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A binding factor for interleukin 2 mRNA

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ABSTRACT

Jurkat cells, a human T lymphocyte line that can be induced to synthesize and secrete interleukin 2, contain a factor that binds interleukin 2 mRNA. Binding can be demonstrated by formation of a complex detectable by gel electrophoresis. The binding is sequence specific and occurs in the 3′-non-coding region, within 160 nt of the end of the coding region, at or near a site on the mRNA that is rich in A and U residues. However, it appears not to be due to known AU binding factors. The factor is protease sensitive and binds non-covalently to interleukin 2 mRNA. It behaves like a protein of molecular weight 50 000–60 000 after UV-induced cross-linking to the mRNA. Preparations of the binding factor also protect interleukin 2 mRNA against degradation by a recently described RNasin-resistant endoribonuclease activity in Jurkat cells. Protection occurs under the same conditions required to generate the gel-retarded complex.

INTRODUCTION

Rates of transcription and degradation control the steady-state levels of cellular mRNAs. The lifetimes of mRNAs in mammalian cells vary from minutes to days, but those encoding proteins that change concentration rapidly are often short lived, allowing for pre-translational control (1,2). The synthesis of the lymphokine interleukin 2 (IL2) and its mRNA is rapidly induced by antigenic or mitogenic stimulators of T helper lymphocytes and upon their removal the mRNA dies away equally rapidly. This is consistent with a relatively short half-life of the mRNA under these conditions, of between 40 and 80 min (3). Other cytokines having short lived mRNAs include GM-CSF (4) and interferon β (5). The stabilities of a number of short lived cytokine mRNAs are regulated. For example, the mRNAs for IL2, IFNγ, GM-CSF and TNFα (but not those for c-Myc and c-Fos) are stabilized by signaling of T helper lymphocytes through the CD28 cell surface structure (6). The same signaling pathway can also stabilize the α and β transcripts of the IL2 receptor (7). Induction of GM-CSF synthesis in some cell lines is caused by stabilization of its mRNA, rather than an increase in its transcription rate (8). Similarly, stabilization of constitutively transcribed mRNA by calcium influx induces IL3 production in a mast cell line (9).

Only limited information is available about the ribonucleases responsible for the degradation of mRNA *in vivo*. All cells contain high levels of RNase A-type RNases or neutral, non-specific RNases, but under reductive intracellular conditions (10,11) these are efficiently inhibited by a tightly bound ubiquitous protein inhibitor (12,13). This inhibitor is commercially available as placental RNasin. The endoribonuclease RNase E, the product of the *ams*/*rne*/*hmp1* gene, is at least partly responsible for the degradation of mRNA in *Escherichia coli* (14,15). The nature of the ribonucleases responsible for mRNA degradation in eukaryotic cells is not known. Given the broad distribution and effectiveness of RNasin, RNases sensitive to it seem unlikely to be directly involved and there is therefore a particular interest in RNasin-resistant RNases as candidate mRNases. The Vhs protein of herpes simplex virus-1 is a known RNasin-insensitive mRNase, which is responsible for the degradation of mRNA upon viral infection (16). Candidate mRNases include an RNasin-resistant exonuclease that preferentially degrades polysome-associated histone mRNA *in vitro* (17) and the RNase in a related system that preferentially attacks polysome-associated c-Myc mRNA (18). We recently described an RNasin-resistant RNase, present in a T lymphocyte cell line, that preferentially attacks IL2 mRNA, relative to β-globin mRNA (19). A *Xenopus* RNase has been described that cleaves the *HoxB7* gene product endonucleolytically (20). A segment of the 3′-NC part of the target mRNA directs the RNase sensitivity in that system.

The regulation of mRNA turnover, particularly of short lived species, may be mediated by factors that recognize sites on the mRNAs. A number of binding factors for short lived mRNAs have been described and some correlations have been made between stability and the presence or absence of factors binding such sites. One of the most thoroughly studied *cis*-acting mRNA motifs is the ‘AU-rich element’ (ARE), which is based on the pentanucleotide AUUUA (21,22). Several different AU binding factors (AUBF), present under different conditions and differing in their fine specificity of binding, have been identified (22–25). The ARE of GM-CSF mRNA destabilizes it in intact cells (4). A coding region site on c-Myc mRNA is responsible for destabilizing it *in vivo* (26,27) and *in vitro* (28) and a specific binding factor for this site has recently been purified and characterized (28).

The RNasin-resistant RNase that we previously described (19) attacks IL2 mRNA at a small number of sites in an *in vitro* assay. Reasoning that this activity might at least reflect the natural sensitivity of IL2 mRNA to degradation, we examined crude

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extracts of T cells for any factors that could interact with IL2 mRNA or alter its sensitivity to RNase. We have discovered a factor that binds to a segment of the 3′-NC region of IL2 mRNA, but it differs from known AU binding factors that also recognize sites in this region. Preparations of the IL2 mRNA binding factor block its degradation by the RNasin-resistant RNase, while not significantly affecting the stability of β-globin mRNA. This report describes the binding and protective properties of the factor(s) specific for IL2 mRNA.

MATERIALS AND METHODS

Cell culture and extract preparation

Cell lines were cultured in 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). Jurkat is a human T leukemia cell line that can be induced to synthesize IL2 (29). Cytosolic extracts of cells (S130) were prepared as described previously (19), aliquoted and stored at –70°C. Briefly, cells were broken in hypotonic medium using a Teflon in glass homogenizer and the S130 fraction was obtained by centrifugation of the cell lysate in a TLA 100.3 rotor (Beckman TL100 Ultracentrifuge) at 53 000 r.p.m. for 2.5 h.

Plasmid DNA constructs and RNA substrates

Full-length IL2 mRNA was generated from the plasmid pHIL2.CA2, a modified version of plasmid pHIL2.GA described earlier (19). This plasmid (see Fig. 1) comprises full-length human IL2 cDNA with an upstream T7 promoter and a 62 nt downstream poly(A) region, within vector pGEM-1. A primer containing a *Pvu*II site, a T7 minimal promoter and 18 nt of the human IL2 5′-NC region was used for the 5′-end of a PCR reaction. The 3′ primer contained an *Eco*RI site, followed by a complement to the 3′-end of IL2 mRNA. The PCR product generated with these primers from pHIL2.GA treated with *Pvu*II and *Eco*RI was cloned into pHIL2.GA from which the segment between the *Pvu*II and *Eco*RI sites had been removed, thereby inserting the complete IL2 sequence between a T7 promoter and a 62 nt poly(A) segment, as shown in Figure 1. Plasmid pHIL2.CA2 was digested with *Bam*HI before transcription to generate IL2 mRNA carrying a poly(A) tail or by *Eco*RI, *Sty*I or *Stu*I to generate shorter RNAs lacking parts of the 3′-NC region. RNA containing only the 3′-NC region was synthesized from a cloned PCR product generated with a 5′ primer comprising a *Pvu*II site, the T7 promoter sequence and 18 nt of IL2 sequence beginning at position 507 (the start of the 3′-NC sequence). The 3′ primer was that used for producing pHIL2.CA2 and the product was also cloned into *Pvu*II/*Eco*RI cut pHIL2.GA to generate pHIL2.3N1. This plasmid is identical to pHIL2.CA2 except that it lacks nt 1–506 of the IL2 sequence (see Fig. 1). RNA containing only the 5′-most 161 nt of the 3′-NC was transcribed from pHIL2.3N1 digested with *Sty*I.

Plasmids for the transcription of intact polyadenylated β-globin mRNA and of the C-terminal 59 amino acids of c-Myc were pSP6β/c (18) and pBSmyc pA.1 (28) respectively, both provided by Dr Jeffrey Ross (University of Wisconsin, Madison, WI). This c-*myc* fragment encodes the coding region determinant (CRD), which binds an mRNA binding and protective factor (28). GAPDH mRNA was synthesized from pKS-GAPDH (30), which was provided by Dr Kathryn Calame (Columbia University, New York, NY). Competitor RNA containing four reiterations of the AUUUA motif (AUR) was made from plasmid pAU (22), provided by Dr James Malter (University of Wisconsin, Madison, WI).

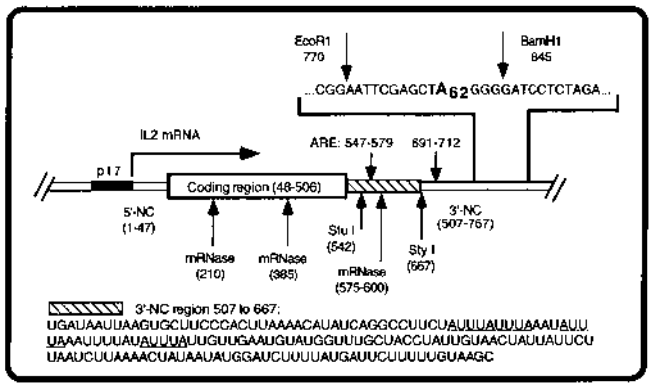

Figure 1. Structure of the human IL2 cDNA used to generate IL2 mRNA substrates. The plasmid pHIL2.CA2 contains a T7 promoter (pT7) followed by the human IL2 cDNA sequence beginning 47 nt upstream of the coding region (43) up to position 767. The 3′-end has been replaced by an *Eco*RI site, then 5 nt and a 62 nt poly(A) segment, as shown. There are two clusters of AU-rich elements (ARE) in the 3′-NC region (ARE). The IL2-selective RNasin-resistant RNase described earlier (19) cleaves at the sites identified as 'mRNase' in the figure, including one just downstream of one of the ARE. The sequence of the 161 nt segment of the 3′-NC (positions 507–667 in the figure), within which the factor studied binds, is shown at the bottom of the figure. Copies of the motif AUUUA within the 161 nt segment, which are associated with instability and the binding of AU binding factors, are underlined. RNAs with 5′-ends at the beginning of the 3′-NC region were generated from plasmid pHIL2.3N1, which is identical to pHIL2.CA2 except that it is missing the region between the T7 promoter and position 506.

Plasmids were copied into RNAs after digestion with the appropriate restriction enzyme. They were labeled with either [α-^{35}S]CTP (for RNase assays) or [α-^{32}P]CTP (for binding studies) and were not capped at the 5′-end, since we have previously shown that capping has no effect on the IL2-selective RNase (19). The assay for degradation of IL2 and β-globin mRNAs was described earlier (19). As before, the assay was supplemented with 0.8 U/μl RNasin (Promega BioTech) to block RNase A-type activity. Samples were worked up as described and analyzed on 1% agarose–2.2 M formaldehyde gels.

Gel filtration chromatography

S130 (1.0 ml at 10 mg/ml protein) was loaded onto a Sephacryl S-200 (Pharmacia) column (54 × 1.0 cm) for separation of the IL2-selective RNase from the inhibitor factor described in this paper. This procedure was done at 4°C. The column was equilibrated with a buffer containing 100 mM NaCl and 25 mM HEPES, pH 7.2. Blue dextran and thymidine were used to determine the void volume and the bed volume respectively and bovine serum albumin (BSA) to establish the position of the 68 000 mol. wt protein. Fractions were assayed for both RNase and the RNase inhibitor.

Mobility shift and UV cross-linking analysis

Ten nanograms (5×10^5 c.p.m.) of ^{32}P-labeled mRNA were heated at 85°C for 5 min and incubated with binding factors at 30°C for 10 min in 10 μl reactions containing 100 mM KAc, 0.50 mM $Mg(Ac)_2$, 2 mM DTT, 10 mM Tris–HCl, pH 7.6, 5%

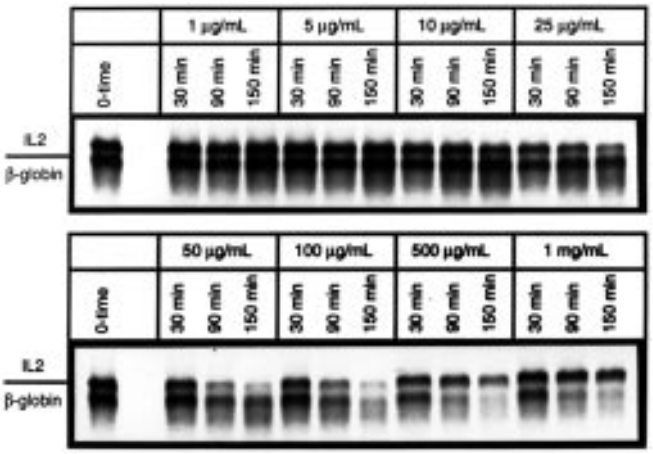

Figure 2. Degradation of IL2 mRNA at increasing concentrations of Jurkat S130. Radioactive full-length poly(A)$^+$ IL2 and β-globin mRNAs were incubated under the conditions of the standard RNase assay with concentrations of Jurkat S130 protein as indicated at the top of each set of the three tracks. Samples taken at the times shown were subjected to agarose gel electrophoresis followed by autoradiography. The results show that increasing the S130 protein concentration up to ~100 µg/ml led to an increasingly rapid rate of degradation of both mRNAs, with IL2 being degraded more rapidly, as described earlier (19). However, at 0.5 and 1.0 mg/ml protein IL2 mRNA was selectively stabilized relative to β-globin.

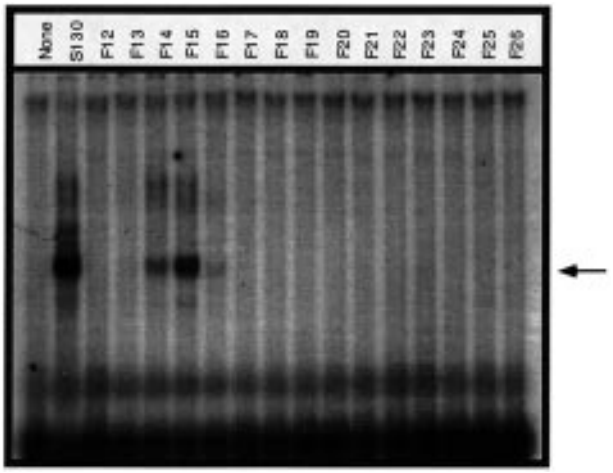

Figure 3. Detection of an IL2 mRNA binding factor. Full-length poly(A)$^+$ IL2 mRNA was incubated either with 1 mg/ml Jurkat S130 or with the same amount of Sephacryl S-200 fractions. After digestion with RNase T1 and addition of heparin the samples were subjected to electrophoresis on a native 6% acrylamide gel. A labeled band (arrow) was observed with both S130 and Sephacryl fractions containing IL2 mRNA protective factor (fractions 14 and 15). On the same column the RNasin-resistant mRNase was centered at fraction 17. In the experiments shown in Figures 4–9 fraction 15 was used as the source of binding factor and fraction 17 as the source of RNasin-resistant mRNase.

glycerol and 1 mg/ml tRNA. RNase T1 was added to a final concentration of 1 U/µl and the sample was incubated for 10 min at 30°C. Heparin was added (final concentration 5 mg/ml) and incubation was continued for 10 min (28). The reaction mixture was then loaded onto a 6% native polyacrylamide gel.

For UV cross-linking analysis the same binding reactions were performed and then the reaction mixtures were exposed to UV light (Stratagene UV Stratalinker Model 1800, run for 5 min at 3 cm) before being loaded onto a 12% polyacrylamide–SDS gel.

RESULTS

Jurkat S130 contains a factor that protects IL2 mRNA from degradation by the IL2-selective RNasin-resistant RNase

The T leukemia cell line Jurkat contains an endoribonuclease that is RNasin resistant and that degrades IL2 mRNA 5–10 times faster *in vitro* than β-globin mRNA (19). It cleaves IL2 mRNA at a small number of sites, including two in the coding region and one in the 3′-NC segment (Fig. 1). This RNase was reproducibly demonstrable in extracts of Jurkat cells when these were tested at moderate protein concentrations. However, assays at increasing concentrations of Jurkat S130 showed an unexpected effect; namely IL2 mRNA was selectively stabilized by high concentrations of S130. Thus at concentrations of S130 protein of ~1 mg/ml IL2 mRNA was almost completely stable, whereas β-globin mRNA was degraded at a rapid rate. In other words, the stability of IL2 mRNA relative to β-globin mRNA was inverted at high S130 concentrations compared with the results obtained at 20- to 100-fold lower protein levels. This suggested the presence of an IL2-selective protective factor in the crude extracts (Fig. 2).

Since β-globin mRNA was degraded even at the highest concentrations of S130, the stabilization of IL2 mRNA was apparently not due to a general inhibition of nuclease activity. The concentration of S130 at which the stabilization of IL2 mRNA became noticeable varied between preparations. In some cases no stabilization was seen up to 1 mg/ml (cf. our earlier work, 19). However, every Jurkat S130 preparation yielded stabilization at some point, usually at or just above 1 mg/ml protein. Stabilization by S130 of Jurkat cells did not depend on stimulation of the cells with mitogens or antigen, but appeared to be a constitutive function.

A factor binding IL2 mRNA can be separated from the IL2-selective RNase

The protection of IL2 mRNA by Jurkat S130 suggested that it contained a component, apart from the RNasin-resistant mRNase, that interacted with IL2 mRNA. Gel shift analysis was therefore performed in an attempt to detect a binding factor for IL2 mRNA. Labeled IL2 mRNA was incubated with S130 at 1 mg/ml (a concentration showing protection of IL2 mRNA) and then subjected to degradation by T1 RNase. After competing out non-specific complexes with heparin the products were analyzed on a native acrylamide gel. A single band was seen with S130 (Fig. 3).

We next attempted to separate the factor responsible for the gel shift from the RNasin-resistant mRNase. We had demonstrated previously that when Jurkat S130 was fractionated on Sephacryl S-200 the RNasin-resistant IL2-selective RNase eluted around the same position as BSA (19). Fractions from a similar column were examined for both the RNasin-resistant mRNase and a factor that would bind IL2 mRNA. The mRNase eluted at the position described earlier, corresponding to fraction 17 in the experiment shown in Figure 3. A gel shift complex was observed with fraction 15 (and to a lesser extent 14) and this shifted band had the same mobility as that seen with S130. This result has been obtained reproducibly, with a two fraction shift between the

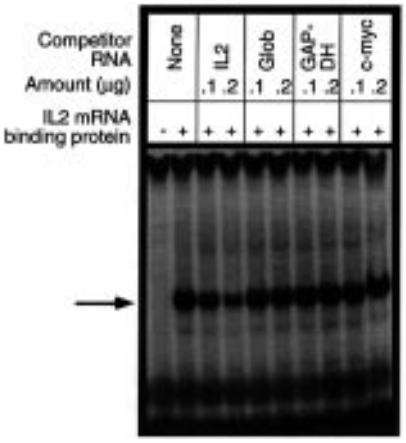

Figure 4. Competition for the IL2 mRNA binding activity. A binding assay similar to that in Figure 3 was carried out in the absence or presence of competing RNAs. Each binding reaction contained 10 ng ^{32}P-labeled IL2 mRNA and the indicated amounts of either full-length IL2 mRNA, full-length β-globin mRNA, full-length (non-adenylated) GAPDH mRNA or the c-Myc CRD RNA fragment. At the level of 0.2 μg competitor the molar ratios of the various competitor RNAs to labeled probe were: IL2, 20:1; β-globin, 21:1; GAPDH, 12:1; c-Myc CRD, 85:1. Only IL2 mRNA showed competition for formation of the complex under these conditions.

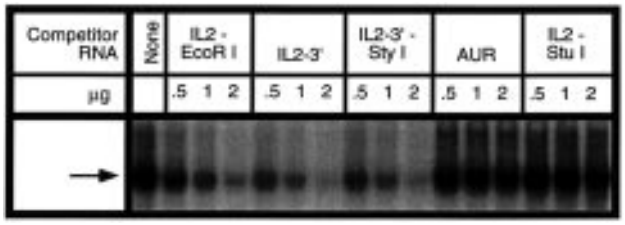

Figure 5. Localization of the binding site on IL2 mRNA. A gel retardation assay for the binding factor was carried out as in Figure 3, using labeled poly(A)$^-$ IL2 mRNA and various unlabeled competing RNAs, present in the amounts indicated. Poly(A)$^-$ IL2 mRNA (IL2-*Eco*RI lanes) competed, as did IL2 RNA containing only the 3′-NC region [positions 507–770, also lacking poly(A)]. IL2 mRNA extending only from the 5′-end of the 3′-NC region to the *Sty*I site (positions 507–667, IL2-3′-*Sty*I in the figure) also competed, but RNA containing four repeats of the ARE motif (AUR), which is known to interact with ARE binding factors (22,31), did not. An IL2 RNA extending from position 1 to 542 (IL2-*Stu*I) also did not compete. At the level of 1 μg the molar ratios for the various competitor RNAs to labeled probe were: IL2-*Eco*RI, 100:1; IL2-3′, 380:1; IL2-3′-*Sty*I, 790:1; AUR, 1700:1; IL2-*Stu*I, 140:1.

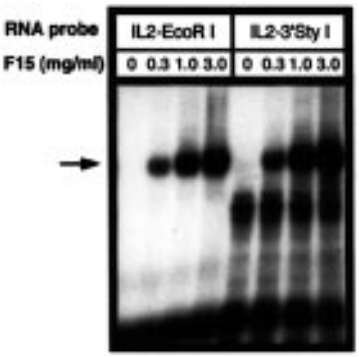

Figure 6. Formation of a binding complex with a 3′-NC IL2 mRNA segment. A gel retardation binding assay was carried out as in Figure 3, using either full-length poly(A)$^-$ IL2 mRNA (IL2-*Eco*RI in the figure) or a segment comprising only positions 507–667 (IL2-3′-*Sty*I) as the labeled target. A retarded band of the same mobility was found for both using Sephacryl S-200 fraction 15 as the source of binding factor. A faster migrating band in the 3′-*Sty*I probe lanes was presumably due to incomplete degradation of the substrate and not to a protein complex, as it was present even when no binding factor was added. It was observed in several experiments and may reflect secondary structure in the shorter substrate.

binding factor and the RNasin-resistant mRNase. The following results were obtained using the peak fractions of binding or RNase activity from Sephacryl S-200 columns. For convenience the source of binding factor is sometimes referred to as ‘fraction 15’ and the RNasin-resistant mRNase as ‘fraction 17’.

The binding factor recognizes a site within the first 160 nt of the 3′-NC region of IL2 mRNA

Sequence-specific competition for the binding factor, as detected by gel shift analysis, was observed (Fig. 4). The addition of unlabeled IL2 mRNA diminished the signal intensity of the shifted band due to saturation of the binding factor. No competition was observed with β-globin, GAPDH or the c-Myc CRD RNAs under the same conditions. It should be noted that the binding reactions routinely contained 1 mg/ml tRNA, compared with 1 μg/ml labeled target mRNA, further supporting the notion of specificity.

To localize the binding sites within IL2 mRNA we synthesized five different RNAs for competition in the gel retardation assay. Labeled full-length IL2 mRNA lacking a poly(A) tail was used as the probe. As shown in Figure 5, full-length IL2 RNA or RNA containing at least the sequence up to the *Sty*I site at position 667 (see Fig. 1) competed efficiently for the binding factor. However, RNA that contained the IL2 sequence from position 1 to the *Stu*I site at position 542 in the 3′-NC, stopping just short of the first ARE, failed to compete. In addition, an RNA containing a 4-fold repeat of an ARE binding motif (AUR) did not affect complex formation, indicating that the binding interaction is not due to ARE binding factors characterized previously (22,31).

As further evidence for the location of the factor binding site, a probe containing only 161 nt of the IL2 mRNA sequence, extending from the end of the coding region (position 507) to the *Sty*I site within the 3′-NC region (position 667), was used for gel shift experiments. This probe formed the same sized complex as full-length IL2 mRNA (Fig. 6). A probe containing the 5′-part of the IL2 mRNA sequence and ending at the *Stu*I site at position 542 did not form a retarded band, nor did the AUR probe (data not shown). These results, together with the competition data in Figure 5, demonstrate that the binding site is in the segment of the 3′-NC region lying upstream of position 667.

The IL2 RNA-binding factor complex was stabilized by UV irradiation followed by electrophoresis in an SDS–polyacrylamide gel (Fig. 7). A labeled complex of apparent molecular weight ~60 000 was formed with Sephacryl S-200 fraction 15 material. The binding factor was competed for by IL2 mRNA containing the entire sequence but lacking the poly(A) tail and also by the sequence terminated at the *Sty*I site (position 667). It was not competed for by either the AUR transcript, the c-Myc CRD transcript or by full-length GAPDH mRNA. Thus there is

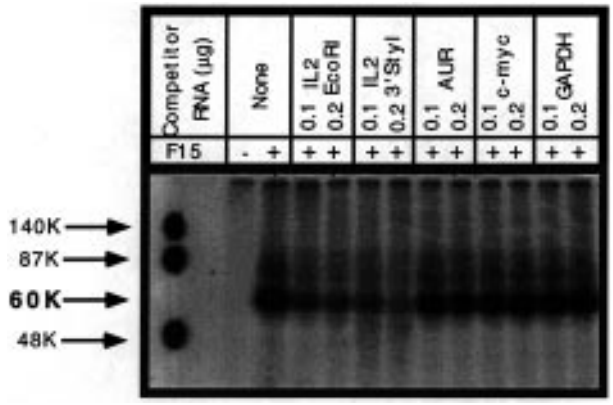

Figure 7. Cross-linking of IL2 mRNA to the binding factor. UV cross-linking of the IL2 mRNA–Sephacryl S-200 fraction 15 complex was carried out using IL2 mRNA lacking poly(A). An SDS-stable band was found at marker protein position 60 kDa. At the level of 0.2 µg competitor the molar ratios of the various competitor RNAs to labeled probe were: IL2, 20:1; IL2 3′-*Sty*I, 96:1; AUR, 340:1; c-Myc CRD, 85:1; GAPDH, 12:1. The formation of this complex was competed for by both full-length poly(A)$^-$ IL2 mRNA (IL2-*Eco*RI) and by RNA extending from position 507 to 667 (IL2-3′-*Sty*I). There was no competition by either the AUR (RNA containing four tandem repeats of the ARE motif), the CRD of c-Myc mRNA or full-length GAPDH mRNA lacking poly(A).

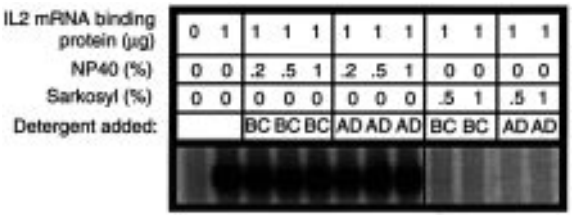

Figure 8. Sensitivity of the IL2 mRNA binding activity to ionic detergent. Only the relevant portion of the autoradiogram of a gel retardation experiment is shown (corresponding to the band at the arrow position in Fig. 3). Either ionic detergent (Sarkosyl) or non-ionic detergent (NP40) was added, either before formation of the complex (lanes labeled B and C) or after (lanes labeled A and D). Sarkosyl, but not NP40, disrupted formation of the complex or its stability after formation.

concordance between formation of the UV cross-linked complex and the gel-retarded complex.

The binding factor complex is non-covalent

The complex between IL2 mRNA and Sephacryl S-200 fraction 15 was not affected by the non-ionic detergent NP40, whether the detergent was present throughout the binding reaction or was added after formation of the complex (Fig. 8). The ionic detergent Sarkosyl, however, prevented formation of the complex and/or destroyed the complex once formed. These results are consistent with the idea that the binding factor is a protein that forms a non-covalent complex with IL2 mRNA.

Protection of IL2 mRNA

The binding factor obtained by Sephacryl S-200 chromatography (fraction 15) blocked the ability of the RNasin-resistant RNase (fraction 17) to degrade IL2 mRNA under conditions leading to

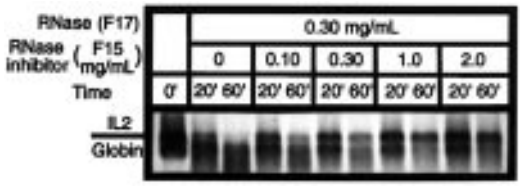

Figure 9. Titration of the IL2 mRNA stabilization factor. Various concentrations of Sephacryl S-200 column fraction 15 (F15; see Fig. 3) were added to the standard RNase assay, using fraction 17 (F17) from the same column as the source of RNase. As in all of the standard RNase assays, RNasin was present to block RNase A-like activity. Degradation of IL2 mRNA was inhibited as the F15 concentration was raised, whereas β-globin mRNA was still degraded even at 2 mg/ml F15 protein.

formation of the complex seen in gel retardation experiments (Fig. 9). Protection was not observed for β-globin mRNA that was present in the same reactions. The protein concentrations stabilizing IL2 mRNA were of the order of 1 mg/ml, similar to those used in binding experiments (cf. Fig. 6). The protective effect of the binding factor was saturable by an excess of IL2 mRNA, but not by β-globin or c-Myc CRD RNA (data not shown). It was destroyed by treatment with proteinase K (data not shown), suggesting that it contains an essential protein component.

DISCUSSION

Strong circumstantial evidence indicates that the factor that binds IL2 mRNA in the gel shift assay also protects it against the RNasin-resistant mRNase described in our previous study (19). Binding and protection are both specific for IL2 mRNA relative to the other RNAs tested, including a sequence that binds AU binding factors (AUBF) (22,31,32). The concentrations of S130 or Sephacryl S-200 fraction 15 protein needed to form the binding complex and to protect against mRNase are similar. Both have properties suggesting they are proteins; the protective factor is sensitive to proteinase K and the binding assay is sensitive to ionic detergents (Fig. 8). The size of the UV cross-linked complex in the binding assay (~60 kDa; Fig. 7) is similar to the size of the binding and protective factors on Sephacryl S-200 (near the elution position of BSA).

The binding factor recognizes a specific segment of the IL2 3′-NC segment, between positions 507 and 667, within which there are two long oligonucleotides lacking G residues. One of these, comprising nt 543–580, contains several AU-rich elements. Under appropriate conditions this fragment binds one of the AUBF (22,33), of mol. wt ~36 000. A second AUBF, AU-B, is found in stimulated normal human T lymphocytes and exhibits a high affinity for ARE in lymphokine mRNAs, but not for those in c-Myc mRNA (33). It is of lower molecular weight. A third component, AU-C, resembles AU-B (24), but is of mol. wt ~43 000. Two immunologically cross-reactive proteins, of mol. wt 37 000 and 40 000, are present in the AU binding factor AUF1 (25). AUF1 binds the 3′-NC regions of c-Myc and GM-CSF RNAs and may be involved in their ARE-dependent degradation. The 37 000 mol. wt protein has been cloned (25). These factors have varying affinities for naturally occurring AREs, but have in common the ability to bind synthetic RNAs containing three or more repeats of the type AUUUA, including the sequence we

have used in this study, which contains four repeats (22). This probe sequence did not compete for binding of our factor to IL2 mRNA (Figs 5 and 7) nor did it form a complex with any factor under the conditions of our experiments (possible reasons for this are discussed below).

Our factor probably binds to one of the two long G-free segments within the region 507–667 of the IL2 RNA probe. These segments have lengths of 38 and 35 nt respectively. The UV cross-linked complex seen in Figure 7 presumably comprises a protein binding factor and one or both of these T1-resistant oligonucleotides. It is noteworthy that binding to IL2 mRNA lacking a poly(A) tail, or indeed a segment carrying only the fragment 507–667, formed a complex with the same mobility as that seen with full-length poly(A)$^+$ mRNA (Fig. 6). The binding factor thus does not resemble poly(A) or poly(U) binding factors (34). However, we have noted an effect of poly(A) on binding of our factor that cannot yet be explained, namely the presence of poly(A), either as part of the IL2 mRNA or unattached, reduces affinity of the factor for the binding site in the 3′-NC region of the IL2 sequence. The saturation level of binding is the same as in the absence of poly(A). One possibility is that the factor is associated with a poly(A) binding factor, which leads to competition between the IL2 3′-NC region site and poly(A). Another possibility is that poly(A) interacts with the factor binding site in the 3′-NC region and thereby blocks factor binding (the binding site is high in A+U). An explanation of the poly(A) effect is currently being sought.

It might seem surprising that we saw no evidence for the ARE binding factors that are known to exist in Jurkat cell extracts and that have an affinity for the 3′-NC region of IL2 mRNA. The reason probably lies in the conditions used. Compared with studies on AUBF mentioned above (24,33) we used a higher temperature (30°C rather than room temperature), a higher salt concentration (100 rather than 40 mM K^+), a lower Mg^{2+} (0.5 rather than 3 mM) and a high concentration of tRNA (1 mg/ml compared with none in the earlier work) to compete for non-specific binding. In comparing our conditions with those of Malter, in which an AU binding protein was first described (22), our binding experiments are higher in salt, lower in Mg^{2+}, 5-fold higher in tRNA and include 5 mg/ml heparin to reduce low affinity complexes. Under our conditions, which we designed to mimic closely those of the functional assay (i.e. inhibition of the RNasin-resistant mRNase), we would have reduced lower affinity binding. This probably accounts for the lack of AUBF activity bound to either the IL2 3′-NC probes or to the AU-rich probe.

There are several other examples of stabilization of mRNAs by binding factors. Stabilization of an mRNA in an *in vitro* polysome-based assay has been shown for c-Myc, where a factor binding to a coding region determinant stabilized the mRNA against degradation (27,28). Using the same system it was found that GM-CSF mRNA is stabilized by an AUBF, presumably interacting with AU-rich 3′-NC sites (31). An endoribonuclease has been purified from *Xenopus* oocytes which cleaves Xlhbox2B mRNA in the 3′-NC region. This attack is blocked by a factor that appears to bind at or near the same sequence recognized by the nuclease, a site that does not contain the classical ARE motif (20). An extract that selectively degrades TGFβ-1 mRNA has been described and also the action of a protective factor that is induced by stimulation of the cells by PMA (35,36). The ability of the factor to stabilize *in vitro* parallels *in vivo* stabilization. Sequence-specific binding factors that may influence mRNA stability include a 65 kDa protein that binds at two sites in the 3′-NC region of β-IFN mRNA (5), an AU-rich region that is involved in destabilizing the mRNA. Another binding factor for a 3′-NC region is found in the tyrosine hydroxylase system (37), where a 75 kDa protein can be cross-linked. Vitellogenin mRNA is stabilized by estrogen, which induces a protein with binding sites in the 3′-NC region of this mRNA (38). A 75 kDa binding factor recognizes a site in the 3′-NC region of the R2 subunit of ribonucleotide reductase. This factor is induced by TGFβ and is not cross-reactive with other short lived mRNAs or poly(A) (39). A 57 kDa PMA-inhibited binding factor apparently destabilizes the ribonucleotide reductase R1 subunit mRNA (40).

There is evidence that for some short lived mRNAs instability sequences identified by functional studies in intact cells are targets for the binding of factors *in vitro*. This is so for c-Fos (41) and c-Myc (28,42). In the study of IL2 mRNA stabilization the linkage between binding and protection *in vitro* and the mechanism of mRNA turnover *in vivo* is not yet established. This work provides a focus for examining such a linkage.

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