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An RNasin-resistant ribonuclease selective for interleukin 2 mRNA

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ABSTRACT

Interleukin 2 (IL2) mRNA has a short half-life in the cytoplasm of T lymphocytes, relative to most mRNA. We have discovered a candidate ribonuclease to account for the rapid turnover of IL2 mRNA in the cytosol of the human T lymphocyte cell line Jurkat. In partially purified form, this RNase is about 7 times as active on IL2 as on β-globin mRNA. Pancreatic RNase, by contrast, does not show a significant preference for IL2 mRNA. Neither 5′ capping, nor polyadenylation of the substrate mRNAs affects their degradation by the IL2-selective mRNase, whose activity is optimal in 0.5 mM Mg^{++} and 100 mM potassium acetate. The mRNase behaves like a protein of molecular weight 60 – 70,000 on gel chromatography, and is unusual in that it is insensitive to placental RNase inhibitor (RNasin). The mRNase cleaves IL2 mRNA at a small number of sites in the coding region, and IL2 mRNA containing only the coding region and 36 nucleotides of the 3′-noncoding region competes efficiently with full-length IL2 mRNA for the mRNase, whereas β-globin mRNA does not.

INTRODUCTION

The level of expression of a gene can be controlled by the rate of synthesis of its mRNA, the efficiency of its translation, and also by the rate of that mRNA's degradation. Different mRNAs in eukaryotic cells can have greatly different rates of degradation, and the degradation rate of a given mRNA can vary widely under different cellular circumstances. The half-life of an average mRNA in mouse L cells and in CHO cells is 8–10 hours (1, 2), whereas the mRNA half-life for c-myc is about 40 minutes (3) and that of globin mRNA may be as long as 60 hours (4). The half-life of c–myc mRNA decreases to about 10 minutes, however, when Daudi cells are treated with interferons (3). The half-lives of the mRNAs of certain cytokines, such as Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and Interleukin 2 (IL2) are relatively short, in the order of 20–60 minutes (5, 6, 7). The half-life of GM-CSF mRNA in B lymphocytes can be increased by 3–4 fold by stimulating the cells with the B cell mitogen LPS (6), while stimulation of human T lymphocytes by cross-linking the CD28 surface antigen stabilizes mRNAs for IL2, Tumor Necrosis Factor α (TNFα), GM-CSF, and Interferon γ (IFNγ) (8). The natural ligand for CD28 has recently been determined to be a cell-surface structure present on B lymphocytes and monocytes, as well as T lymphocytes. In mice, it is identified by monoclonal antibody B7/BB1. Cell signaling through the CD28 molecule appears to be the first example of enhancement of a lymphokine response by stabilization of the relevant mRNA (9, 10, 11).

There may, in principle, be two complementary aspects that determine the half-life of an mRNA. First, the secondary and tertiary structure of an mRNA may make it more or less susceptible to a given RNase. Secondly, there may be special RNases, or factors altering the reactivities of common RNases, that affect the degradation of specific mRNAs. Such nuclease systems must still discriminate between mRNAs to perform their function of specific degradation. Evidence for both aspects exists. The 3′ noncoding region (3′-NC) of GM-CSF mRNA has been shown to influence the half-life of mRNA molecules. When a 51-bp segment from the 3′-NC of GM-CSF mRNA was transplanted into the 3′-NC of β-globin mRNA, the intracellular half-life of the hybrid globin mRNA was found to decrease to less than 30 minutes (12). The transplanted segment contains an AU-rich element (ARE) whose presence in the 3′-NC region of mammalian mRNAs has been found to be correlated with a relatively short half-life (13). This motif, UUAUUUAU, occurs in the 3′-NC of the mRNAs of various inflammatory mediators, including interferons, IL1, IL2, and TNFα, and also c-fos mRNA. Evidence for the existence of specific RNases includes the demonstration of an exonuclease that preferentially degrades H4 histone mRNA, relative to β-globin mRNA (14). Crude cellular extracts have been prepared that preferentially degrade mRNAs containing the ARE on the one hand (15), or c-myc mRNA on the other (16), although such activities have not been either purified or characterized.

The biochemical characterization of specific mRNases has not yet been possible, since there are few well-documented cell-free preparations capable of carrying out such activities. One of the most intriguing of such systems is that described by Ross and colleagues, in which mRNA bound to polysomes is subjected to degradation *in vitro*. In this system, the addition of a cytosolic

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component (S130) greatly enhances the selective degradation of c-myc mRNA (17). The component in S130 has recently been identified as a factor that binds ARE in RNA, but is itself not the RNase – the latter is contributed by the polysome preparation on which the c-myc mRNA is isolated (18). Whether this system is responsible for degrading GM-CSF mRNA *in vivo* is not known, but an observation that supports this conclusion is that GM-CSF mRNA competes for the binding of the ARE-binding factor, whereas mRNAs not bearing the ARE in their 3'NC do not.

There is almost no evidence as to whether different short-lived mRNAs are degraded by the same mechanism(s) in eukaryotic cells. It would be extremely helpful to have soluble systems for studying this process for any short-lived mRNA. Although the polysome-based system of Ross et al. (19) has provided useful information on c-myc degradation, it has been difficult to resolve further. We have been interested in the turnover of IL2 mRNA, as this is both rapid and subject to variation in intact T lymphocytes. IL2 also contains the AU octamer motif characteristic of the ARE in its 3'-NC region. We have therefore investigated the degradation of synthetic IL2 mRNA by extracts of the human T cell line Jurkat. As a control, we have used the degradation of synthetic human β-globin mRNA. By reducing the contributions of nonspecific RNases using RNasin, we have found that such extracts carry out the selective degradation of IL2 mRNA. The selective RNase activity has been partially characterized, and distinguished from the bulk of nonspecific cellular RNases.

MATERIALS AND METHODS

Preparation of cell extracts

The human T leukemia cell line Jurkat was cultured in suspension medium RHFM (RPMI 1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, 20 mM sodium bicarbonate, 0.34 mM pyruvate, 0.02 M HEPES buffer, 9% fetal bovine serum). Cells were harvested at a density of 5×10^5 per mL, washed once with phosphate-buffered saline (PBS – 0.15 M NaCl, 0.034 M sodium phosphate, pH 7.2–7.4), resuspended in a hypotonic buffer (1 mM potassium acetate, 1.5 mM $Mg(Ac)_2$, 2 mM dithiothreitol, 10 mM Tris-HCl, pH 7.6) and then broken in a Teflon-glass homogenizer. The cell lysate was centrifuged for 15 min. at 11,500 rpm in an SS34 Sorvall rotor to precipitate the nuclei. The supernatant fraction was centrifuged in a TLA100.2 rotor (Beckman TL100 Ultracentrifuge), at 53,000 rpm for 2.5 hr. The supernatant was the S130 fraction In some preparations the pellet containing the polysomes was resuspended in a small volume of the same hypotonic buffer described above. Both S130 and resuspended polysomes were stored at −70°C.

In vitro synthesis of ^{35}S-labeled mRNA

The plasmid pHIL2-GA contains the human IL2 cDNA (5'-NC, coding, 3'-NC), followed by a segment of 62 A residues in the coding strand, within a pGEM1 vector. It was prepared by subcloning an EcoR I fragment of human IL2 cDNA in the plasmid pGEMA, which is a pGEM1 vector (Promega BioTech) containing the poly A segment. pGEMA was produced by first cleaving pGEM1 with Sac I and extending the overhanging 3' ends with terminal deoxynucleotidyl transferase and dATP. Poly dT was annealed to the oligo A regions by cooling the mixture from 65°C and incubating for 30 min. at room temperature. Poly dA was then added, the sample was incubated at room temperature for 30 min., and then treated with the Klenow DNA polymerase I fragment plus dATP and dTTP to close any gaps and generate flush ends. The DNA was treated with Sma I to remove the dA:dT segment from one end of the linear plasmid DNA, and the large fragment was isolated by gel electrophoresis. Finally, the pGEMA plasmid was circularized by blunt-end ligation with T4 polynucleotide ligase. The EcoR I fragment of human IL2 cDNA was then inserted into the EcoR I site of pGEMA.

The structure of pHIL2-GA is shown in Figure 1. The complete IL2 cDNA sequence follows the T7 promoter, and the IL2 3'-NC is followed by an EcoR I site, then 10 nucleotides of plasmid sequence, followed by 62 A residues. Before copying pHIL2-GA into full-length IL2 mRNA with RNA polymerase, it was linearized with BamH I, which cleaves 3 nucleotides beyond the end of the poly A segment. Human IL2 mRNA was synthesized *in vitro* using the T7 RNA polymerase according to the procedure described by the supplier (Promega BioTech). Capped mRNA was synthesized by including $m^7G(5')ppp(5')G$ (Pharmacia) nucleotide in the reaction. Radioactive label was provided as ^{35}S–rCTP (New England Nuclear). Capped β-globin mRNA was made from pSP6β/c (20), kindly provided by Dr. Jeffrey Ross, University of Wisconsin. The β-globin transcript generated from this plasmid with SP6 RNA polymerase contains approximately 40 adenylate residues following the 3' NC segment. The specific activity of ^{35}S-labeled mRNAs was about 2.5×10^7 cpm/μg.

Variant (shortened) IL2 mRNAs were generated by cleavage of the plasmid pHIL2-GA with restriction enzymes Sty I or Stu I, which cleave within the 3'-NC region, followed by enzymatic copying with T7 RNA polymerase. The relevant sites are indicated in Figure 1. Poly A^- IL2 mRNA was generated from a plasmid containing the same insert as pHIL2-GA, but lacking the poly A:T region.

In vitro mRNA degradation assay

The standard reaction contained 20 ng (5×10^5 cpm) of synthetic mRNA and 0.5 μg of S130 extract, incubated at 37°C in 50 μL reactions containing final concentrations of 100 mM KAc, 0.50 mM $Mg(Ac)_2$, 2 mM DTT, 10 mM Tris-Cl, pH 7.6 and 0.8 U/μL RNasin (Promega BioTech). Agarose gel electrophoresis was carried out on 10 μL aliquots that had been withdrawn at various times and quickly mixed with 100 μL of stopping buffer: 10 mM Tris-Cl pH 7.6, 1 mM EDTA, 0.35 M NaCl, 2% N-Lauroylsarcosine. Following extraction with phenol and then chloroform/isoamyl alcohol (24:1 v/v), samples were precipitated with ethanol. The dissolved pellets were subjected to electrophoresis on 1% agarose—2.2 M formaldehyde gels equilibrated with 50 mM boric acid—5 mM sodium borate running buffer. For quantification, the bands corresponding to full-length, intact mRNAs were excised from the dried gels and the radioactivity determined by scintillation counting in toluene-Omnifluor (NEN).

The degradation of mRNA *in vivo* is usually treated as a first-order reaction (26, 27):

$$(1)\ \log\ (R/R_0) = -k\ t$$

where R/R_0 is the fraction of remaining undegraded RNA and k is a constant. However, since the degradation of mRNA is enzymatic, it is more logical to describe the kinetics of disappearance of full-length molecules by equation (2):

$$(2)\ dR/dt = -V\ R/(K_m + R)$$

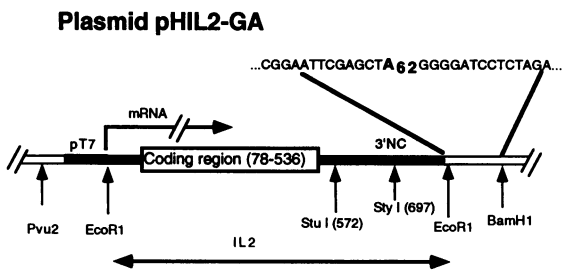

Figure 1. Structures of plasmid pHIL2-GA. A complete human IL2 cDNA was inserted at the EcoR I site of plasmid pGEM1, in the orientation shown. The T7 promoter (pT7, 17 nucleotides preceding the start site of the transcript) is situated immediately to the left of the left-hand EcoR I site. Ten nucleotides after the right-hand EcoR I site, there is a stretch of 62 A residues in the coding strand, followed by 3 G residues and a BamH I site, where the plasmid was linearized before transcription into full-length, polyadenylated mRNA. The rest of the pGEM1 multiple cloning site is distal to the BamH I site. Cleavage at Stu I and Sty I within the 3'-noncoding region deletes one, or both, AU-rich elements (ARE) from the product mRNAs (see Figure 10).

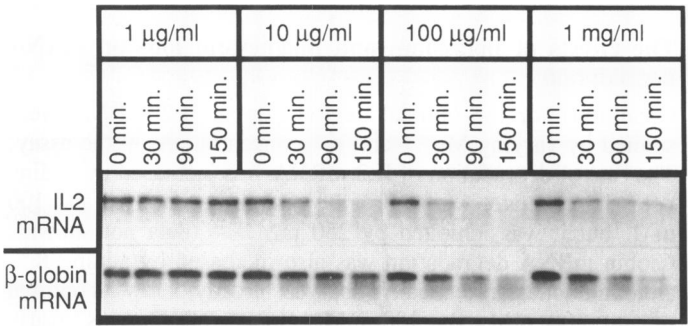

Figure 2. Titration of S130 in the mRNase reaction. IL2 and β-globin mRNAs were incubated with between 1 µg/mL and 1 mg/mL of Jurkat cell S130 extract under standard assay conditions. Aliquots were removed and subjected to agarose gel electrophoresis at the times indicated. The figure shows the region of the full-sized mRNA bands only.

where V is the maximal reaction rate at saturating substrate, and K_m applies to that particular RNA substrate. It is awkward to use equation (2) to plot the remaining concentration of full-length RNA (R). Since it reduces to the first-order form

$$dR/dt = -V\ R/K_m$$

for RNA at concentrations well below the K_m of the mRNase, the treatment of decay as a first order process, using equation (1), may be justified. Several sets of data have been plotted as first-order decay (i.e., according to equation 1) in this paper, based on this assumption.

At RNA concentrations approaching or exceeding K_m, equation (1), which predicts that the decay rate is independent of RNA concentration, is no longer valid. When the starting concentration R_0 is high relative to K_m, as in experiments where high concentrations of non-radioactive 'competitor' mRNA is added, equation (2) predicts a zero-order reaction, with the rate of degradation being described by equation (3):

$$(3)\ R/R_0 = 1-V\ t/R_0$$

In other words, addition of very high levels of substrate mRNA in 'competition experiments' reduces the rate of degradation carried out by a saturable enzyme, when this is measured by the remaining fraction of full-length molecules (R/R_0). This is the basis for experiments described in Figure 8.

Sephacryl S-200 chromatography

A Sephacryl S-200 (Pharmacia) column (54×1.0 cm) was equilibrated and eluted with 100 mM NaCl−25 mM HEPES, pH 7.2 buffer. The void volume (V_o) was determined with Blue dextran, and the total column volume (V_t) with thymidine. In addition, the elution position of bovine serum albumin (BSA), Mr 68,400, was also established. A 1.0 mL sample of S130 extract (10 mg protein) was loaded onto the column, eluted with the same buffer, and 1.4 mL fractions were collected. These procedures were done at 4°C. Fractions were assayed for RNase activity in the standard assay, in the presence or absence of 0.8 U/µL RNasin. The peak fraction of RNasin-resistant activity (Figure 5) was pooled as Fraction 2, and used for some experiments.

Primer labeling and extension

Primers complementary to various regions of IL2 mRNA were 5'-end labeled with T4 polynucleotide kinase. About 200 ng of each primer was incubated with 50 µCi γ-^{32}P-ATP, and 10 units T4 polynucleotide kinase in 50 mM TrisCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, 0.1 mM spermidine, for 45 minutes at 37°C. The reaction was then heated to 68° for 10 minutes, and applied to a G50 spin column. The final specific activity of the primers was about 5×10^6 cpm/µg. Antisense primers used in these experiments were as follows: A22 (18mer with 5' end at position 206), A21 (18mer with 5' end at position 482), A15 (21mer with 5' end at position 530), A20 (18mer with 5' end at position 719), and A19 (20mer with 5' end at position 884). The coordinates of the mRNA used in these experiments were such, that the coding region began at position 78 and ended at position 536, and the poly A tract ended at position 872, just to the left of the BamH I cleavage site at 885.

To carry out mapping of cleavage sites, unlabeled IL2 mRNA was degraded under standard conditions with 20 µg/mL S130 for various times. Each aliquot was extracted with phenol-chloroform and precipitated with ethanol. Samples were resuspended in 30 µL hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide) with 1×10^5 cpm of ^{32}P-labeled primer. The mixture was heated to 85° for 10 min., and then incubated at 37° overnight. Samples were ethanol precipitated and resuspended in 20 µL of reverse transcriptase buffer (50 mM TrisCl, pH 8.3, 7.5 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 U/µL RNasin, 0.5 mM each dNTP) and 100 units of 'Superscript' reverse transcriptase was added. Incubation followed at 37° for one hour. The products were precipitated with ethanol, resuspended in formamide loading buffer, and subjected to electrophoresis, following denaturation, in 7 M urea−6% acrylamide gels.

RESULTS

Selective degradation of IL2 mRNA *in vitro*

A crude cytosolic extract (S130) was prepared from Jurkat cells by removing nuclei and then subjecting the cytoplasm to centrifugation at 130,000×g for 150 minutes. This S130 extract was added to labeled human IL2 and β-globin mRNAs and incubated under various conditions. At the outset, the incubation conditions were based on the work of Ross and colleagues for studying mRNA degradation on polysomes (14, 19), and

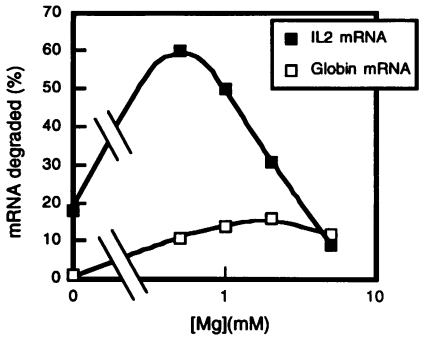

Figure 3. Dependence of mRNase activity in S130 on Mg^{++} ion. Standard conditions were used to assay mRNase activity on IL2 or β-globin mRNA, with KAc at 100 mM. The Mg^{++} concentration (as the acetate salt) was varied as indicated. The 0 mM reaction contained 2 mM EDTA. The disappearance of full-length mRNAs was determined by cutting bands out of agarose gels and counting. The data represent the IL2 and β-globin mRNA degraded in 60 minutes.

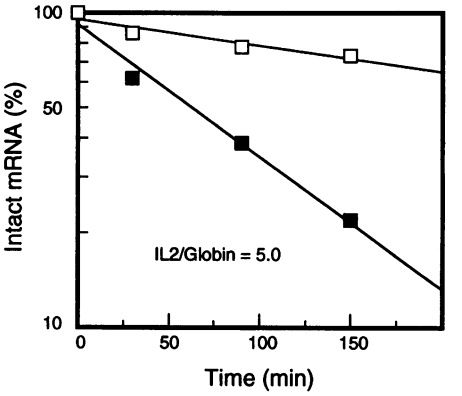

Figure 4. Quantitative analysis of IL2 and globin mRNA by Jurkat cell S130. Synthetic IL2 or β-globin mRNAs were subjected to RNase activity in 10 μg/mL of S130 extract under the standard assay conditions. The mRNA decay was analyzed by the gel electrophoresis assay, and plotted in first-order fashion. Best-fit curves were calculated in each case and the relative slopes determined.

contained an energy-generating system (ATP, GTP, creatine kinase and creatine phosphate). However, the relevant mRNase activity in S130 was found to be independent of the energy-generating system (data not shown), and it was subsequently deleted from the assay.

Addition of increasing concentrations of S130 protein to the assay led to the selective degradation of IL2 mRNA, relative to β-globin (Figure 2), that was evident at about 10 μg/mL of S130 protein. At 100 μg/mL S130, degradation of IL2 was also preferential, but this preference began to diminish when the S130 concentration was raised to 1 mg/mL. The loss of selectivity was primarily due to a decrease in degradation of IL2 mRNA at high S130 concentrations. Further work (in preparation) has confirmed that there is a selective inhibitor of IL2 mRNA degradation that is evident at high protein concentrations. Subsequent experiments were carried out at concentrations of S130 where IL2 mRNA was selectively degraded (10–25 μg/mL protein).

The IL2-selective mRNase activity was most clearly evident in the presence of the general RNase inhibitor RNasin. However, in crude S130 extracts, degradation was selective even in the absence of added RNasin, presumably because S130 contains a sufficient level of a similar RNase inhibitor. When studies were extended to more purified preparations (below), it was necessary to add RNasin, because the endogenous general RNase inhibitor was removed by chromatography on Sephacryl. All assays, unless indicated otherwise, contained commercial RNasin.

Table I. RNase Activity of Polysomes and S130

mRNA	Relative first-order rate constant: S130	Polysomes	S130 + Polysomes
IL2	3.18	4.84	6.57
Globin	0.60	2.17	3.80
IL2/Globin	5.3	2.2	1.7

IL2 or β-globin mRNA were subjected to RNase assay under standard conditions (RNasin present), using either 10 μg/mL of S130, or an aliquot of polysomes corresponding to the equivalent number of cells. The first order rate constant was obtained by plotting the loss of intact mRNA (gel electrophoresis assay) against time, as described in Figure 4.

The effects of potassium and magnesium ions on mRNA degradation

Conditions for the optimal degradation of IL2 mRNA were studied by varying Mg^{++} and K^{+} concentrations in the assay. The rate of degradation of IL2 mRNA was increased by adding potassium acetate, with a broad optimum between 10 and 100 mM, but it was inhibited by 250 mM K^{+} (data not shown). Globin mRNA degradation was also increased by adding K^{+}, although not as dramatically, and was already below its optimal rate at 100 mM. By 250 mM, globin mRNA was nearly completely resistant, although IL2 was still degraded at a slow rate.

As can be seen in Figure 3, Mg^{++} was required to maximize mRNase activity, with the optimal concentration for IL2 mRNA degradation being around 0.5–1.0 mM. At 5 mM Mg^{++}, IL2 mRNA was almost completely resistant to degradation. The relatively low rate of degradation of β-globin mRNA did not show a marked optimum under the conditions of the assay, and it was not inhibited significantly at 5 mM Mg^{++}, making it quite different from IL2 mRNA degradation. At 0.5–1.0 mM Mg^{++}, the rate of IL2 mRNA degradation by 10 μg/mL S130 was in the order of 5-fold greater than that of β-globin mRNA.

Based on these results, the standard assay for selective degradation of IL2 mRNA contained 100 mM KAc and 0.5 mM $Mg(Ac)_2$.

Quantitation of mRNase selectivity

Figure 4 represents the quantitative analysis of IL2 and β-globin mRNA degradation by S130 under the standard assay conditions, treating the results as first-order kinetic processes (see Materials and Methods). The loss of full-length RNA molecules was measured by cutting bands from gels following electrophoresis. The data were plotted according to equation (1) (first order kinetics), from which first-order decay constants were derived. IL2 mRNA was degraded at least 5 times faster than β-globin mRNA by S130, a result that has been obtained repeatedly. Whether the residual activity on β-globin mRNA was due to contaminating nonspecific RNase, or to an intrinsic sensitivity of this mRNA to the same RNasin-resistant activity that degrades IL2 mRNA, is not known.

Table II. Activity of RNase A on IL2 and Globin mRNA

RNase A Conc. (pg/mL)	Relative first-order rate constant: IL2	Globin	IL2/Globin
2	8.1	6.8	1.19
4	11.5	6.7	1.72
		Ave:	1.5

IL2 or β-globin mRNA were subjected to the standard RNase assay conditions (with no RNasin), except that pancreatic RNase A was used instead of S130, at the concentrations indicated. The degradation reactions showed the first-order behavior seen in Figure 4, yielding the rate constants shown.

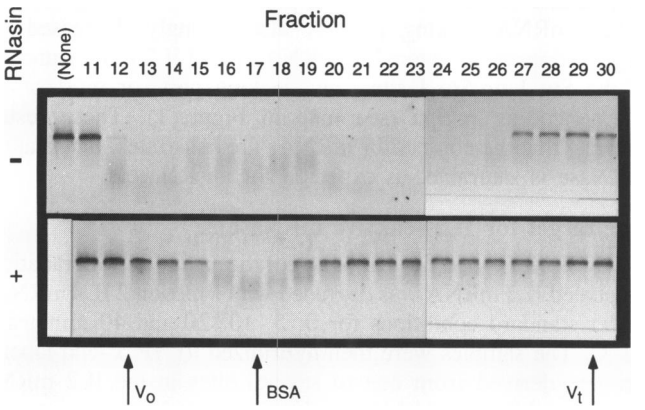

Figure 5. Gel filtration chromatography of S130. A sample of Jurkat S130 extract was fractionated on a Sephacryl S-200 column, as described in the Materials and Methods. Fractions (1.4 mL) were assayed for RNase activity on IL2 mRNA with (+) and without (−) 0.8 U/μL of RNasin present. Samples taken at 60 min. were analyzed by agarose gel electrophoresis. The left-most track in the top row of fractions contains the starting amount of undegraded IL2 mRNA (no sample is present in this track in the bottom row). The horizontal lines seen near the bottom of the tracks representing fractions 24−30 are due to the bottom of the gel in this region of the electropherogram. The RNasin-resistant activity ran as a single peak with apparent size similar to that of BSA marker.

Selective mRNase activity in polysomes and S130

Although the primary intention of this word was to seek a soluble, specific mRNase, assays were also carried out on polysomes prepared from the same cells, since earlier results indicate that polysomes could be a source of mRNase activity (14, 19). The labeled synthetic mRNAs were added to resuspended polysomes that had been precipitated during the centrifugation step in preparing S130. Using polysomes or S130 from equivalent numbers of Jurkat cells, it was found that both contained RNase activity that was RNasin-resistant. Results were plotted in first-order kinetic fashion, and the derived first-order rate constants are compared in Table I. Although polysomes appeared to contain more total RNase activity than S130 did, the specificity was less, with a ratio of IL2/globin degradation being 2.2 and 5.3 for polysomes and S130, respectively. An equal combination of both fractions also had a low specificity (1.7). Therefore, all subsequent work has been carried out with S130, since polysomes offer no marked advantage in terms of selective ribonuclease activity, and would require extraction to obtain a soluble activity.

To determine whether a nonspecific RNase distinguishes IL2 from β-globin mRNA, assays were carried out with pancreatic RNase A, as shown in Table II. This enzyme showed a selectivity

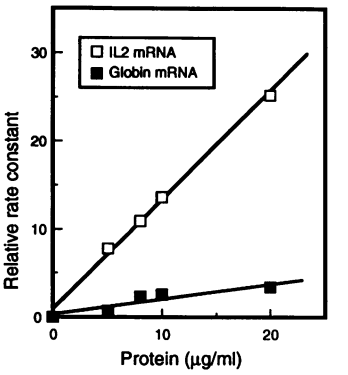

Figure 6. The activity of Fraction 2 mRNase on IL2 and β-globin mRNAs. Fraction 17 from the Sephacryl S-200 column described in Figure 5 was assayed for RNase activity using synthetic IL2 or β-globin mRNA under standard assay conditions, including RNasin. Samples were taken at 15, 45 and 90 minutes, and subjected to gel electrophoresis. The decay of the mRNAs was determined by cutting out the full-length mRNA bands from the gels, and plotting their first-order decay, as in Figure 4. The first-order decay constants determined by least squares regressions of these data are shown as a function of protein concentration in the assay. The ratio of the slopes of the lines (IL2/β-globin) is 7.2 .

for IL2 mRNA of only about 1.5-fold, significantly lower than that of the RNasin-resistant activity in S130 (5-fold or greater). Pancreatic RNase A degradation of both IL2 and β-globin mRNAs was totally RNasin-sensitive, as expected.

Separation of selective mRNase activity from bulk cellular RNase

As a first step in purifying a selective, RNasin-resistant mRNase activity from the bulk RNase activity in S130, and as a way of confirming that it represents a unique activity therein, S130 was subjected to gel filtration chromatography on Sephacryl S-200, as shown in Figure 5. The total RNase activity, as reflected by the degradation of IL2 mRNA in the absence of RNasin, was spread throughout the chromatogram, with the major part being apparently smaller than the marker BSA. Only a small component of the total RNase activity was RNasin-resistant. This was present in a single peak with a mobility similar to that of the marker BSA (MW 68,400), and away from the bulk of total RNase activity.

The peak of RNasin-resistant activity from the S-200 column, which we refer to as Fraction 2, was assayed on β-globin and IL2 mRNA, using the gel electrophoresis assay. The fraction of full-length RNA remaining at 15, 45, and 90 minutes in the presence of various concentrations of the partially purified RNase was determined and represented in first-order plots. These kinetic plots were good fits (correlation coefficients between .905 and .994). The first order constants determined by linear regression of these data are shown as a function of protein concentration in Figure 6. The rate constants for mRNase activities under standard assay conditions were a linear function of protein concentration, and the relative rate of IL2 degradation was 7.2 times faster than that of β-globin.

The selectivity for IL2 mRNA does not depend on the 5′-cap nucleotide, nor on 3′-polyadenylation

Since the degree of polyadenylation of the mRNAs differed slightly (62 residues in IL2, 40 in β-globin), and both are less polyadenylated than natural mRNAs, we tested the effect of

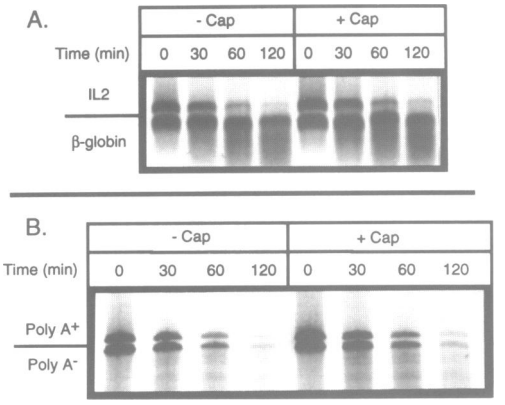

Figure 7. Effects of 5′-capping and 3′-polyadenylation on IL2-selective mRNase activity. (A) IL2 and β-globin mRNAs were synthesized as in the standard methodology, with or without the 'capping' ^{7}Me-G nucleotide. The mRNA samples were then subjected to the standard degradation reactions using Fraction 2. Neither the rate of IL2 mRNA degradation, nor the ratio between IL2 and β-globin was significantly affected by omission of the 5′ cap nucleotide. (B) IL2 mRNA was synthesized from either the standard plasmid, which generates 62 A residues at the 3′ end (Figure 1), or from a plasmid lacking the A:T region. Both were synthesized with or without the 5′-cap nucleotide present in the reaction. Samples were analyzed at various times by polyacrylamide gel electrophoresis. The larger band represents the polyadenylated IL2 mRNA, the smaller band, non-polyadenylated IL2 mRNA. The rates of degradation, both absolute and relative, were not significantly different.

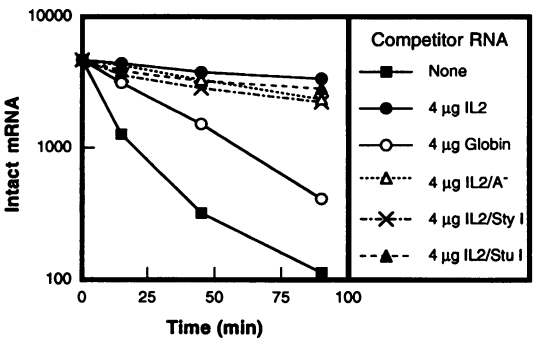

Figure 8. Competition for the IL2-selective mRNase. Unlabeled competitor RNAs were generated as described in Materials and Methods, and added to the standard assay containing 20 ng of radioactive IL2 (polyadenylated and capped). Each reaction contained 10 µg/mL of Fraction 2 mRNase, under standard assay conditions. Poly A^{-} IL2 mRNA was synthesized as described in Materials and Methods. The RNAs generated after cleavage of plasmid pHIL2-GA with Stu I and Sty I (see Figure 1) were 572 and 697 nucleotides in length, respectively, and cut out one, or both, of the ARE from the mRNA transcript (Figure 10). Samples were subjected to agarose gel electrophoresis. The ordinate represents the counts in the full-length bands cut from the gel.

deleting the poly A region from IL2 mRNA. In addition, the degree of capping at the 5′-end by 7-Methyl G was not controlled, and may have been responsible for different sensitivities. As seen in Figure 7, however, leaving out the cap nucleotide entirely had no discernible effect on either the rate of IL2 mRNA degradation or on the selectivity of IL2 relative to β-globin mRNA (panel A). Furthermore, IL2 mRNA lacking poly A appeared to be degraded at the same rate as that bearing the A_{62} tract, whether capped or not (panel B).

The IL2-selective mRNase is saturable

The target site and specificity of the IL2-selective mRNase was examined by adding various unlabeled competitor mRNAs at high concentrations to the standard assay, using Fraction 2 enzyme (Figure 8). The standard assay contains 20 ng each of the labeled substrate mRNAs. The addition of 4 mg of IL2 mRNA to the 50 µL reaction, a 200-fold increase in substrate concentration, greatly reduced the rate of degradation (measured on the total pool of mRNA substrate), as predicted by equation (3) (Materials and Methods) for RNA concentrations that exceed K_m. Beta-globin mRNA was much less effective as a competitor. However, it did diminish the rate of IL2 mRNA degradation, suggesting that the same enzyme that degrades IL2 mRNA may also be responsible for the low level of β-globin mRNA degradation.

IL2 mRNA lacking poly A also strongly decreased the degradation rate of intact IL2 mRNA, as did IL2 RNA truncated at the Sty I or Stu I sites, and lacking part, or most of the 3′−noncoding region (see map in Figure 1). These results indicate that the competitor mRNAs are substrates, and that the mRNase is saturable, as expected for an RNase.

The target for IL2-selective mRNase

To look for specific endonucleolytic sites of degradation, unlabeled IL2 mRNA was degraded with Fraction 2 IL2 mRNase under standard conditions for 0, 5, 10, 20 and 40 minutes at 37°C. The samples were then hybridized to ^{32}P 5′-end-labeled primers derived from one of several sites in the IL2 mRNA sequence. After overnight hybridization, the primer was extended in a reverse transcriptase reaction for 1 hour, precipitated, and analyzed by urea-polyacrylamide gel electrophoresis. The results are shown in Figure 9.

Primer A15, a 21mer ending at position 530 in the synthetic IL2 mRNA, generated a number of bands upon extension, only 3 of which were dependent on prior degradation with Fraction 2 mRNase (Figure 9). The end points of the extended primers mapped to positions 240, 415, and 430, respectively, within the coding region. A similar experiment was performed with primer A21 (5′ end at position 482), which indicated cut sites at positions 240, 400, and 417 (data not shown). Of the other primers used, A19, produced one (weak) degradation-dependent band, that mapped to position 634 (Figure 9). Primer A20 indicated a site at position 605 (data not shown), in approximate agreement. The summary of primer extension analysis is as shown in Figure 10. The predominant sites are in the region of 400−430 (3 or 4 bands), which is centered 75% of the distance through the coding region of the primary translation product.

DISCUSSION

This paper describes an mRNase activity in the cytosolic fraction of Jurkat cells that has a selectivity for IL2 mRNA relative to β-globin mRNA. The activity cleaves IL2 mRNA predominantly at a small cluster of sites in the 3′ half of the coding region. Weaker cleavage was also seen at position 240 (35% of the way through the coding region), and around position 605−635 in the 3′-NC region. The latter site, which was very weak and difficult to detect, lies just to the right of one of the AU-rich elements (ARE) (Figure 10), that may be involved in cytokine mRNA instability. In addition, truncated IL2 mRNA that ends at position 572, just outside the coding region, is an efficient competitor for the mRNase activity (Figure 8), consistent with the notion

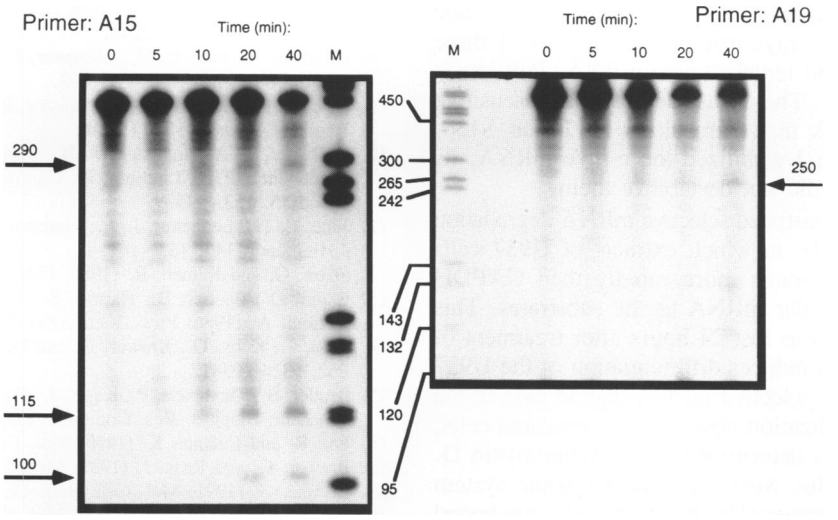

Figure 9. Determination of cleavage sites in IL2 mRNA. Unlabeled IL2 mRNA was treated with Fraction 2 mRNase in a standard reaction for the times indicated (0 to 40 minutes), and then subjected to primer extension analysis. Results are shown for the primers A15 (5′ end at position 530) and A19 (5′ end at position 884). In the A15 sample, 3 bands were found only after treating the mRNA with S130, and these were 290, 115, and 100 nucleotides long, respectively, using the marker bands in lane M (denatured Hae III-treated PM2 DNA). Only one, weak, mRNase-dependent band was found with primer A19, and this was of length 250.

that the major sensitive sites lie to the left of this position. Neither a 5′-^{7}MeG cap, nor polyadenylation at the 3′ end, had any effect on the rate of IL2 mRNA degradation. Selectivity (IL2/β-globin mRNA degradation rate) also did not depend on either of these features.

The IL2-selective mRNase is insensitive to RNasin, which blocks the major, nonspecific RNase activities in crude extracts. The Sephacryl results indicate that a low molecular weight cofactor is not necessary for the IL2-selective mRNase activity, and that it resides in a protein of MW 60−70,000, larger than the bulk of RNasin-sensitive cellular RNase activity.

The reactivity of the mRNase on β-globin may be due to a nonspecific contaminating RNase, but several lines of evidence suggest that it is the IL2-selective mRNase itself that carries out the low level of degradation of β-globin mRNA. In the first place, both activities have the unusual property of being resistant to RNasin. Secondly, the relative activity of Fraction 2 on the two mRNAs is similar to that of the crude S130. Although IL2 mRNAs was more efficient, β-globin mRNA did compete for the IL2-degrading activity (Figure 8). In further experiments (not shown), degradation of β-globin mRNA by Fraction 2 was competed for by IL2 mRNA, suggesting that the two mRNAs are targets for the same mRNase.

The IL2 mRNase activity is saturable, and IL2 mRNA and its 5′ half are much more effective than β-globin mRNA in saturating it. From the data used to generate Figure 8, it is possible to make a rough estimate of the K_m for IL2 mRNA (additional data not shown were obtained for 2 μg IL2 mRNA in the 50 μL reaction). The standard assay contains about 1.3×10^{-9} M IL2 mRNA. At 2 and 4 μg added as competitor, the concentrations are 100 and 200−fold higher, respectively. Plotting the resulting data in double reciprocal fashion leads to a predicted K_m of 2.5×10^{-8} M. Thus, the assumption used in the Materials and Methods, that the standard reaction is being carried out at an mRNA substrate concentration well below the K_m, is valid, and a first-order plot is reasonable for the standard assay. A more interesting calculation is to determine the *in vivo* level of IL2 mRNA relative to this K_m. We have previously determined (7) that a stimulated EL4.E1 cell contains about 600 molecules of IL2 mRNA. This translates into a concentration of about 2×10^{-9} M, or 10-fold lower than the K_m for the mRNase activity studied here.

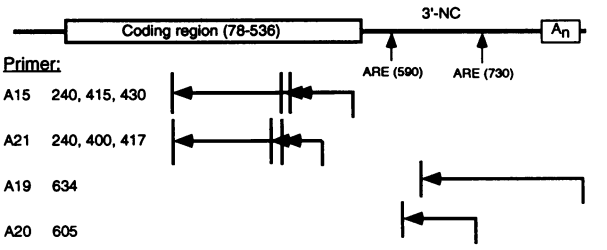

Figure 10. The experiments shown in Figure 9, plus results with the additional primers A21 (5′ end at position 482) and A20 (5′ end at position 719) were carried out. The apparent end-points of the various extended primers are mapped (short vertical lines at the arrowheads). No extended products were found with a primer whose 5′ end was at position 206 (data not shown).

Previous work has documented an RNase activity in crude cell extracts that preferentially degrades a synthetic globin mRNA bearing multiple repeats of the octanucleotide motif UUAUUUAU (15). In that work, the rates of degradation of the control (normal globin) mRNA were not given, and it is thus difficult to determine what the actual ratio of preference for the modified mRNA was. In addition, only the TCA-soluble counts released were measured, and there were no data regarding initial endonucleolytic cleavage of the mRNA. The earlier results also differed from ours in that RNase activity was more-or-less proportional to the amount of crude extract added, and that commercial RNasin at least partially inhibited it. More importantly, the principle target sequence in the IL2-selective mRNase appears not to involve the ARE present in the 3′-NC region of the mRNA.

The work of Pei and Calame (16) described an RNase in crude cell extracts that degrades c-myc mRNA lacking exon 1 three times more slowly than full length c-myc mRNA. Polysomes contained a similar activity. The RNase activity was insensitive to commercial RNasin, as in our results with Jurkat S130. Although removal of exon 1 stabilized the c-myc mRNA, its addition to other mRNAs did not destabilize them.

Another system that demonstrated selective mRNA degradation was described recently (22), in which extracts of U937 cells degraded TGF-β1 mRNA much more rapidly than GAPDH mRNA, using isolated cellular mRNA as the substrates. This selective mRNase activity was lost 24 hours after treatment of the cells with PMA, which induces differentiation of the U937 promonocytes. The loss of selective mRNA degradation in the extracts mirrored the stabilization observed in stimulated cells, when mRNA turnover was determined using Actinomycin D.

The starting point for this work was the polysome system described by Ross and colleagues (19), in which polysome-bound mRNAs encoding c-myc is preferentially degraded upon incubation *in vitro*. In that system, removal of the poly A end of mRNA is an early, and limiting event, that is controlled by poly A-binding protein (23). Although the major nucleolytic activity is associated with polysomes, an S130 component destabilizes c-myc, and this destabilizer is missing in cycloheximide-treated cells (17). A factor that binds AU-rich RNA has been purified from S130, and shown to have an enhancing activity toward c-myc degradation on polysomes *in vitro* (18). The S130 factor described in that work, however, does not have the RNase activity itself. A similar report describes the presence of an AU-motif-binding factor in T cells that have been stimulated through the T cell receptor complex (24). This factor's presence is inversely related to stabilization of mRNAs by PMA stimulation, and may be the same as the factor described by Brewer (18).

The cytosolic S130 activity that degrades IL2 mRNA may be present at similar, or even higher levels on polysomes, but the S130 preparation is probably a more tractable source for purification. It represents a sequence-specific, endoribonuclease activity with a selective preference for degrading IL2 mRNA, relative to β-globin mRNA. Its distribution in other cell types, and its possible regulation through factors known to influence IL2 mRNA turnover, such as the stimulation of T lymphocytes with antibody to the surface antigen CD28 (8) or treatment of the cells with cycloheximide (25), remain to be determined.

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