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A Scientific Journey Through the 2-5A/RNase L System

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Abstract

The antiviral and antitumor actions of interferons are caused, in part, by a remarkable regulated RNA cleavage pathway known as the 2-5A/RNase L system. 2′-5′ linked oligoadenylates (2-5A) are produced from ATP by interferon-inducible synthetases. 2-5A activates pre-existing RNase L, resulting in the cleavage of RNAs within single-stranded regions. Activation of RNase L by 2-5A leads to an antiviral response, although precisely how this happens is a subject of ongoing investigations. Recently, RNase L was identified as the hereditary prostate cancer 1 gene. That finding has led to the discovery of a novel human retrovirus, XMRV. My scientific journey through the 2-5A system recounts some of the highlights of these efforts. Knowledge gained from studies on the 2-5A system could have an impact on development of therapies for important viral pathogens and cancer.

Keywords

2-5A; RNase L; interferon; cancer; virus

1. Background on the 2-5A/RNase L system

Over the past thirty-years, investigations into the mechanisms of interferon (IFN) action have elucidated how antiviral innate immunity operates within and between mammalian cells. The focus of my work over this period has been, and continues to be, probing the biology and biochemistry of the 2-5A/RNase L system, and studying its roles in health and disease. My scientific journey, from basic research to clinical studies, are summarized in this monograph. The 2-5A/RNase L system is one the principal pathways by which IFNs suppress viral infections. Exposure of cells to IFNs induces expression of genes that result in an “antiviral state”. Type I IFNs bind to the IFN-α receptor 1 (IFNAR1) and IFNAR2 polypeptide chains on cell surfaces initiating JAK-STAT signaling to the IFN stimulated genes (ISGs)[1]. Included among over a hundred different ISGs are genes encoding 2-5A synthetases (OAS)[2,3]. The OAS genes are a family of ISGs that function in the 2-5A/RNase L system (Fig. 1). In humans there are three functional OAS genes (*OAS1-3*), resulting in 8 to 10 OAS isoforms due to alternative mRNA splicing[4]. In mice, in addition to *OAS2* and *OAS3* there are 7 separate *OAS1* genes, including *OAS1b*, the flavivirus resistance gene (*Flv*^{r}][5-8]. When stimulated by dsRNA, the functional OAS proteins produce a series of short 5′-phosphorylated, 2′,5′-linked oligoadenylates collectively referred to as 2-5A [$p_x5'A(2'p5'A)_n$; x = 1-3; n≥2] from ATP[9]. The first molecular clone for an OAS was obtained by Michel Revel's lab[10]. Biochemical characterization of OAS proteins by Ara Hovanessian's lab[11-14] and Ganes Sen's lab [15-19] and a crystal structure by Rune Hartmann and Vivien Yee (in collaboration with Ganes Sen and Just Justesen)[20] have led to functional and structural insight into the OAS family of

proteins. Because dsRNA is a frequent viral pathogen associated molecular pattern, 2-5A often accumulates in IFN-treated and virus-infected cells[21-23]. The principal species of 2-5A found in such cells is the trimeric form, $p_3(A2'p5')_2A$[21]. 2-5A is a transient signaling molecule that is degraded within minutes by the combined action of 2′-phosphodiesterase and 5′-phosphatase(s) [24,25]. The only well-established function of 2-5A is activation of the latent endoribonuclease, RNase L[26]. To do so, 2-5A must have at least one 5′-phosphoryl group, the internucleotide linkages must be 2′ to 5′ and the nucleotides must by adenylyl residues for optimal activity[27]. RNase L is activated by subnanomolar levels of 2-5A resulting in the cleavage of single-stranded regions of RNA, preferentially after UpUp and UpAp dinucleotides [28,29]. In addition, single-stranded regions of RNAs that are partially double-stranded are preferentially cleaved by RNase L[30]. Ribosomal RNAs in intact ribosomes are cleaved at specific sites providing an index of prior RNase L activity and a frequent characteristic of virus-infected cells (Fig. 1)[31].

2. Monitoring the presence and activation of RNase L in intact cells

I entered ongoing studies on the 2-5A system as a postdoctoral fellow in Ian M. Kerr's lab in late 1979. By that time, members of the Kerr lab (notably Ara Hovanessian and Bryan Williams with collaborator Michael Clemens) had firmly laid the groundwork by discovering 2-5A synthetase (OAS) activity[32], elucidating the chemical structure of 2-5A[9], and demonstrating that isolated and purified 2-5A activated the 2-5A dependent RNase[33](now referred to as “RNase L”; the “L” stands for “latent”). Complementary work in the labs of Peter Lengyel, Michel Revel, Corrado Baglioni and Charles Samuel gave impetus to these early studies[34-37]. I came to the Kerr lab because of my interest in unusual nucleotide regulators and my fascination with Ian Kerr's remarkable discovery of 2-5A[9,38]. The discovery of 2-5A followed Ian Kerr's observation of an IFN-induced increase in the sensitivity of protein synthesis to inhibition by dsRNA[39-41]. My transition was an easy one in that I was immediately able to go to work because all of the cell-free system components were in place in Eppendorf tubes in the −70C° freezer. The labs in the National Institute for Medical Research (NIMR) in Mill Hill, London were somewhat Spartan at that time, but it was a great place to do a post-doc because of the wonderful group of scientists that were assembled there, particularly in the area of virology. Not to mention the history, after all in the mid-1950's the NIMR is where A. Isaacs and J. Lindenmann discovered IFN. [For readers who have never had the pleasure, the NIMR also appears in the film *Batman Begins* (source: Wikipedia.org)]. I made many good and longtime friends from my days at the NIMR, including George Stark, who was pursuing collaborative studies with Ian Kerr at the time[42]. Our lab group moved to the Imperial Cancer Research Fund (now Cancer Research UK) labs in Lincoln's Inn Fields in the summer of 1980.

My first task was to develop methods for detecting and characterizing the RNase and to collaborate in the development of quantitative and highly sensitive methods for conveniently measuring 2-5A from intact cells. It was not possible at the time to synthesize high specific activity 2-5A using OAS and radiolabeled ATP because the K_m was too large. Therefore, using enzymatically synthesized and purified 2-5A, a high specific activity, radiolabeled probe [$p_3(A2'p5')_3A$-^{32}pCp] was made using T4 RNA ligase and purified by HPLC[25]. The ^{32}P-labeled 2-5A probe (>10^6 Ci/mol) was used to detect the RNase on filters and in gels[43] and it was an essential component of the “radiobinding” assay used to measure nanomolar levels of 2-5A from IFN treated and virus infected cells[21]. The model system for these early studies involved IFN treatment of mouse L fibroblasts followed by infections with the picornavirus, encephalomyocarditis virus (EMCV). Detection of activated RNase L within cells could be conveniently monitored by the specific breakdown in the rRNA[44]. By demonstrating IFN- and virus-dependent activation of both OAS and RNase L, these and prior studies (with Bryan Williams, Matty Knight, Jane Cayley, Danny Wreschner and other members of the Kerr lab)

firmly established the 2-5A/RNase L system as a participant in the antiviral actions of IFNs [21,28,45,46].

3. Molecular cloning of RNase L

By the beginning of 1982, I was preparing to start my career as Assistant Professor at the Uniformed Services University of the Health Sciences in Bethesda in Robert Friedman's pathology department. The long and torturous journey of cloning RNase L began soon thereafter (described in [47]). We tried everything, including a heroic effort by Carl Dieffenbach using an antibody-based approach, but what eventually succeeded (by Bret Hassel and Aimin Zhou) was screening phage lifts of an IFN induced cDNA library with radiolabeled 2-5A probe[26](Fig. 2). What this taught me about research is never give up if the goal is worthwhile.

After a sabbatical year in Bryan Williams' lab at the Hospital for Sick Children in Toronto, I continued studies on RNase L to the present at the Cleveland Clinic, which assembled a dream team of interferon mavens including Bryan Williams, Ganes Sen, George Stark, Andy Larner, Tom Hamilton, Ernest Borden and Richard Ransohoff. In addition, a long-term and productive collaboration with chemist, Paul Torrence at the National Institutes of Health continued after the move to Cleveland. These efforts were aimed at directing RNase L to cleave target RNAs in vivo [48,49]. Cloning of RNase L provided the first real insight into the enzyme and contributed to, or was complemented by, many interesting and important findings by several different labs. For instance, Peter Walter discovered that the IRE1 protein kinases, which function in the unfolded protein response (UPR) in organisms from yeast to humans, were also ribonucleases after finding homology with the RNase L catalytic domain[50]. Those results, in turn, led to elucidation of the UPR pathway involving a unique RNA splicing pathway [51]. Therefore, RNase L is not alone, but rather it is the founding member of a family of stress-response ribonucleases[52,53]. In addition, the deduced amino acid sequence of the 2-5A binding domain led the groups of K. Nakamura and Y. Kitade to solve the crystal structure of this RNase L domain complexed with 2-5A[54]. The structure of the complete RNase L, with or without bound 2-5A, remains to be solved. Meanwhile, in complementary work, Catherine Bisbal and co-workers were successful in cloning the RNase L inhibitor (RLI), an ATP binding cassette protein also known as ABCE1[55]. Khalid Khabar showed that RNase L could affect the stability of PKR mRNA and levels of PKR, demonstrating that the two pathways are in a type of balance[56]. Studies by the groups of Robert Suhadolnik[57,58] and Bernard LeBleu [59,60] described up-regulation of the 2-5A system and accumulation of a 37 kDa polypeptide from RNase L in PBMC of patients with chronic fatigue syndrome. In addition, Lawrence Kuo's group at Merck Research Laboratories performed outstanding enzymology and biophysical studies of RNase L[61-64].

RNase L is a fascinating enzyme with an interesting arrangement of structural and functional domains and an intriguing mode of action (Fig. 3). Biochemical characterization of RNase L in my lab was performed by Beihua Dong[52,65-68]. From the N-to the C-terminus, there are nine ankyrin repeats, several protein kinase-like motifs and the ribonuclease domain. What distinguishes RNase L from all other ankyrin-repeat proteins is that ankyrin repeats 2 and 4 of RNase L constitute the 2-5A binding site[54]. In the absence of 2-5A, inhibitory domains in the ankyrin and protein kinase motifs suppress the ribonuclease domain while also maintaining RNase L as a monomer, probably due to masking of the interaction sites. 2-5A binding to the ankyrin region is believed to induce a conformational shift that releases internal interactions, first allowing the monomers to attract, and then to dimerize. In the dimer the nuclease domains are no longer repressed by internal interactions and are thus able to cleave RNA[52].

4. Involvement of RNase L in the antiviral action of IFNs

It is currently unknown precisely how the 2-5A system restricts viral infections in vivo. To address this question, we generated RNase L gene knockout mice[69]. The RNase L-deficient mice which have enhanced susceptibility to infections by the picornaviruses, EMCV or Coxsackievirus B4[69,70], herpes simplex virus 1 (HSV-1)[71], and the flavivirus, West Nile virus[72]. Interestingly, there are presently a few instances in which RNase L actually promotes viral diseases and/or replication, rather than blocking it[73,74]. For example, Leslie Schiff's group has shown that RNase L and PKR contribute to reovirus-mediated shut-off of host protein synthesis and these proteins can, with some viral strains, actually promote viral replication [75]. In a collaboration with Bryan Williams, we generated the triply deficient (TD) mice, lacking RNase L, PKR, and Mx1[76]. These TD mice retained a significant IFN antiviral response, demonstrating that there is much more to IFN action than these genes. The TD and *Rnasel*$^{-/-}$ mice are currently in widespread many different labs investigating antiviral innate immunity. In addition, Bret Hassel's group reported with us that the *Rnasel*$^{-/-}$ mice have extended lifespans and that RNase L has a role in cell senescence[77]. Therefore, the *Rnasel*$^{-/-}$ mice may be useful in aging research.

RNase L could eliminate a single-stranded RNA virus by as few as one RNA cleavage event per genome, in which case the infected cells might survive[78]. However, we and others have shown that activation of RNase L beyond an ill-defined threshold causes apoptosis[69, 79-83]. Therefore, activation of RNase L could lead to elimination of virus-infected cells under some circumstances. RNase L mediated apoptosis is the result of a JNK-dependent stress-response pathway leading to cytochrome c release from mitochondria and caspase-dependent apoptosis[80,81].

By using radioactively labeled 2-5A, we showed that mice expressed RNase L in every tissue type that was analyzed[69]. In addition, RNase L pre-exists in a wide range of different human cell types and tissues due to the presence of several different tissue specific promoter elements in the *RNASEL* gene[84]. In contrast, no 2-5A binding proteins were detected in several different organs of *Rnasel*$^{-/-}$ mice[69]. Therefore, the ability to bind 2-5A with high affinity (K_D = 40 pM)[85] is a unique biochemical property of RNase L. As a result, 2-5A is an unambiguous signal for initiating RNA cleavage through activation of RNase L.

We have recently shown that RNase L not only degrades RNA, it also induces at the transcriptional levels the expression of many genes[86]. For instance, the macrophage inhibitor cytokine-1 (MIC-1) gene is induced by RNase L in a pathway involving stress-activated kinases. Induction of the MIC-1 gene in response to 2-5A required stimulation of the MAP kinases, JNK and ERK, and the catalytic function of RNase L. There are significant gaps in our understanding of the transcriptional signaling pathways mediated by 2-5A activation of RNase L. For example, how RNase L activity stimulates JNK and ERK and the downstream events to gene induction are unknown. However, JNK is activated in response to different treatments that cleave or modify rRNA in intact ribosomes (including ricin A, alpha sarcin, uv light, and RNase L)[81,87,88]. Because some of the RNase L-induced genes have antiviral functions, this phenomenon could be contributing to the antiviral effect of 2-5A. In summary, RNase L could be causing its antiviral effects through a combination of direct cleavage of viral RNA, inhibition of protein synthesis through the degradation of rRNA, induction of apoptosis, and induction of other antiviral genes.

5. The role of RNase L in hereditary prostate cancer and discovery of a human retrovirus

One of the remarkable aspects of being a scientist, is that you never know where your scientific journey will lead. RNase L was initially proposed to be a candidate tumor suppressor by us and Peter Lengyel based on its involvement in the antiproliferative activity of IFN and on the location of *RNASEL* at chromosome 1q25, a region deleted or rearranged in some breast cancers [89-91]. However, specific evidence of a role for RNase L in the suppression of cancer had to wait almost 10 years, until the long sought, hereditary prostate cancer 1 (HPC1) was mapped to the RNase L gene by the group of Jeff Trent[92]. Several germline mutations or variants in *HPC1/RNASEL* have been observed in hereditary prostate cancer[92-95] (reviewed in ref. [96]), including a common (35% allelic frequency) missense variant of RNase L, in which a G to A transition at nucleotide position 1385 (G1385A) results in a glutamine instead of arginine at amino acid position 462 (R462Q). Remarkably, in collaboration with Graham Casey, a large, controlled sib-pair study implicated the RNase L “Q” variant in up to 13% of unselected prostate cancer cases[95]. One copy of the mutated gene increased the risk of prostate cancer by about 50%, whereas individuals that were homozygous for the mutation had a two-fold increased risk of prostate cancer. The RNase L “Q” variant had a 3-fold decrease in catalytic activity compared to the wild-type enzyme[95,97]. Although several genetic and epidemiologic studies support the involvement of *RNASEL* (and notably the R462Q variant) in prostate cancer etiology [92-95], others did not [98-100], suggesting that either population differences or environmental factors such as infections may modulate the impact of *RNASEL* on prostatic carcinogenesis.

While the anti-apoptotic phenotype of RNase L deficiency could potentially suppress cancer, RNase L is also a principal mediator of the antiviral action of IFN. This led us to consider the possibility that the putative linkage of RNase L alterations to HPC might reflect enhanced susceptibility to a viral agent. To test our hypothesis, in collaboration with Don Ganem, Joseph DeRisi and Eric Klein we examined wild-type and RNase L variant (R462Q) prostate tumors for evidence of viral sequences, by hybridization to a DNA microarray composed of the most conserved sequences of all known human, animal, plant and bacterial viruses[101-103]. We reported in 2006 that 40% (8 of 20) of all tumors homozygous for the R462Q allele harbored the genome of a novel gammaretrovirus, called XMRV, which is closely related to xenotropic MuLVs[103]. In contrast, retroviral sequences were present in <2% of tumors bearing at least one copy of the wild-type allele (1 of 66). In addition, virus-harboring cells were detected within infected prostatic tumor tissues by fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC) for gag protein. The infected cells were stromal, fibroblastic and hematopoietic elements, rather than epithelial cancer cells. Therefore, any effect of XMRV on prostate cancer development would have to be indirect involving alterations in the tumor microenvironment. These findings were the first detection of xenotropic MuLV-like agents in humans, and reveal a strong association between infection with the virus and defects in RNase L activity. Therefore, results are a direct validation that RNase L functions as an antiviral gene in humans.

Recently, we have assembled a replication-competent viral molecular clone of XMRV[104]. XMRV replicated more efficiently in prostate cancer LNCaP cells harboring a epigenetic silencing of JAK1[105] and a mutation in one allele of *RNASEL*, than in DU145 prostate cancer cells which contain only wild type RNase L. Viral replication was sensitive to IFN, and reduction in levels of RNase L decreased the IFN antiviral effect, as predicted based on the presence of XMRV in prostate tumors with mutated RNase L. In addition, expressing of the human xenotropic MuLV receptor, XPR1, rendered hamster cells susceptible to XMRV infections. In collaboration with Samson Chow, XMRV integration sites were mapped in human DNA isolated directly from prostate tissue, thus validating the humans have been

infected with this virus. XMRV has been recently added to a list of only several other viruses that are known to be associated with human cancers[106].

6. Summary

The 2-5A/RNase L system, a prime mediator of antiviral innate immunity, is a fascinating and unique biochemical pathway of higher vertebrates. Recent studies in humans have demonstrated that germline mutations in *RNASEL* can enhance the risk of prostate cancer and have led to the discovery of the novel human retrovirus, XMRV. Because RNase L can inhibit a range of different types of viruses, it is also a target for future drug development efforts. Where else the journey will lead from here is uncertain, but there never seems to be a dull moment when investigating the 2-5A/RNase L system.

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Biography

Robert H. Silverman, Ph.D. is Professor at The Lerner Research Institute, Cleveland Clinic (Cancer Biology), at Case Western Reserve University (Biochemistry and Molecular Biology & Microbiology) and Cleveland State University (Chemistry). Dr. Silverman received his B.Sc. from Michigan State University and his Ph.D. in Molecular, Cellular and Developmental Biology from Iowa State University. His postdoctoral training was at the Roche Institute for Molecular Biology, New Jersey and at the National Institute for Medical Research and Imperial Cancer Research Fund Laboratories, London. He was Professor in the Department of Pathology

at the Uniformed Services University of the Health Sciences in Bethesda prior to joining the Cleveland Clinic in 1991. In 1993 he received the Milstein Award from the International Society of Interferon and Cytokine Research. Understanding the molecular mechanisms of interferon action against viruses and cancer cells is the focus of Dr. Silverman's laboratory.

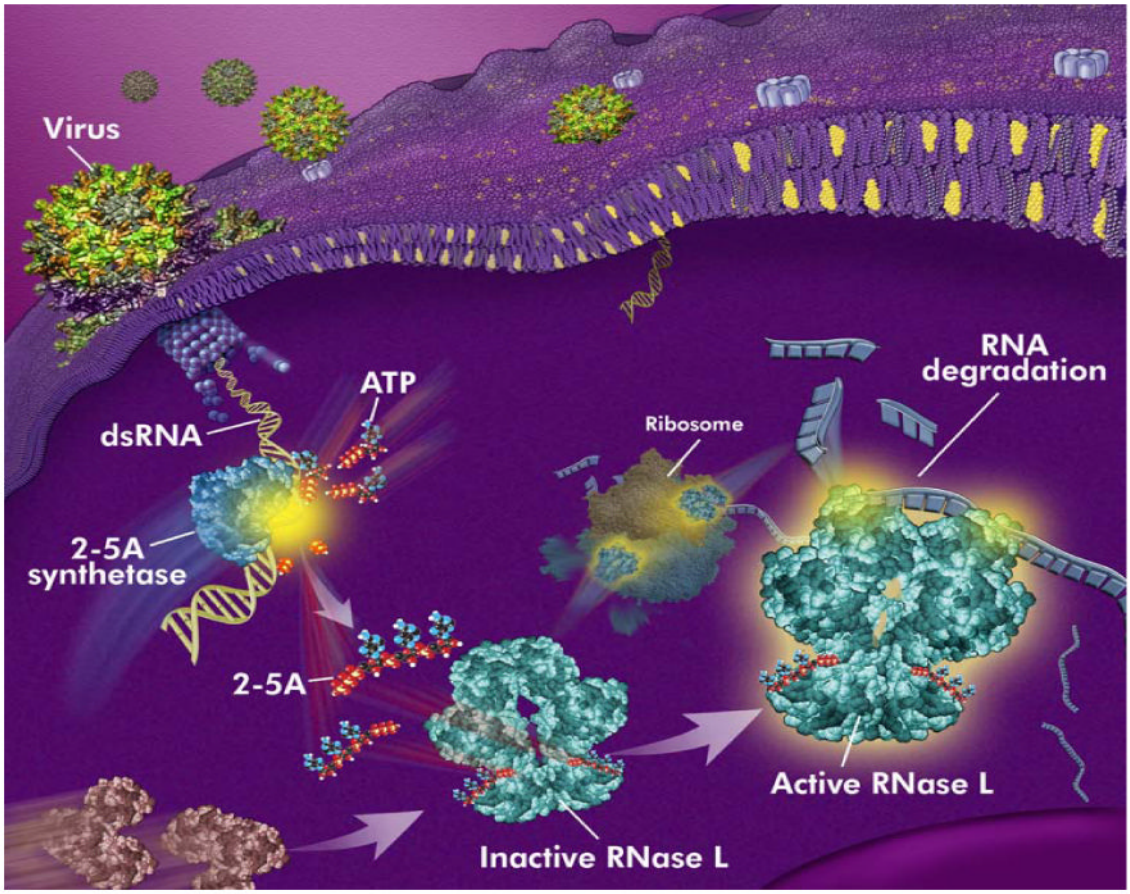

Figure 1. The 2-5A/RNase L pathway is a classic antiviral innate immune pathway

The viral pathogen associated molecular pattern, dsRNA, activates IFN induced 2-5A synthetase (also known as oligoadenylate synthetase or OAS). This results in the synthesis of 2-5A from ATP. The 2-5A bindings to inactive monomeric RNase L, forcing the formation of activated dimers of RNase L. The resulting degradation of single stranded loop regions in RNA, including rRNA in intact ribosomes, produces a potent antiviral response in the IFN treated and virus infected cell.

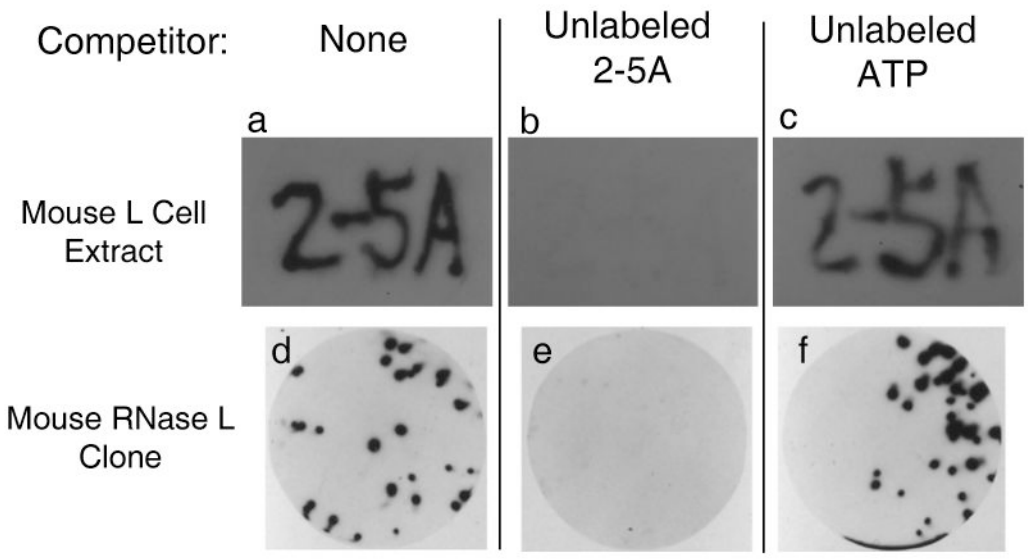

Figure 2. Cloning of RNase L with radiolabeled 2-5A as probe (as described in ref. [26])

(a-c) Post-mitochondrial supernatant fraction of mouse L cells was used to write the word "2-5A" on nitrocellulose filters. Subsequently, the filters were incubated in a solution of the 2-5A probe (a) without additions, (b) with unlabeled 2-5A as competitor, and (c) with unlabeled ATP. The filters were washed, dried and used to expose x-ray film. The results on autoradiography show that binding was prevented with unlabeled 2-5A but not with ATP, thus demonstrating the feasibility of the approach. The first clone for a partial murine RNase L was obtained by screening a cDNA expression library of IFN and cycloheximide treated mouse L cells. The third round screening was done (d) without competitor, (e) with unlabeled 2-5A as competitor, or (f) with unlabeled ATP.

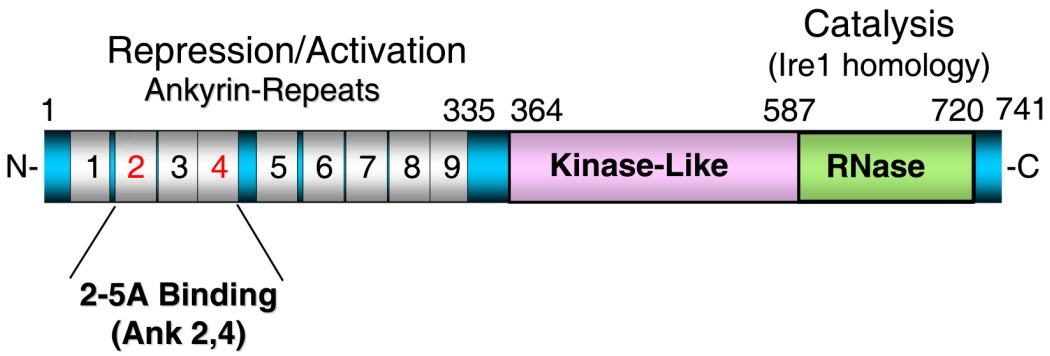

Figure 3. The domain structure of RNase L

Ank, ankyrin repeat; Ire1homology, nuclease domain similar to Ire1 kinase/endoribonucleases.