leu Lys Ala Ile Ile Lys Pro Gln Tyr Val Ala Arg Phe Leu Arg Leu Ala    180

AAG GGG ACA GTG GGA TCT ATT TTG GAC CGA AAA GAT GAA ACA AAG ACA CAG GCA    594
Lys Gly Thr Val Gly Ser Ile Leu Asp Arg Lys Asp Glu Thr Lys Thr Gln Ala    198

ATT GTA TGT CAG CAG CTT GAT TTA ACC CAC CTA AAA GAA CGA AAT GTT GAA GAT    648
Ile Val Cys Gln Gln Leu Asp Leu Thr His Leu Lys Glu Arg Asn Val Glu Asp    216

CTT TCA GGA GGA GAG TTG CAG AGA TTT GCT TGT GCT GTC GTT TGC ATA CAG AAA    702
Leu Ser Gly Gly Glu Leu Gln Arg Phe Ala Cys Ala Val Val Cys Ile Gln Lys    234

GCT GAT ATT TTC ATG TTT GAT GAG CCT TCT AGT TAC CTA GAT GTC AAG CAG CGT    756
Ala Asp Ile Phe Met Phe Asp Glu Pro Ser Ser Tyr Leu Asp Val Lys Gln Arg    252

TTA AAG GCT GCT ATT ACT ATA CGA TCT CTA ATA AAT CCA GAT AGA TAT ATC ATT    810
Leu Lys Ala Ala Ile Thr Ile Arg Ser Leu Ile Asn Pro Asp Arg Tyr Ile Ile    270

GTG GTG GAA CAT GAT CTA AGT GTA TTA GAC TAT CTC TCC GAC TTC ATC TGC TGT    864
Val Val Glu His Asp Leu Ser Val Leu Asp Tyr Leu Ser Asp Phe Ile Cys Cys    288

TTA TAT GGT GTA CCA AGC GCC TAT GGA GTT GTC ACT ATG CCT TTT AGT GTA AGA    918
Leu Tyr Gly Val Pro Ser Ala Tyr Gly Val Val Thr Met Pro Phe Ser Val Arg    306

GAA GGC ATA AAC ATT TTT TTG GAT GGC TAT GTT CCA ACA GAA AAC TTG AGA TTC    972
Glu Gly Ile Asn Ile Phe Leu Asp Gly Tyr Val Pro Thr Glu Asn Leu Arg Phe    324

AGA GAT GCA TCA CTT GTT TTT AAA GTG GCT GAG ACA GCA AAT GAA GAA GAA GTT   1026
Arg Asp Ala Ser Leu Val Phe Lys Val Ala Glu Thr Ala Asn Glu Glu Glu Val    342

AAA AAG ATG TGT ATG TAT AAA TAT CCA GGA ATG AAG AAA AAA ATG GGA GAA TTT   1080
Lys Lys Met Cys Met Tyr Lys Tyr Pro Gly Met Lys Lys Lys Met Gly Glu Phe    360

GAG CTA GCA ATT GTA GCT GGA GAG TTT ACA GAT TCT GAA ATT ATG GTG ATG CTG   1134
Glu Leu Ala Ile Val Ala Gly Glu Phe Thr Asp Ser Glu Ile Met Val Met Leu    378

GGG GAA AAT GGA ACG GGT AAA ACG ACA TTT ATC AGA ATG CTT GCT GGA AGA CTT   1188
Gly Glu Asn Gly Thr Gly Lys Thr Thr Phe Ile Arg Met Leu Ala Gly Arg Leu    396

AAA CCT GAT GAA GGA GGA GAA GTA CCA GTT CTA AAT GTC AGT TAT AAG CCA CAG   1242
Lys Pro Asp Glu Gly Gly Glu Val Pro Val Leu Asn Val Ser Tyr Lys Pro Gln    414

AAA ATT AGT CCC AAA TCA ACT GGA AGT GTT CGC CAG TTA CTA CAT GAA AAG ATA   1296
Lys Ile Ser Pro Lys Ser Thr Gly Ser Val Arg Gln Leu Leu His Glu Lys Ile    432

AGA GAT GCT TAT ACT CAC CCA CAA TTT GTG ACC GAT GTA ATG AAG CCT CTG CAA   1350
Arg Asp Ala Tyr Thr His Pro Gln Phe Val Thr Asp Val Met Lys Pro Leu Gln    450

ATT GAA AAC ATC ATT GAT CAA GAG GTG CAG ACA TTA TCT GGT GGT GAA CTA CAG   1404
Ile Glu Asn Ile Ile Asp Gln Glu Val Gln Thr Leu Ser Gly Gly Glu Leu Gln    468

CGA GTA CGT TTA CGC CTT TGC TTG GGC AAA CCT GCT GAT GTC TAT TTA ATT GAT   1458
Arg Val Arg Leu Arg Leu Cys Leu Gly Lys Pro Ala Asp Val Tyr Leu Ile Asp    486

GAA CCA TCT GCA TAT TTG GAT TCT GAG CAA AGA CTG ATG GCA GCT CGA GTT GTC   1512
Glu Pro Ser Ala Tyr Leu Asp Ser Glu Gln Arg Leu Met Ala Ala Arg Val Val    504

AAA CGT TTC ATA CTC CAT GCA AAA AAG ACA GCC TTT GTT GTG GAA CAT GAC TTC   1566
Lys Arg Phe Ile Leu His Ala Lys Lys Thr Ala Phe Val Val Glu His Asp Phe    522

ATC ATG GCC ACC TAT CTA GCG GAT CGC GTC ATC GTT TTT GAT GGT GTT CCA TCT   1620
Ile Met Ala Thr Tyr Leu Ala Asp Arg Val Ile Val Phe Asp Gly Val Pro Ser    540

AAG AAC ACA GTT GCA AAC AGT CCT CAA ACC CTT TTG GCT GGC ATG AAT AAA TTT   1674
Lys Asn Thr Val Ala Asn Ser Pro Gln Thr Leu Leu Ala Gly Met Asn Lys Phe    558

TTG TCT CAG CTT GAA ATT ACA TTC AGA AGA GAT CCA AAC AAC TAT AGG CCA CGA   1728
Leu Ser Gln Leu Glu Ile Thr Phe Arg Arg Asp Pro Asn Asn Tyr Arg Pro Arg    576

ATA AAC AAA CTT AAT TCA ATT AAG GAT GTA GAA CAA AAG AAG AGT GGA AAC TAC   1782
Ile Asn Lys Leu Asn Ser Ile Lys Asp Val Glu Gln Lys Lys Ser Gly Asn Tyr    594

TTT TTC TTG GAT GAT tagactgactctgagaatattgataagccatttattaaaaggagtatttac   1848
Phe Phe Leu Asp Asp                                                       599

tagaatttttgtcatataaaacttgaatcaggatttatgccccacatactctggaacttgaagtataata   1920
tacttaatataacatacaaaaagccagttgggttctaaattgtagttgaaacacagaaaatgccactttttgt   1992
tcctgaagaggctctttgtgccataatattctaaaatgaagacatttcaagctatacaaattacttccaagt   2064
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cattttttaaatcttgaaaaggtttctgtacttacctggtttgccaagtatgccagtgtaatgaaactgcc   2208
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tttgttttcagtctttgcactatattaccagtatatggtttccgaggaagattatctactgcaaaacaccac   2352
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tttgtgagtttggatatagagcacattatctaaaccatttttgtagttccaaaaacccatctaaatttcttga   2856
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tatgagtgaggaagagtgtttactaataaatgactggggcaagcaaaattgaggaggaaattagaaactgt   3000
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aatatacagaatggaataaaaaaatgatctcccttttattaccctcccaaaggttaccagcgtttgaatttaa   3216
taatgtatattcttttcatgcttttttctgtgcacttacctaagtgtgaatatgtaaagggtttgttttgtat   3288
acaaatgggattatactaaaataagtaatgcctatttttaaggatagttaaatttgtgaatgatcatttca   3360
aatatattgaataaaataagcaaaagctattgtttatttactgatcctgaaaaaaaaaaaaaaaaaaaaaaa   3432
aaaaaaaaaaaaaaaaaa                                                        3451
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FIG. 1. **Cloning of the full-length cDNA encoding the RLI.** *A*, the H2ABP cDNA (2861 bp) was isolated from a Daudi cells λ-Zap expression library by screening with 2–5ApCp. The full-length cDNA clone RLI (3568 bp) was isolated using anchored PCR with poly(G) cDNA and an internal primer determined from the H2ABP sequence. The additional fragment (924 bp) (noted *PCR* on the scheme) was added to H2ABP in the *Bgl*II site. The RLI cDNA contains 707 additional base pairs on the 5′ side of H2ABP. *B*, nucleotide sequence and deduced amino acid sequence of the RLI DNA. *Numbers* on the right of the sequence indicate the position of nucleotides and amino acids, respectively. The *box with black squares* indicates the position of the ferredoxin-like sequence. *Open boxes* indicate the two P-loop motifs. The *gray box* shows a potential PKC phosphorylation site. The internal repeats of 128 amino acids are *underlined* in *bold*. In the 3′-non-coding region, the AATAAA polyadenylation signal sequences are *underlined*, and the ATTTA putative instability sequences are *double underlined*.

cells were seeded at 10^5 cells/well. Cells were treated 24 h later with different dilutions of Huα/β interferon for 20 h. Cells were then infected with vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMCV) at a multiplicity of infection of 1.0 for 1 h at 37 °C in RPMI medium supplemented with 5% (v/v) fetal calf serum. Unadsorbed viruses were removed by three washings with RPMI containing 10% (v/v) fetal calf serum. Virus titers were determined 18 h later by an end point assay as described previously (33).

RESULTS

Expression Cloning of Human RLI—Human Daudi cells were chosen as a source of mRNA because they contain a high basal level of 2–5A binding activity. The cDNA library (λ-Zap) was screened with a radiolabeled 2–5ApCp probe using the technique we have developed for the renaturation of RNase L activity on filter (15 and "Experimental Procedures"). We identified one positive clone among 10^6 plaques. This clone named H2ABP for human 2–5A binding protein contains a 2861-bp insert. It has an initiator ATG at residue 225 and is translated as a 39-kDa protein in rabbit reticulocyte extract or in wheat-germ extract. It is in an open reading from the first nucleotide. The full-length cDNA was, therefore, isolated by anchored PCR with the internal primer described in Fig. 1*A*. A 924-bp fragment which overlaps the H2ABP clone by 217 bp was obtained. This sequence was added to H2ABP by cloning in the *Bgl*II site. The sequence of the complete 3568-bp clone (RLI) is presented in Fig. 1*B*.

Sequence Analysis of RLI—RLI cDNA has a 5′-non-coding region of 118 nucleotides, a predicted open reading frame extending until nucleotide 1914, and a long 3′-untranslated region of 1654 bp. The open reading frame of 599 amino acids encodes a polypeptide of predicted molecular weight 67,515. The ATG at the beginning of the open reading frame has the characteristics of a "strong" initiator codon, *e.g.* a purine in position −3 and a G in +4 (34). RLI contains two repeated ATP/GTP-binding sites or P-loop motifs (35): one from residues 110 to 117 and another from residues 379 to 386. Interestingly, RLI has a complete homology with the $CX_2CX_2CX_3C$:4Fe4S-Ferredoxin motif between amino acids 55 and 66 (see Ref. 36 for a review). Another remarkable feature is an internal repeat of 128 amino acids with 53% homology starting at amino acids 200 and 440 (Fig. 1*B*). RLI has a high number (32) of thiol groups and is very rich in leucine residues. Its estimated isoelectric point is 8.7.

No significative homology with other cDNAs in the EMBL and Genbank data bases has been found.

Regulation of RLI RNAs Expression by IFN—The cDNA of RLI hybridized with two cellular mRNAs of 3.5 and 2.8 kb in HeLa cells (Fig. 2*A*). The same result was obtained in Daudi and CEM cells (data not shown). Huα/β IFN treatment did not regulate the two mRNAs which hybridize with the RLI clones, even at high concentration. There was no quantitative variation of these mRNAs as a function of the duration of IFN treatment. An induction of the 15-kDa protein, which has been described to be induced by IFN treatment (37), was observed in the same conditions thus confirming that cells responded normally to IFN treatment.

To approach the difference between the 2.8- and 3.5-kb mRNAs, we made use of a 762-bp probe (*A* in Fig. 2*B*) specific of the 5′ end of RLI and a 764-bp probe (*B* in Fig. 2*B*) representing its 3′ end; their hybridization positions are indicated in Fig. 1*A*. As shown in Fig. 2*B*, probe A hybridizes with the two mRNAs while probe B hybridizes uniquely to the larger mRNA. These two mRNAs therefore differ in their 3′-untranslated region. The similarity between the sizes of the two natural mRNAs and those of the cDNAs we have cloned is purely coincidental.

In a chase experiment using actinomycin D, the half-life of the RLI mRNAs was estimated to be 4 h in HeLa cells (data not shown).

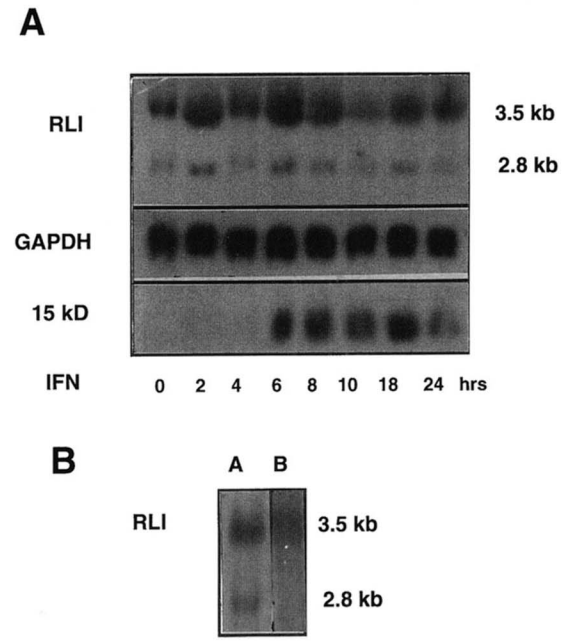

FIG. 2. **Regulation of RLI mRNA expression by human α/β-IFN.** *A*, Northern blot analysis of total cytoplasmic RNA extracted from non-treated HeLa cells or from cells treated with 10^3 units/ml of Huα/β IFN for 2, 4, 6, 8, 10, 18, or 24 h. RNAs (20 μg) were fractionated on a 1.2% (w/v) agarose gel and hybridized with the RLI cDNA probe, with a human 15-kDa probe or with a human GAPDH probe. The filter was submitted to autoradiography for 5 h for the GAPDH probe and for 12 h for the other probes. *B*, Northern blot analysis of total cytoplasmic RNA extracted from HeLa cells. RNAs (20 μg) were fractionated on a 1.2% (w/v) agarose gel and hybridized with the *A* and *B* RLI cDNA probes described in Fig. 1*A*.

In Vitro Expression and Properties of RLI—The biological properties of the RLI clone were studied after expression in two cell-free translation systems, *e.g.* in a wheat-germ S30 (which is deficient in 2–5A/RNase L system) and in a rabbit reticulocyte lysate (in which the 2–5A/RNase L system is present).

The expression of the full-length RLI cDNA in wheat-germ or in rabbit reticulocyte extracts gave rise to a single polypeptide of 68 kDa as expected (Fig. 3A, *lanes 2* and *4*). The translation of 2DR cDNA gave rise to a single polypeptide of 80 kDa (Fig. 3*A*, *lanes 1* and *3*). The level of expression was a little lower in wheat-germ extract than in reticulocyte lysate for the two clones. No post-translational modifications of these polypeptides were observed when they were translated in the presence of canine pancreatic microsomal membranes (data not shown). The 2DR and RLI cDNA translation products were not recognized by the mAb3-specific monoclonal antibody that we have developed against RNase L (18). As shown in Fig. 3*B*, and as expected, only the 80 kDa polypeptide (RNABP) was revealed in reticulocyte extract, either before or after translation (compare *lanes 1* to *lanes 2* and *3*). Likewise mAb3 did not recognize any proteins in the wheat-germ extract in which the 2–5A/RNase L system, and consequently RNABP is absent.

RLI slightly bound 2–5ApCp when the translation incubations in wheat-germ extracts were spotted on a nitrocellulose sheet and incubated with the 2–5ApCp probe (Fig. 3*C*) by the protocol used to screen the λ-Zap library ("Experimental Procedures" and data not shown). Surprisingly, we observed a decreased binding of 2–5ApCp by the endogenous RNase L when RLI was expressed in a rabbit reticulocyte lysate. At variance, an increase in the binding of 2–5ApCp was clearly observed when 2DR was expressed in the wheat-germ or in the rabbit reticulocyte extracts (Fig. 3*C*).

No binding of 2–5ApCp by *in vitro* expressed RLI (in wheat-

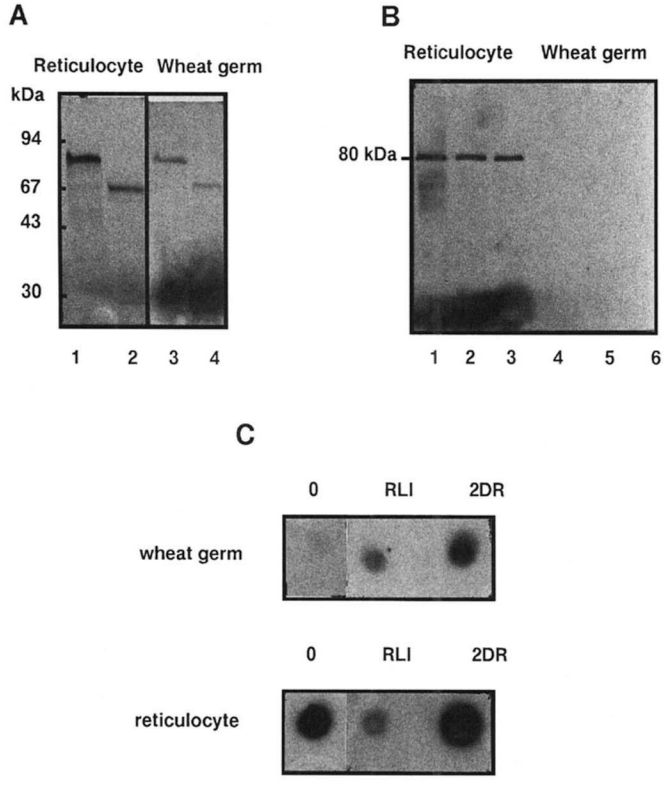

FIG. 3. **In vitro translation of RLI cDNA.** *A*, RLI (*lanes 2* and *4*) or 2DR (*lanes 1* and *3*) cDNAs were transcribed and translated *in vitro* in reticulocyte (*lanes 1* and *2*) or in wheat-germ (*lanes 3* and *4*) extracts in the presence of [^{35}S]methionine. The products were analyzed by SDS-PAGE and autoradiography. *B*, reticulocyte extracts with no mRNA (*lane 1*), after translation of 2DR (*lane 2*), or RLI (*lane 3*), and wheat-germ extracts with no mRNA (*lane 4*), after translation of 2DR (*lane 5*), or RLI (*lane 6*) were analyzed by SDS-PAGE and Western blot with the mAb3 monoclonal antibody specific of the RNase L complex. *C*, translation reactions of 2DR or RLI in wheat-germ or reticulocyte extracts and control extracts were spotted on nitrocellulose. No RNase L, 2DR, or RLI in wheat germ (*0*); 0.8×10^{-15} mol of RLI in wheat germ or in reticulocyte extracts (*RLI*); 1.3×10^{-15} mol of 2DR in wheat germ or in reticulocyte extracts (*2DR*); 1.3×10^{-15} mol of endogenous RNase L in reticulocyte extracts (*0*, *RLI*, and *2DR*) (see "Experimental Procedures"). The nitrocellulose sheet was≤ hybridized with a 2–5ApCp probe and analyzed by autoradiography.

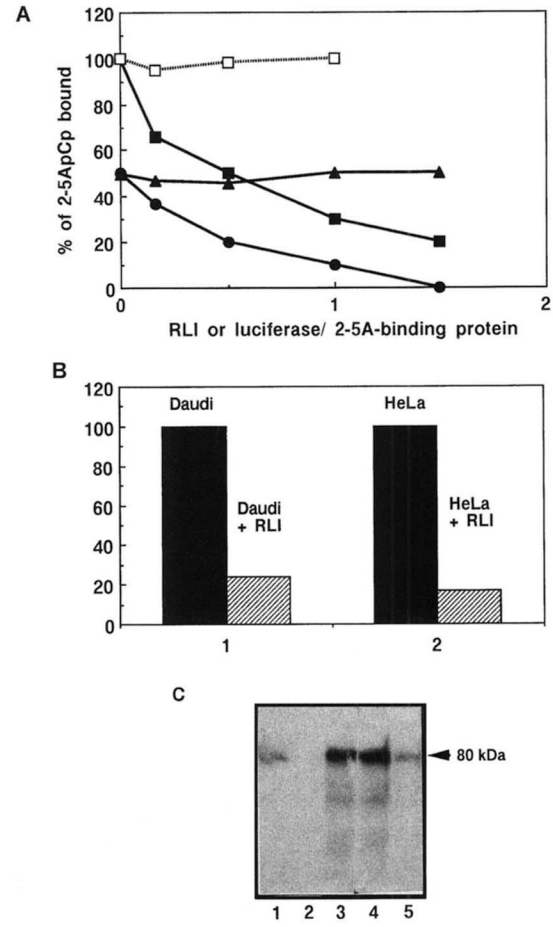

FIG. 4. **Inhibition of 2–5A-binding on RNase L by RLI.** *A*, mRNAs coding for luciferase, RLI, or 2DR were translated in reticulocyte or wheat-germ extracts. Various concentrations of the translation products were added to a reticulocyte or to a wheat-germ extract, or to an extract in which the 2DR mRNA has already been expressed. This gave rise to different ratios between RLI (or luciferase), endogenous RNase L, and *in vitro* expressed 2DR, as indicated. The extracts were then tested in a 2–5A binding assay. The results are expressed as percent of 2–5ApCp bound. 100% is the binding obtained when 2DR alone was expressed. Reticulocyte + luciferase (▲), reticulocyte + RLI (●), reticulocyte + 2DR + RLI(■), wheat germ + 2DR + RLI (□). *B*, RLI translated in reticulocyte extract was added to Daudi (*1*) or HeLa cell extracts (*2*). The extracts were then tested in a 2–5ApCp binding assay. The results are expressed in percent of 2–5ApCp bound. 100% is the 2–5ApCp binding observed in Daudi or HeLa cell extracts supplemented with a reticulocyte lysate but not with RLI. *C*, covalent 2–5ApCp binding in reticulocyte extract by endogenous RNase L or by *in vitro* expressed 2DR in the following conditions: *1*, reticulocyte extract alone; *2*, with translated RLI; *3*, with translated 2DR; *4*, with translated 2DR and luciferase; *5*, with translated 2DR and RLI.

germ extracts) could be detected in the conditions described for the radiobinding (25) or for the radiocovalent assays (26) (data not shown), thus confirming that the affinity of RLI for 2–5A is poor. On the contrary the expression of RLI in reticulocyte extracts led to a large dose-dependent decrease in the radiobinding and radiocovalent binding of 2–5ApCp by endogenous RNase L in reticulocyte lysate (Fig. 4, *A* and *C*). As expected, the expression of 2DR alone in wheat-germ or in reticulocyte extracts increased 2–5ApCp binding in both assays (Fig. 4, *A* and *C*). The co-expression of RLI gave rise to an inhibition of 2–5ApCp binding by 2DR whether using the radiobinding or the radiocovalent assay (Fig. 4, *A* and *C*). As a control no inhibition of endogenous RNase L in reticulocyte extract or of expressed 2DR was observed upon addition of an irrelevant protein like luciferase (Fig. 4, *A* and *C*). The same phenomenon was observed in other cell extracts. As an example, the addition of RLI to Daudi or HeLa cell extracts inhibited the binding of 2–5ApCp by RNase L (Fig. 4*B*). It is interesting to note that the translation of RLI in wheat-germ extracts did not inhibit the binding of 2–5ApCp by 2DR (Fig. 4*A*). This difference between the two translation systems has not been elucidated but could reflect differences in the post-translational modifications of the protein; another hypothesis is discussed later.

The RLI-mediated inhibition of 2DR activity did not result from a competition for 2–5A. Indeed, the inhibition of 2–5ApCp binding by 2DR was not modified if the experiment was performed with increasing concentrations of 2–5ApCp provided while the ratio between 2DR and RLI was not modified (data not shown). It is important to notice here that the initial truncated H2ABP clone exhibited the same properties than the full-length one; it inhibited 2–5ApCp binding by endogenous RNase L or by *in vitro* expressed 2DR (data not shown).

RLI Antagonizes the 2–5A-dependent Nuclease Activity of Endogenous RNase L or Cloned 2DR—Since RLI inhibited 2–5ApCp binding, it was expected to behave as a RNase L antagonist. We, therefore, tested whether the expression of RLI also inhibited its nucleolytic activity in a cell-free assay. The rRNAs and their specific degradation pattern that constitute an index of 2–5A/RNase L activity (50) were studied in rabbit

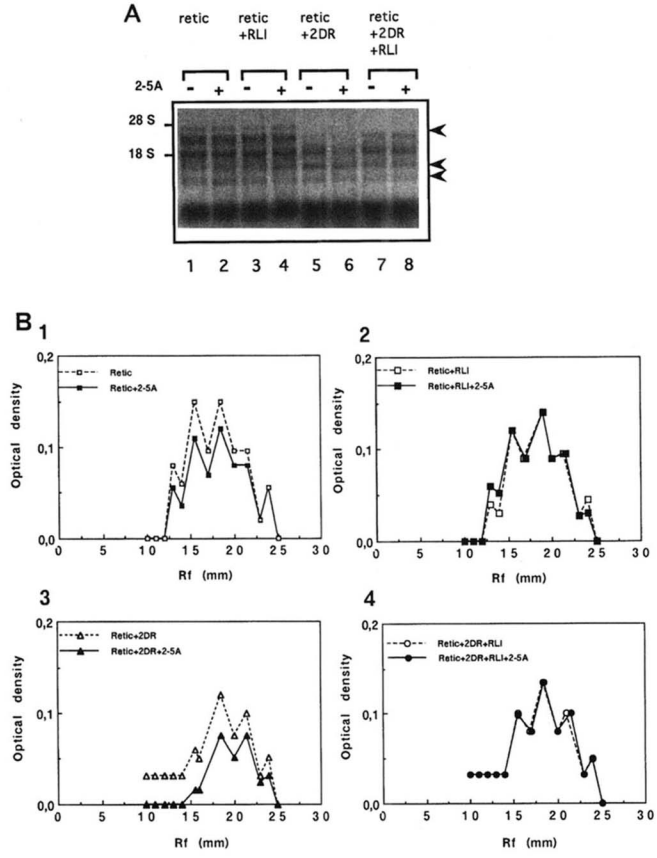

FIG. 5. **Inhibition of RNase L activity by RLI.** *A*, reticulocyte extracts alone (*lanes 1* and *2*) and supplemented with RLI mRNA (*lanes 3* and *4*), 2DR mRNA (*lanes 5* and *6*), or 2DR and RLI mRNAs (*lanes 7* and *8*) were incubated 60 min at 30 °C to generate the recombinant proteins. They were then supplemented (*lanes 2*, *4*, *6*, and *8*) or not (*lanes 1*, *3*, *5*, and *7*) with 2–$5A_4$ and further incubated for 30 min at 30 °C. rRNAs were analyzed on a 1.2% (w/v) agarose gel. Intact 28 S and 18 S rRNAs migration is indicated on the left of *A*. Major rRNAs degradation products are indicated by *arrows* on the right of *A*. *B*, the rRNAs and their degradation products were quantified by image analysis and represented on diagrams. *Diagram 1*, reticulocyte alone; *diagram 2*, reticulocyte with RLI; *diagram 3*, reticulocyte with 2DR; *diagram 4*, reticulocyte with 2DR and RLI.

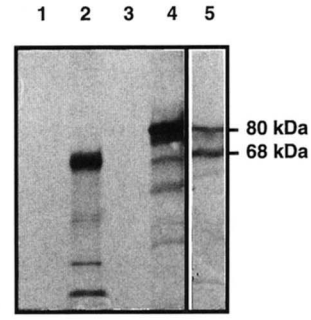

FIG. 6. **Immunoprecipitation of RLI and 2DR by mAb3.** RLI or 2DR were translated in reticulocyte or wheat-germ extracts in the presence of [^{35}S]methionine. mAb3 (1/1000) was then added in the translation incubation. The mAb3·protein complex was precipitated with protein A-Sepharose and analyzed on SDS-PAGE. *1*, RLI translated in wheat-germ extract. *2*, RLI translated in reticulocyte extract. *3*, 2DR translated in wheat-germ extract. *4*, 2DR translated in reticulocyte extract. *5*, co-immunoprecipitation of 2DR and RLI by mAb3 in reticulocyte extract.

reticulocyte extracts supplemented or not with RLI, 2DR, and 2–5A (Fig. 5). Total RNAs from the extracts were fractionated by agarose gel electrophoresis (Fig. 5*A*) and their degradation products were quantified by image analysis (Fig. 5*B*). RLI and 2DR have not yet be purified and were therefore expressed by the *in vitro* translation of their mRNAs in reticulocyte lysates. The 2–5A/RNase L pathway is functional in these extracts even in the absence of exogenous 2–5A as evident from the occurrence of rRNAs degradation products (as marked by *arrows* in Fig. 5*A*). Wheat-germ extracts in which the 2–5A pathway is not functional could not be used as a source of recombinant nuclease since cloned 2DR is inactive even though it is capable of binding 2–5A (22). Likewise, we did not succed in expressing RLI as a functional protein in a wheat-germ extract (see above). Nevertheless, the addition of 2–5A in the rabbit reticulocyte extract gave rise to a slight but reproducible increase of rRNA degradation which no longer occurred where RLI was also present (Fig. 5*A*, *lanes 2* and *4*, and Fig. 5*B*, *diagrams 1* and *2*).

Similar observations have been made in extracts supplemented with cloned 2DR. Here again rRNA degradation had already taken place in the control extract and exogenous 2–5A largely increased degradation (Fig. 5*A*, *lanes 5* and *6*, and Fig. 5*B*, *diagram 3*). The latter was antagonized by the co-expression of RLI (Fig. 5*A*, *lanes 7* and *8* and Fig. 5*B*, *diagram 4*). The degradation of rRNAs observed in extracts which were not supplemented with 2–5A was only partially inhibited by RLI. Whatever the explanation it is clear that RLI expression antagonizes the 2–5A-dependent nucleolytic activity of RNase L whether endogenous to the reticulocyte extract or provided from cloned 2DR.

The inhibition of RNase L by RLI seems specific because we observed no inhibition of pancreatic RNase or nuclease T2 (data not shown).

Association of RLI with RNase L—We have previously established that the protein which binds 2–5A (2–5ABP) was co-immunoprecipitated with a RNABP by mAb3 in a high molecular weight complex. mAb3 is a specific monoclonal antibody which recognizes only RNABP and neutralizes RNase L activity (18). *In vitro* translated 2DR was immunoprecipitated with RNABP as expected from our previous results (18) (Fig. 6, *lane 4*). Likewise, *in vitro* translated RLI was co-immunoprecipitated with RNABP from reticulocyte extracts (Fig. 6, *lane 2*). When present together RLI and 2DR were also immunoprecipitated by mAb3 (Fig. 6, *lane 5*). On the contrary, when the two proteins were translated in wheat-germ extract (in which RNABP is absent, see Fig. 3*B*) they were not immunoprecipitated by mAb3 (Fig. 6, *lanes 1* and *3*).

RLI Does Not Degrade 2–5A—The RLI-induced inhibition of 2–5ApCp binding by endogenous RNase L or by *in vitro* expressed 2DR could result from 2–5ApCp degradation. In order to eliminate this possibility, 2–5ApCp was incubated with a reticulocyte extract alone or with a reticulocyte extract supplemented with translated 2DR or RLI, at 4 °C (in the radiobinding assay condition) or at 37 °C for increasing periods of time. Undegraded 2–5ApCp was quantified by acrylamide-urea gel electrophoresis (32). A dephosphorylation of 2–5ApCp was observed (at 4 or 37 °C) in the reticulocyte extract, whether incubated alone or supplemented with RLI (Fig. 7). The degradation of 2–5ApCp was not increased when RLI was added (compare, for example, *lanes 1* and *2*, or *4* and *5* in Fig. 7) in experimental conditions in which an inhibition of RNase L was observed. In a separate experiment 2–$5A_4$ was incubated without terminal pCp and analyzed by high performance liquid chromatography on a C18 μBondapack column (11). Once again, no increased degradation was observed upon RLI addition (data not shown).

Separation of RLI and RNase L Reactivates RNase L—To determine whether RLI stably modifies the 2–5A binding capacity of RNase L, the two proteins were dissociated by gel electrophoresis and the capacity of RNase L to bind 2–5ApCp

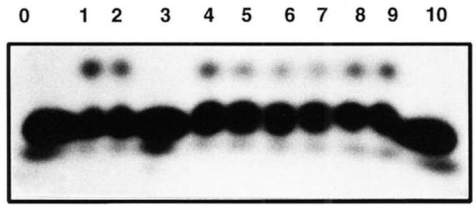

FIG. 7. **Stability of 2–5ApCp in reticulocyte extracts with or without RLI.** 2–5ApCp (*lanes 0, 3,* and *10*) was incubated with reticulocyte extract alone (*lanes 1, 4, 6,* and *8*) or with reticulocyte extract and RLI (*lanes 2, 5, 7,* and *9*) for 15 min at 4 °C (*lanes 1* and *2*), or for 15 min (*lanes 4* and *5*), 30 min (*lanes 6* and *7*), 60 min (*lanes 8* and *9*) at 37 °C.

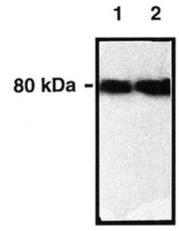

FIG. 8. **2–5A binding activity of RNase L on filter.** *1*, reticulocyte-extract alone, or *2*, with translated RLI was analyzed by SDS-PAGE, transferred to nitrocellulose sheet, and incubated with 2–5ApCp as indicated under "Experimental Procedures." After washing, the membrane was submitted to autoradiography.

was determined by the Western blot assay (15). As shown in Fig. 8, no more difference in the binding of 2–5ApCp was observed whether the reticulocyte extract had been incubated alone (Fig. 8, *lane 1*) or had been supplemented with RLI mRNA (Fig. 8, *lane 2*). This should be compared with the data reported in Fig. 4 where a 80% inhibition of 2–5ApCp binding was observed when RLI and RNase L were not separated.

Inhibition of the 2–5A/RNase L Pathway in Intact Cells—RLI inhibits 2–5ApCp binding and behaves as a RNase L inhibitor in cell-free extracts. We therefore tested whether its overexpression antagonized the IFN-regulated 2–5A/RNase L pathway in intact cells. HeLa cells were stably transfected with the H2ABP cDNA clone which codes for a truncated but fully functional protein as already mentioned.

The 2–5ApCp binding by RNase L in the H2ABP/pcDNAIneo selected clone is reduced by 30% as compared to non transfected HeLa cells or to cells transfected with the vector alone (data not shown). This clone (H2ABP/pcDNAIneo) and a clone transfected with the empty vector (pcDNAIneo) were compared for the antiviral effect of IFN against VSV and EMCV (Fig. 9).

IFN treatment of the pcDNAIneo cells resulted in a dose-dependent reduction in EMCV yield (Fig. 9*A*). The antiviral activity of IFN at concentrations as high as 1000 units/ml was significantly reduced in H2ABP/pcDNAIneo cells (Fig. 9*A*), in keeping with a reduced level of 2–5ApCp binding by RNase L. In contrast, IFN protection from VSV challenge was similar in the H2ABP/pcDNAIneo cells and in control pcDNAIneo cells (Fig. 9*B*). These results indicate that RLI inhibits the IFN activation of RNase L pathway *in vivo* as well. Moreover they confirm that the 2–5A system is a potent inhibitor of EMCV replication and that anti-VSV effect of IFN must predominantly be mediated by another pathway (6).

DISCUSSION

The human 2–5A-dependent RNase L inhibitor described in this paper was cloned by the screening of an expression library with 2–5ApCp. Its 2–5A-binding characteristics differ from those of the 2DR polypeptide recently cloned by Zhou *et al.* (1993) (22) since RLI does not bind 2–5ApCp under conditions classically used to characterize RNase L, *e.g.* 2–5A binding and covalent binding. Our experimental conditions differ from those used by Zhou *et al.* (1993) (22) as following (i) the λ-Zap library was prepared from Daudi cells with no IFN induction, (ii) the filters were screened without guanidine denaturation of proteins, (iii) 2–5ApCp was prepared from natural 2–5A rather than from bromo-substitued 2–5A (the two have different affinities for RNase L, 38).

The expression of this clone inhibits the activation of RNase L by 2–5A and leads to the inhibition of its endoribonuclease activity. This clone was therefore termed RLI for RNase L Inhibitor.

RLI mRNAs Are Not Regulated by IFN—The RLI cDNA hybridizes with two mRNAs of 3.5 and 2.8 kb (Fig. 1) which differ in their 3′-untranslated regions. The biological significance of these two mRNAs has not been investigated. Several putative AUUUA instability sequences (39) are present in their 3′-untranslated region, perhaps indicating differences in their post-transcriptional regulation. Their half-lives in HeLa cells following actinomycin-D treatment are, however, similar.

Even at high dose (10^3 units/ml) IFN does not regulate RLI mRNAs abundance. This contrasts with the fact that IFN regulates the 2–5A pathway with a large increase in the level of 2–5A synthetases and a 3-fold increase in RNase L mRNA (22). The ratio between the activator (2–5A), RNase L, and the inhibitor will therefore be shifted toward activation after IFN treatment. Further investigations are, however, required to confirm if these two proteins always display opposite regulation in circumstances under which RNase L activity is modified.

Consensus Sequences in RLI cDNA—The cDNA of RLI exhibits a few interesting features. RLI contains two phosphate-binding loop (P-loop) motifs between amino acids 110–117, and 379–386 (Fig. 1*B*). These P-loop motifs are conserved in most adenine and guanine nucleotide-binding proteins, such as adenylate kinase, RecA protein, ras oncogene product p21, heterodimeric G proteins, elongation factors, or proteins involved in active transport (see Ref. 35 for a review).

Interestingly, the P-loop motif of RLI and of 2DR is repeated. Whether a P-loop is required for 2–5A binding by RLI as demonstrated for 2DR (22) has not yet been established. The first P-loop is certainly not required for 2–5A binding or for RNase L inhibition by RLI since these two properties remain in the truncated (H2ABP) form of RLI.

Another interesting motif is the $CX_2CX_2CX_3C$ ferredoxin-like sequence between the amino acids 55 and 66 (Fig. 1*B*). This ferredoxin-like sequence is found in proteins with very different functions and in particular in proteins which interact with nucleic acids such as bacterial endonuclease III. Kuo *et al.* (1992) suggested that endonuclease III is the prototype of a new class of iron-sulfur proteins wherein the primary rôle of the iron-sulfur cluster is to position conserved basic residues for interaction with the phosphate backbone of the DNA substrate (40). The iron-sulfur cluster plays a rôle analogous to the zinc atom in "zinc-finger" proteins (41). This cysteine-rich region in RLI could be involved in binding of RLI with nucleic acids. Alternatively, this cysteine-rich region could mediate the formation of heterodimers with RNase L. We indeed demonstrated here that RNase L can be immunoprecipitated in association with RLI (Fig. 6). Finally, it is worth mentioning here that RNase L behaves as a high molecular weight complex in non-denaturing conditions (19).

Inhibition of RNase L Activity by RLI Is Due to the Association of the Two Proteins—The inhibition of RNase L activity by RLI is not due to the degradation of 2–5A. RLI does not increase the degradation rate of 2–5ApCp in a reticulocyte extract (Fig. 7) in the conditions where an inhibition of 2–5A binding is observed (Fig. 4).

The radiobinding and covalent binding assays are routinely

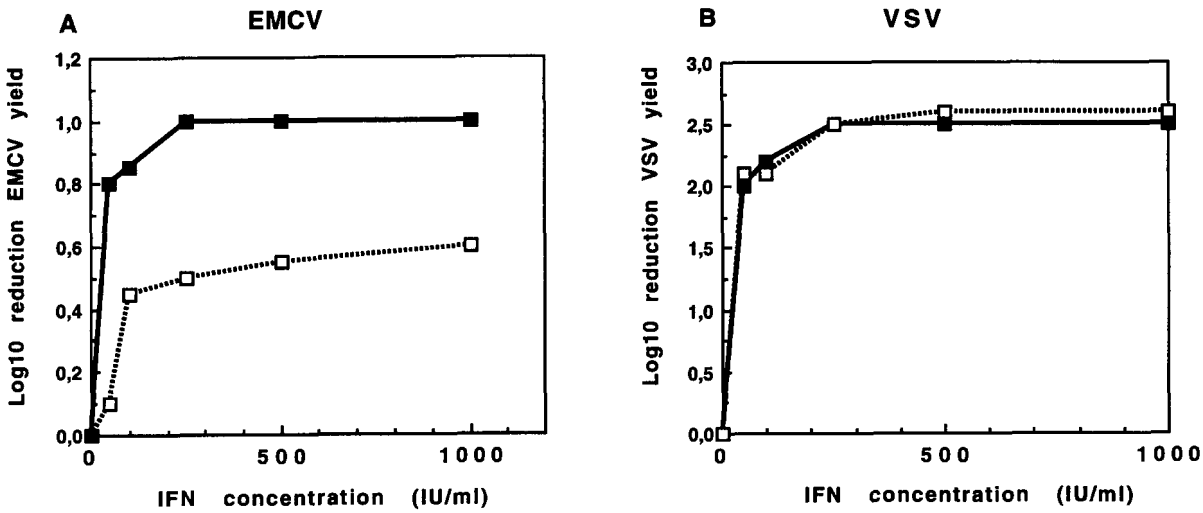

FIG. 9. **Expression of the truncated active RLI supresses a part of the anti-EMCV activity of IFN.** Control pcDNAIneo cells (■) or H2ABP/pcDNAIneo cells (□) were incubated for 20 h in the absence or presence of Huα/β IFN at the indicated concentration and infected thereafter with EMCV (*A*) or VSV (*B*). Virus was harvested 18 h later and titrated on indicator cells as indicated under "Experimental Procedures."

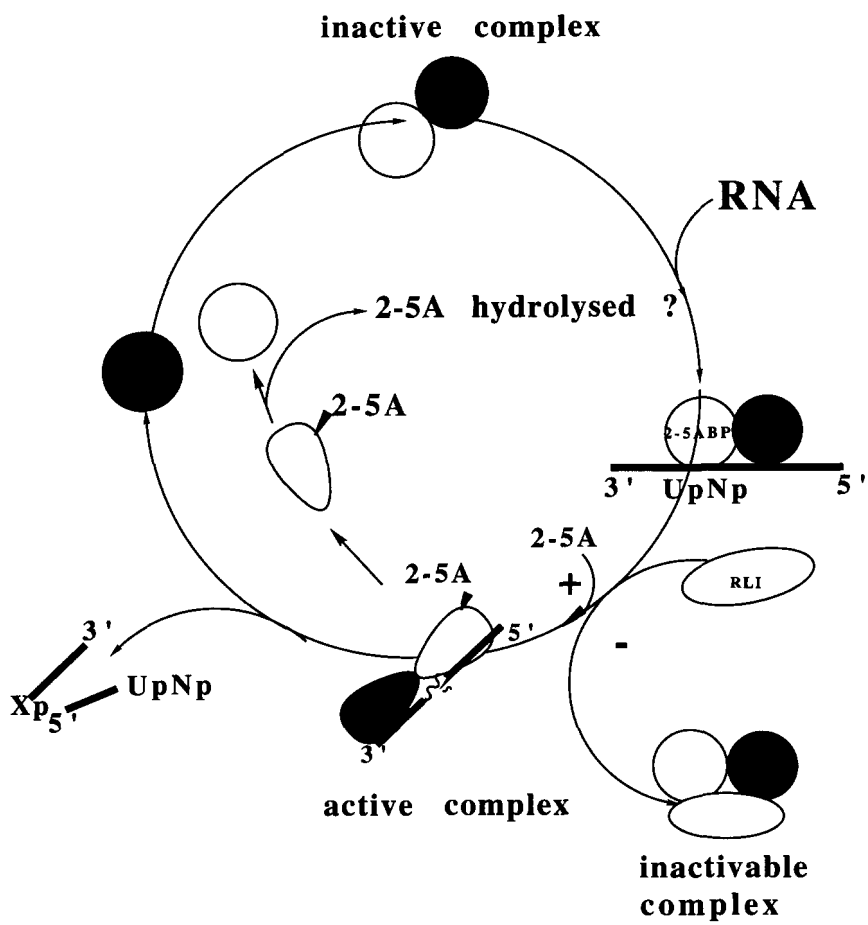

FIG. 10. **Hypothetical scheme of RNase L activation by 2–5A and its inhibition by RLI.**

performed with an 8-fold excess of 2–5ApCp. A competition between RLI and RNase L for the binding to 2–5ApCp seems therefore unlikely in these conditions. Moreover, increasing the concentration of 2–5ApCp while keeping constant the ratio between the two proteins does not modify inhibition of the 2–5ApCp binding by 2DR. This confirms that the ratio between RNase L and its RLI inhibitor is critical while the concentration of 2–5ApCp is not a limiting factor in these particular experimental conditions.

The inhibition of RNase L by RLI is probably due to a direct interaction of the two proteins and not to a stable modification of RNase L. The two proteins are indeed immunoprecipitated in a complex with the RNase L associated RNABP which we have already described (Fig. 6). This is not surprising since (i) mAb3 recognizes a single RNABP which is associated with the 2–5ABP in the RNase L protein complex and inhibits the 2–5A-

dependent RNA cleavage by RNase L (18). (ii) Hassel *et al.* (1993) have described sequences allowing interactions between 2DR and other proteins (6), and (iii) analogous sequences exist in RLI cDNA (Fig. 1). The immunoprecipitation of 2DR in association with RNABP was also expected since it probably reflects the organization of the natural RNase L complex. On the other hand it seems logical that a regulatory protein (RLI) could be associated with the protein it regulates (RNase L). Interestingly, the immunoprecipitation of 2DR and RLI only happens when RNABP is present and when these proteins are associated. This does not occur in wheat-germ extract where RNABP is absent (Fig. 3). In addition RLI translated in wheat-germ extract does not inhibit RNase L activity.

The inhibition of RNase L activity by RLI therefore seems to require a direct association between the two proteins (as tentatively illustrated in Fig. 10). This hypothesis is further supported by the fact that the electrophoretic dissociation of the two proteins (RLI and RNase L) restores the 2–5A binding capacity of RNase L (Fig. 8). Some unknown factors remains. For example, we do not know whether RLI interacts directly with 2–5ABP (or 2DR) or by the intermediate of RNABP (18). The physiological role of RNABP itself remains to be elucidated since cloned 2DR behaves as a fully active 2–5A dependent nuclease (22). Antibodies directed against each protein as well as cloned functional proteins will hopefully allow determination of the role of each component of the putative 2–5A-RNase L complex. Work along those lines is now in progress in our laboratory.

The human Daudi cell RLI is active on RNase L from other human cells (HeLa) or from other species (rabbit) in keeping with the finding by Zhou *et al.* (1993) that 2DR is a well conserved protein (22).

Expression of RLI Inhibits the 2–5A/RNase L Pathway in Intact Cells—The inhibitor of RNase L is also active in intact cells (Fig. 9). Its overexpression in HeLa cells partly antagonizes the antiviral effect of IFN against EMCV but not VSV as expected (6). This confirms and extends previous studies showing that the 2–5A system is an important component of the antiviral activity of IFNs on picornaviruses. The overexpression of a cDNA coding for the 40 kDa form of 2–5A-synthetase largely reduces picornavirus yield (42–44).

The inhibition of the anti-EMCV activity of IFN in RLI-transfected cells is only partial which could be due to the remaining RNase L activity in this clone. This could reflect the partial inhibition of nuclease activity observed *in vitro* (Fig. 5). Likewise the inhibition of RNase L pathway during EMCV infection needs a high level of expression of a truncated form of 2DR behaving as a dominant negative competitor (6). It was also demonstrated that a 2–5A inhibitor analog inhibited IFN protection against EMCV by 10-fold only (45). Another possibility is therefore that a second pathway like the IFN-induced protein kinase (PKR) is also required for a full expression of the IFN antiviral activity against picornaviruses. It has indeed been shown that PKR is also implicated in the molecular mechanism of EMCV inhibition (46).

Nevertheless, it is apparent from this study that RLI is active in intact cells and *in vitro* in keeping with a major rôle of the 2–5A/RNase L in the antiviral action of IFN against picornaviruses but not on VSV replication.

Biological Role of RLI—Hassel *et al.* (1993) have recently confirmed the central role played by RNase L and the 2–5A system in the antiviral and in the antiproliferative effects of IFNs, and they have postulated its implication in the control of mRNA stability (6). 2–5A and RNase L are known to vary in various physiological conditions as IFN treatment, cell growth arrest, or hormone status. Several authors (see Ref. 24 for a review) have underlined the importance of nucleases in the regulation of mRNA stability and consequently in gene regulation. As cited in the introduction, the 2–5A system is a well characterized system of RNA degradation whose activation was believed to be controlled mainly by 2–5A synthetase(s) and 2–5A. RLI might constitute an important additional element for the down-regulation of the 2–5A pathway. RI or RAI for ribonuclease/angiogenin inhibitor was, to our knowledge, the only other known RNase inhibitor cloned (47, 48). RI belongs to a highly diversified protein superfamily with a common repetitive module. RLI shows no sequence homology with this inhibitor which is not surprising since RNase L is very different from placental ribonuclease and angiogenin. It is of note, however, that both RNase inhibitors are very rich in leucine residues and in thiol groups.

These two systems (RLI and RI) can also be compared in their mode of action. The equilibrium between RI and its RNase target plays a central role in the regulation of mRNA turnover and protein biosynthesis, the two partners being always present at the same time in the cell (49). In this paper we demonstrate at least *in vitro* that the inactivation of RNase L by RLI depends of the ratio between the two proteins. IFN treatment, which increases RNase L, will modify the ratio between the two proteins and shift the balance toward activation of the 2–5A/pathway.

RLI adds a new and potentially important level of control and regulation of the 2–5A/RNase L pathway. Studies of its regulation will hopefully be helpful in understanding the regulation and the biological role of the 2–5A pathway.

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