MOLECULAR AND CELLULAR BIOLOGY, June 1991, p. 3288–3295
0270-7306/91/063288-08$02.00/0

Vol. 11, No. 6

An Inducible Cytoplasmic Factor (AU-B) Binds Selectively to AUUUA Multimers in the 3′ Untranslated Region of Lymphokine mRNA

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Received 17 December 1990/Accepted 22 March 1991

Considerable evidence suggests that the metabolism of lymphokine mRNAs can be selectively regulated within the cytoplasm. However, little is known about the mechanism(s) that cells use to discriminate lymphokine mRNAs from other mRNAs within the cytoplasm. In this study we report a sequence-specific cytoplasmic factor (AU-B) that binds specifically to AUUUA multimers present in the 3′ untranslated region of lymphokine mRNAs. AU-B does not bind to monomeric AUUUA motifs nor to other AU-rich sequences present in the 3′ untranslated region of c-*myc* mRNA. AU-B RNA-binding activity is not present in quiescent T cells but is rapidly induced by stimulation of the T-cell receptor/CD3 complex. Induction of AU-B RNA-binding activity requires new RNA and protein synthesis. Stabilization of lymphokine mRNA induced by costimulation with phorbol myristate acetate correlates inversely with binding by AU-B. Together, these data suggest that AU-B is a cytoplasmic regulator of lymphokine mRNA metabolism.

Although the primary control of eukaryotic gene expression occurs at the transcriptional level, it has become clear that posttranscriptional mechanisms also play important roles in gene regulation. For instance, the efficiency of nuclear RNA splicing and processing has been suggested to play a regulatory role in the expression of some genes (21). Within the cytoplasm, selective mRNA compartmentalization (30), translation (4, 6, 15), and degradation (3, 12, 19, 20, 27) can each influence the final level of gene expression. In order for gene expression to be regulated selectively at any level, the cell must have a means to discriminate between genes or their products. For example, selective gene expression is regulated at the transcriptional level in part by interactions between sequence-specific nuclear DNA-binding proteins (transcription factors) and sequence motifs within promoter/enhancer regions (for a review, see reference 18). This study was undertaken to determine whether similar sequence-specific cytoplasmic RNA-binding factors are involved in regulating cytoplasmic mRNA metabolism.

Transcripts from many transiently expressed genes, including lymphokine genes and the proto-oncogenes c-*myc* and c-*fos*, contain AU-rich sequences in their 3′ untranslated regions (UTRs) and are rapidly degraded in the cytoplasm (5, 16, 22–24). Several studies have demonstrated that the presence of AU-rich sequences in the 3′ UTRs of a variety of eukaryotic mRNAs correlates with rapid mRNA degradation (for a review, see reference 8). Deletion of AU-rich sequences from the c-*fos* (31) or c-*myc* (10) 3′ UTR confers stability upon transcripts produced from transfected constructs. Furthermore, introduction of a 51-nucleotide AU-rich sequence from the granulocyte-macrophage colony-stimulating factor (GM-CSF) 3′ UTR into the 3′ UTR of the rabbit β-globin gene confers instability upon the otherwise stable β-globin mRNA (23). Thus AU-rich sequences present in the 3′ UTRs of certain mRNAs may function as destabilizing elements that target these mRNAs for rapid cytoplasmic degradation.

Although 3′ AU-rich sequences may function as destabilizing elements in lymphokine as well as proto-oncogene transcripts, there is evidence that cells can differentially regulate the stability of lymphokine and proto-oncogene transcripts. Degradation of a number of lymphokine mRNAs was shown to be regulated differentially from c-*myc* mRNA in human T cells (16). Furthermore, Schuler and Cole (22) described a monocytic cell line that displayed specific cytoplasmic stabilization of GM-CSF mRNA while c-*myc* and c-*fos* mRNAs remained unstable. Transcripts produced in this cell line from hybrid constructs containing the 3′ UTR of the GM-CSF, c-*myc*, or c-*fos* genes linked to a reporter gene showed the same stability pattern as the endogenous transcripts: the GM-CSF hybrid transcript was stable while the c-*myc* and c-*fos* hybrid transcripts were unstable. In contrast, the same hybrid GM-CSF construct produced unstable transcripts in two other cell lines. This observation suggests that GM-CSF mRNA stability is regulated by a *trans*-acting factor(s) that can discriminate between the 3′ UTR sequences in GM-CSF mRNA and the 3′ UTR sequence in c-*myc* or c-*fos* mRNA.

In this work we assayed cytoplasmic extracts from purified human T cells for factors that bind to lymphokine RNA but not c-*myc* RNA. We identified a novel cytoplasmic factor (AU-B) that binds to AU-rich sequences present in the 3′ UTR of interleukin-2 (IL-2), tumor necrosis factor alpha (TNF-α), and GM-CSF mRNAs but does not bind to AU-rich sequences present in the 3′ UTR of c-*myc* mRNA. This factor is not present in unstimulated T cells but is induced by T-cell receptor (TCR)-mediated stimulation and displays kinetics that parallel the expression of lymphokine genes. The finding that this factor binds to 3′ lymphokine RNA but not 3′ c-*myc* RNA suggests that this factor could be used by the cell to distinguish lymphokine RNA from c-*myc* RNA

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and thus differentially regulate the metabolism of these transcripts. We also detect an ubiquitous factor (AU-A) that resembles the AU-binding factor reported by Malter (17). However, we found that this activity is localized primarily within the nucleus, is not regulated in an activation-dependent manner, and does not distinguish between lymphokine and c-*myc* sequences.

MATERIALS AND METHODS

Preparation of T-cell extracts. Human T cells were purified by a previously described negative selection procedure (11) and were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U of penicillin G per ml, 100 μg of streptomycin per ml, and 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). T cells were stimulated with an immobilized monoclonal antibody (G19-4) directed against the CD3 component of the TCR/CD3 complex as previously described (9) (referred to hereafter as α-CD3). Cytoplasmic extracts were prepared from unstimulated and α-CD3-stimulated T cells by lysis in an extraction buffer containing 0.2% Nonidet P-40, 40 mM KCl, 10 mM HEPES (pH 7.9), 3 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 5% glycerol, 8 ng of aprotinin per ml, 2 ng of leupeptin per ml, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Nuclei were removed by centrifugation at 14,000 × *g* for 2 min in an Eppendorf microfuge, and cytoplasmic extracts were immediately frozen on dry ice and were stored at −70°C. The nuclei were extracted for 30 min at 4°C on a rocker in a buffer containing 20 mM HEPES (pH 7.9), 0.42 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 25% glycerol. This nuclear extraction mixture was spun at 14,000 × *g* for 10 min in an Eppendorf microfuge, and the supernatants were dialyzed against a solution containing 20 mM HEPES (pH 7.9), 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 20% glycerol. The nuclear extracts were frozen on dry ice and stored at −70°C.

In vitro transcription reactions. The following DNA fragments were cloned into the polylinker region of the pGEM3Z or pGEM7Zf(+) vector (Promega) and used as templates for in vitro transcription reactions: a 585-bp fragment of human TNF-α cDNA beginning 5′ at the internal *Eco*RI site and containing most of the 3′ UTR, a 1.0-kb fragment of human c-*myc* cDNA beginning 5′ at the internal *Cla*I site and containing part of the coding region and all of the 3′ UTR, a 242-bp *Sau*3A cDNA fragment from the IL-2 3′ UTR, a 500-bp *Sac*I-*Nar*I fragment of genomic human TNF-α DNA containing exon 1, a 400-bp *Pst*I-*Eco*RI fragment of chicken Oct-1 cDNA, an oligonucleotide containing the sequence 5′-ATTTATTTATTTATTTATTTA-3′ (5-AU) found in the GM-CSF 3′ UTR, and an oligonucleotide containing the sequence 5′-ATTTA-3′ flanked by polylinker sequences. In vitro transcription reactions were performed according to Promega instructions, using SP6 or T7 RNA polymerase. Labeled RNA transcripts were produced by inclusion of [α-^{32}P]UTP (800 Ci/mmol) in the reaction, and the resulting transcripts had a specific activity of approximately 3 × 10^8 cpm/μg.

Analysis of RNA-protein interactions. T-cell cytoplasmic extracts (1 to 20 μg of protein) were incubated at room temperature for 30 min with ^{32}P-labeled RNA (0.4 to 4.0 fmol) in a buffer containing 40 mM KCl, 10 mM HEPES (pH 7.9), 3 mM $MgCl_2$, 1 mM DTT, and 5% glycerol. The volume of each reaction was 20 μl. RNase T_1 (1 U/μl, final concentration) was added to cleave unbound RNA, and heparan sulfate (5 mg/ml, final concentration) was added to reduce nonspecific binding. The reaction mixtures were cross-linked with 254-nm UV radiation, using a Stratalinker cross-linking apparatus (energy = 250 mJ/cm^2). This method requires only 60 to 90 s and gives highly reproducible results, in comparison with previously described UV cross-linking procedures which often require 30 to 60 min of irradiation to achieve observable results. The reaction mixtures were then separated by electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels under reducing conditions. Gels were dried and exposed to film at −70°C.

Northern (RNA) blot analysis. Purified human T cells were cultured at 2 × 10^6 cells per ml for 6 h with an immobilized α-CD3 antibody alone or with α-CD3 antibody plus phorbol myristate acetate (PMA). Dactinomycin (10 μg/ml) was then added to the cultures, and cells were harvested after 0, 15, 45, 75, or 105 min. Total cellular RNA was isolated (7) and equalized as described previously (28). Northern blots were prepared and hybridized sequentially with ^{32}P-labeled nick-translated probes; the GM-CSF probe was a 700-bp *Eco*RI-*Hin*dIII cDNA fragment (32), the c-*myc* probe was a 1.0-kb *Cla*I-*Eco*RI cDNA fragment (1), and the HLA probe was a 1.4-kb *Pst*I fragment from the HLA B7 gene (25). After hybridization, blots were washed and exposed to film at −70°C.

RESULTS

Identification of an RNA-binding activity that binds to 3′ TNF-α RNA. Since sequences in the 3′ UTR of lymphokine transcripts are important for their rapid degradation in the cytoplasm (22, 23), we hypothesized that *trans*-acting factors exist that bind to these sequences. To test this hypothesis, cytoplasmic extracts prepared from purified resting human T lymphocytes were incubated with a ^{32}P-labeled riboprobe consisting of 585 bases present in the 3′ UTR of TNF-α mRNA as described in Materials and Methods. Following successive incubations with RNase T_1 and heparan sulfate, the reaction mixtures were cross-linked with UV radiation and separated by electrophoresis on 10% SDS-polyacrylamide gels. RNA-binding activity in the extracts could be identified as radiolabeled bands displaying altered mobility. A major complex (complex A) was detected in extracts from unstimulated T cells when 3′ TNF-α RNA was used as a probe (Fig. 1). Although this complex may be composed of one or more factors, the RNA-binding component(s) of this complex will be referred to as factor A. Factor A was easily visualized when [^{32}P]UTP was used to label the 3′ TNF-α probe but was poorly visualized when [^{32}P]CTP was used (Fig. 1a), suggesting that factor A binds to a region rich in U nucleotides but poor in C nucleotides. To assess further the specificity of this binding, unlabeled RNA was used to compete for this complex. Unlabeled 3′ TNF-α RNA efficiently competed for this complex while a similarly sized transcript from the Oct-1 gene did not, suggesting that the interaction was specific (Fig. 1b). An unlabeled ribo-oligonucleotide containing the sequence 5′-AUUUAUUUAUUUAUUUAUUUA-3′ (5-AU) found in 3′ GM-CSF RNA was also used as a competitor. This sequence is the core element of the 51-bp GM-CSF AU-rich sequence shown by Shaw and Kamen (23) to influence mRNA stability. This element was also present in the genetic constructs used by Schuler and Cole (22) to demonstrate that GM-CSF mRNA stability is regulated by *trans*-acting factors. As seen in Fig. 1b, this GM-CSF 5-AU sequence effectively competed for binding of factor A to 3′ TNF-α RNA. Together, these data suggest that

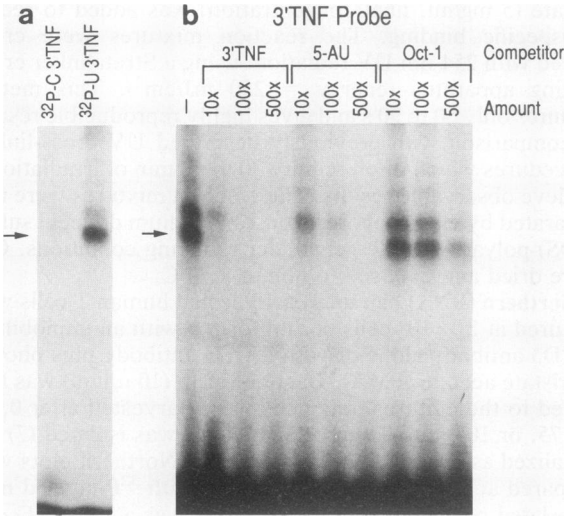

FIG. 1. Identification of an RNA-binding factor (factor A) that binds to 3′ TNF-α AU-rich sequences. (a) Cytoplasmic extracts (8 μg of protein) were incubated with 0.4 fmol of transcripts consisting of sequences present in the 3′ UTR of TNF-α mRNA labeled with either [^{32}P]CTP (^{32}P-C 3′TNF) or [^{32}P]UTP (^{32}P-U 3′TNF). (b) Cytoplasmic extracts (8 μg of protein) were incubated with 4 fmol of [^{32}P]UTP-labeled transcripts consisting of sequences present in the 3′ UTR of TNF-α mRNA (3′TNF Probe) in the absence of competitor transcripts or in the presence of a 10-fold (10×), 100-fold (100×), or 500-fold (500×) molar excess of the following unlabeled competitor transcripts: transcripts consisting of sequences present in the 3′ UTR of TNF-α mRNA (3′TNF), transcripts consisting of the 5-AU sequence present in the 3′ UTR of GM-CSF mRNA (5-AU), or transcripts derived from Oct-1 sequences (Oct-1). Following successive incubations with RNase T_1 and heparan sulfate, the reaction mixtures were UV cross-linked at 250 mJ/cm^2 on a Stratalinker cross-linking apparatus and were separated by electrophoresis on 10% SDS-polyacrylamide gels. Arrows indicate the position of the major RNA-binding complex (referred to in the text as complex A).

factor A binds to the AU-rich sequences found in 3′ TNF-α mRNA.

Factor A binds to 3′ lymphokine as well as 3′ c-*myc* AU-rich sequences. Schuler and Cole (22) have shown that GM-CSF and c-*myc* mRNA degradation is differentially regulated in monocytic cell lines, and our laboratory has shown that degradation of a number of lymphokine mRNAs is regulated differentially from c-*myc* mRNA in human T cells (16). These observations predict the existence of *trans*-acting factors that differentially recognize lymphokine and c-*myc* mRNA. Therefore we performed experiments to determine whether factor A can distinguish lymphokine RNA from c-*myc* RNA. As seen in Fig. 2a, binding by factor A to the 3′ TNF-α riboprobe is effectively competed for by 3′ TNF-α or 3′ c-*myc* RNA but not by irrelevant RNA (Oct-1). This suggests that factor A has specificity for 3′ c-*myc* as well as 3′ TNF-α sequences. In fact, factor A binds directly to ^{32}P-labeled 3′ c-*myc* RNA, and this binding is competed for by unlabeled 3′ c-*myc* or 3′ TNF-α RNA but not Oct-1 RNA (Fig. 2b). In addition to binding to ^{32}P-labeled 3′ TNF-α and 3′ c-*myc* RNA probes, factor A also binds to the 3′ IL-2 riboprobe and to the GM-CSF 5-AU ribo-oligonucleotide probe (Fig. 2c). Although multiple bands were detected with each of these probes, only factor A binding to these probes was reproducibly competed for by the GM-CSF 5-AU ribo-oligonucleotide but not by a control ribo-oligonucleotide containing the sequence 5′-AUUUA-3′ (1-AU) flanked by polylinker sequences. Table 1 shows for each probe the predicted sequences of the RNase T_1-protected fragments to which this factor binds. Although factor A may bind to other AU-rich sequences present in these probes, experiments performed using truncated transcripts or transcripts produced from synthesized oligonucleotides indicate that factor A can bind to the GM-CSF 5-AU and c-*myc* sequences listed in Table 1. The differences in electrophoretic mobility of complex A in Fig. 2 are due to differences in the lengths of the protected RNA fragments. After subtracting the molecular weight of the bound RNA fragment, factor A has a molecular size of approximately 34 kDa. Since factor A binds to 3′ AU-rich regions of lymphokine as well as c-*myc* transcripts, this factor alone cannot explain how a cell distinguishes lymphokine transcripts from c-*myc* transcripts.

A novel AU-binding factor that binds to 3′ lymphokine but not 3′ c-*myc* RNA is induced by T-cell activation. Although unstimulated T cells do not express lymphokine genes or the c-*myc* proto-oncogene, stimulation of T cells through the TCR/CD3 complex induces expression of these genes (16). We hypothesized therefore that *trans*-acting factors which regulate the expression of these genes at the level of mRNA stability might be present only in activated cells. To test this hypothesis, cytoplasmic extracts from unstimulated T cells or from T cells that had been stimulated for 6 h with an α-CD3 monoclonal antibody were assayed for RNA-binding activity. We found that α-CD3 stimulation induced a 30-kDa factor (factor B) which bound to the GM-CSF ribo-oligonucleotide probe but not to the 3′ c-*myc* probe (Fig. 3a). Complex B is relatively difficult to visualize, possibly because different RNA-protein interactions become cross-linked by UV irradiation with different efficiencies. It is possible that spatial positioning of reactive groups on factor B and its bound RNA is not optimal for efficient cross-linking. Factor B appeared to bind to the GM-CSF 5-AU riboprobe with higher affinity than factor A, since the appearance of factor B binding activity following α-CD3 stimulation resulted in a decrease in factor A binding to the GM-CSF 5-AU riboprobe when the binding reactions were performed using a limiting GM-CSF 5-AU probe concentration (Fig. 3a). However, no decrease in factor A binding to the 3′ c-*myc* probe was observed following α-CD3 stimulation under the same conditions. This result suggests that factor A was present at equivalent concentrations in extracts from unstimulated and α-CD3-stimulated T cells and that factor B did not compete with factor A for the 3′ c-*myc* probe. Independent experiments showed that factor B has approximately a 10-fold-greater relative affinity for the GM-CSF 5-AU probe than does factor A (data not shown). In addition to binding to the GM-CSF 5-AU probe, factor B also bound to 3′ TNF-α and 3′ IL-2 riboprobes (Fig. 3b). Together, these data suggest that factor B bound specifically to 3′ lymphokine but not 3′ c-*myc* AU-rich sequences.

Factor B binding is specifically competed for by 3′ lymphokine sequences but not by 3′ c-*myc* sequences. Competition experiments were performed to confirm our conclusion that factor B binds to 3′ lymphokine but not 3′ c-*myc* RNA sequences. Extracts from unstimulated T cells or T cells that had been stimulated for 6 h with monoclonal antibody α-CD3 were incubated with the GM-CSF 5-AU ribo-oligonucleotide probe (Fig. 4). As in the foregoing experiments, factor B binding was induced by α-CD3 stimulation, and the appearance of factor B binding in extracts from α-CD3-stimulated cells correlated with decreased factor A binding to this probe. Factor A binding activity in extracts from α-CD3-

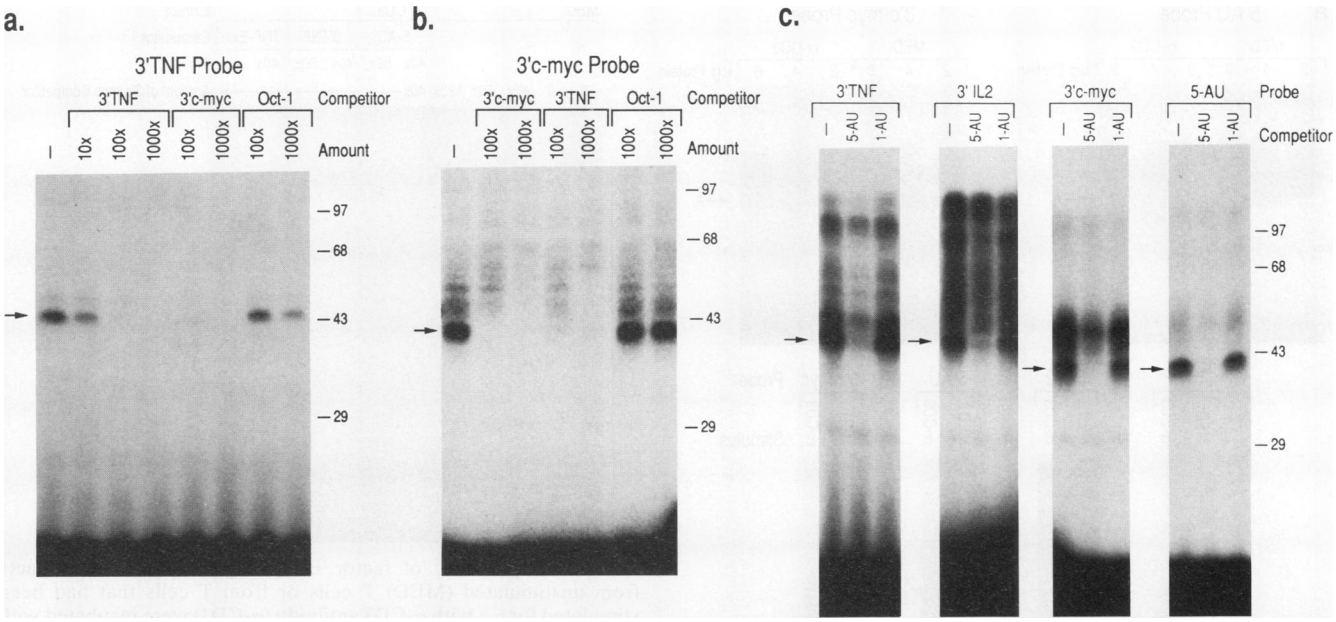

FIG. 2. Specificity of factor A binding. (a) Cytoplasmic extracts (20 μg of protein) from unstimulated T cells were incubated with 0.4 fmol of [^{32}P]UTP-labeled transcripts consisting of sequences present in the 3′ UTR of TNF-α mRNA (3′TNF Probe) in the absence of competitor RNA or in the presence of a 10-fold (10×), 100-fold (100×), or 1,000-fold (1000×) molar excess of the following unlabeled competitor transcripts: transcripts consisting of sequences present in the 3′ UTR of TNF-α mRNA (3′TNF), transcripts containing the 3′ UTR of c-*myc* mRNA (3′c-myc), or transcripts derived from Oct-1 sequences (Oct-1). (b) Cytoplasmic extracts (13 μg of protein) from unstimulated T cells were incubated with 0.4 fmol of [^{32}P]UTP-labeled transcripts containing the 3′ UTR of c-*myc* mRNA (3′c-myc Probe) in the absence of competitor transcripts or in the presence of a 100-fold (100×) or 1,000-fold (1000×) molar excess of the following unlabeled competitor transcripts: transcripts containing the 3′ UTR of c-*myc* mRNA (3′c-myc), transcripts consisting of sequences present in the 3′ UTR of TNF-α mRNA (3′TNF), or transcripts derived from Oct-1 sequences (Oct-1). (c) Cytoplasmic extracts (2 μg of protein) from unstimulated T cells were incubated with 4 fmol of [^{32}P]UTP-labeled transcripts in the absence of competitor transcripts or in the presence of a 20-fold molar excess of unlabeled GM-CSF 5-AU or 1-AU competitor transcripts. The following ^{32}P-labeled transcripts (probes) were used: transcripts consisting of sequences present in the 3′ UTR of TNF-α mRNA (3′TNF), transcripts containing sequences present in the 3′ UTR of IL-2 mRNA (3′IL2), transcripts containing the 3′ UTR of c-*myc* mRNA (3′c-myc), and transcripts containing the 5-AU sequence found in the 3′ UTR of GM-CSF mRNA (5-AU). Following successive incubations with RNase T_1 and heparan sulfate, the reaction mixtures were UV cross-linked and were separated by electrophoresis on 10% SDS-polyacrylamide gels under reducing conditions. In panels a and b, the reaction mixtures were UV cross-linked for 30 min on a UV light box; in panel c, the reaction mixtures were UV cross-linked at 250 mJ/cm^2 on a Stratalinker cross-linking apparatus. The migration of protein molecular weight standards is shown to the right in kilodaltons. The arrows indicate the position of complex A migration.

stimulated cells was competed for with unlabeled 3′ c-*myc* RNA. However, factor B binding to the GM-CSF 5-AU probe was not competed for by 3′ c-*myc* RNA. The ability of factor B to bind to the GM-CSF 5-AU probe in the presence of a 120-fold molar excess of 3′ c-*myc* RNA demonstrates that factor B binds independently from factor A and that the factor B complex does not arise from a modification of factor A after binding. Since 3′ c-*myc* RNA competed for factor A binding but not factor B binding to the GM-CSF 5-AU probe, competitions with unlabeled GM-CSF 5-AU, 3′ TNF-α, or TNF exon 1 RNA sequences were performed in the presence of a 40-fold molar excess of 3′ c-*myc* RNA. GM-CSF 5-AU or 3′ TNF RNA effectively competed for factor B binding, but TNF exon 1 RNA did not. Together, these data suggest that factor B binding is specific for 3′ lymphokine AU-rich sequences and that factor B does not bind to 3′ c-*myc* sequences. Thus, factor B could potentially be used by the cell to distinguish lymphokine from c-*myc* transcripts and differentially regulate their metabolism.

In independent experiments using an RNA gel retardation assay under nondenaturing conditions, a GM-CSF 5-AU binding activity was detected in resting T cells and its level increased following α-CD3 stimulation. All of the GM-CSF 5-AU binding activity in resting cells was inhibited by unlabeled 3′ c-*myc* RNA. In contrast, the GM-CSF 5-AU binding activity in α-CD3-stimulated cells was only partially inhibited by unlabeled 3′ c-*myc* RNA but was completely inhibited by unlabeled GM-CSF 5-AU RNA (data not

TABLE 1. Factor A binding to RNase T_1 fragments

Transcript	Predicted RNase T_1 fragment to which factor A binds	*n*
3′ TNF	AUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUACAG	37
3′ IL-2	CCUUCUAUUUAUUUAAAUAUUUAAAUUUUAUAUUUAUUG	39
GM-CSF 5-AU	UAUUUAUUUAUUUAUUUAUUUACUCG	26
3′ c-*myc*	CUUACCAUCUUUUUUUUUUCUUUAACAG	28

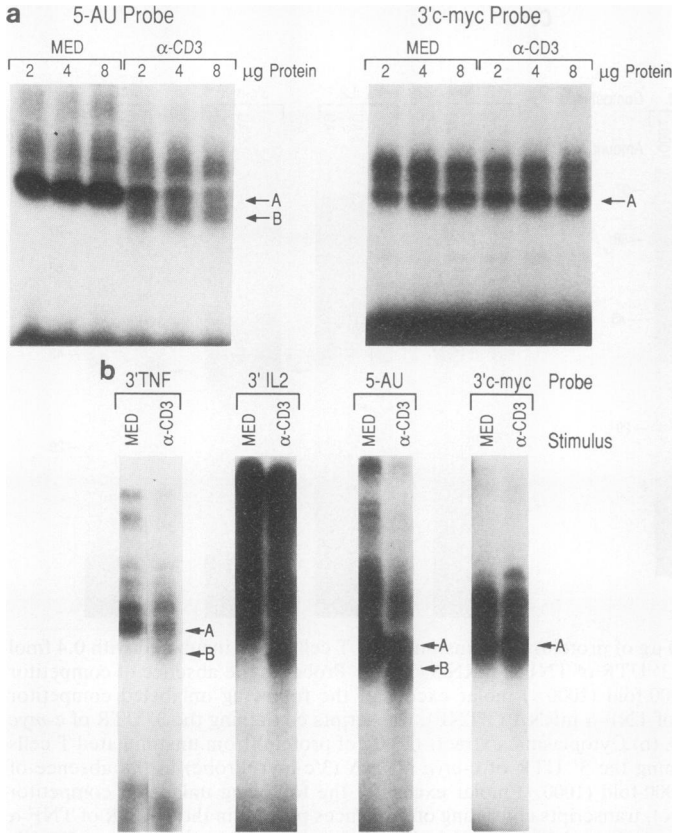

FIG. 3. Induction by α-CD3 stimulation of a second AU-specific RNA-binding factor (factor B). (a) Cytoplasmic extracts (2 to 8 μg of protein) from unstimulated (MED) T cells or from T cells that had been stimulated for 6 h with α-CD3 antibody (α-CD3) were incubated with 3 fmol of [^{32}P]UTP-labeled GM-CSF 5-AU riboprobe (5-AU Probe) or c-*myc* 3′ UTR riboprobe (3′c-myc Probe). (b) Cytoplasmic extracts (8 μg of protein) from unstimulated (MED) T cells or from T cells that had been stimulated for 6 h with α-CD3 antibody (α-CD3) were incubated with 3 fmol of the indicated [^{32}P]UTP-labeled riboprobes. Following successive incubations with RNase T_1 and heparan sulfate, the reaction mixtures were UV cross-linked at 250 mJ/cm^2 on a Stratalinker cross-linking apparatus and were separated by electrophoresis on 10% SDS-polyacrylamide gels. Arrows A and B indicate the positions of migration of complexes A and B, respectively.

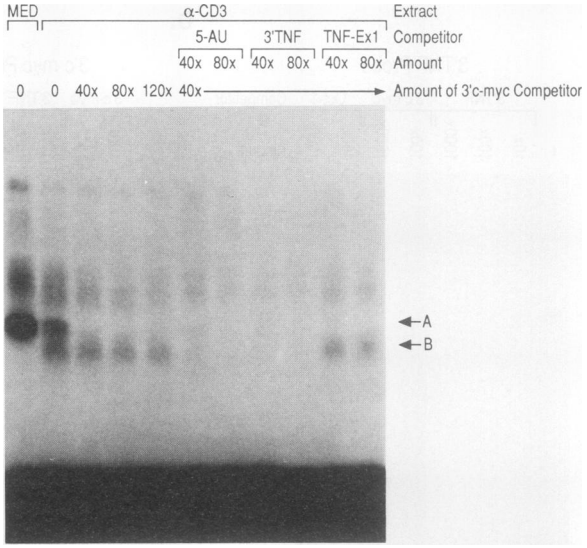

FIG. 4. Specificity of factor B binding. Cytoplasmic extracts from unstimulated (MED) T cells or from T cells that had been stimulated for 6 h with α-CD3 antibody (α-CD3) were incubated with 4 fmol of [^{32}P]UTP-labeled GM-CSF 5-AU riboprobe. A 40-fold (40×), 80-fold (80×), or 120-fold (120×) molar excess of unlabeled c-*myc* 3′ UTR competitor RNA (3′c-myc Competitor) was included in the indicated reactions. A 40-fold molar excess of c-*myc* 3′ UTR RNA competed for complex A but not complex B. Therefore competition of complex B was carried out in the presence of a 40-fold molar excess of c-*myc* 3′ UTR RNA as well as a 40-fold (40×) or 80-fold (80×) molar excess of GM-CSF 5-AU (5-AU), TNF-α 3′ UTR (3′TNF), or TNF exon 1 (TNF-Ex1) competitor RNA. Arrows A and B indicate the positions of migration of complexes A and B, respectively.

shown). These data suggested that complexes A and B comigrated in RNA retardation gels. Since complexes A and B could be separated following UV cross-linking on SDS-polyacrylamide gels, binding activity was assessed by this method in all subsequent experiments.

Kinetics of factor B RNA-binding activity following α-CD3 stimulation. If factor B is involved in the regulation of lymphokine mRNA metabolism, then the kinetics of factor B induction might be expected to parallel the expression of lymphokine mRNA. For example, following α-CD3 stimulation, IL-2 and GM-CSF mRNA expression is observable at 3 h, peaks at 6 h, and decreases to undetectable levels by 24 h (16, 29). As seen in Fig. 5, factor B binding to the GM-CSF 5-AU probe appears within 3 h following α-CD3 stimulation, peaks at 6 h, and is gone by 24 h. The induction of factor B activity following α-CD3 stimulation requires new translation and new transcription, since cycloheximide (Fig. 5) and dactinomycin (data not shown) both block the induction of this complex. As was also seen in Fig. 3 and 4, the appearance of factor B binding resulted in a decrease in factor A binding to the GM-CSF 5-AU probe as a result of the higher relative affinity of factor B for the GM-CSF 5-AU probe. Factor A binding to the 3′ c-*myc* probe did not decrease at the 3- or 6-h time point, suggesting that the amount of factor A did not change during this time period. Thus, the time course of the appearance of factor B binding parallels the induction of lymphokine genes following α-CD3 stimulation and is consistent with factor B functioning as a transiently induced regulatory protein.

Factor B is a cytoplasmic factor composed of protein. If factor B were a cytoplasmic factor that regulates lymphokine mRNA metabolism, then one might expect to find it predominantly in the cytoplasm. Therefore cytoplasmic and nuclear extracts from α-CD3-stimulated T cells were assayed for factor A and factor B RNA-binding activities. As seen in Fig. 6a, factor A binding activity is more abundant in nuclear extracts than in cytoplasmic extracts, whereas factor B activity is visualized only in cytoplasmic extracts. In extracts from resting T cells, factor A activity is also more abundant in nuclear extracts than in cytoplasmic extracts (data not shown).

The observation that the protein synthesis inhibitor cycloheximide blocks the induction of factor B binding raised the possibility that factor B could be an induced protein. To define more clearly the composition of factors A and B, cytoplasmic extracts from unstimulated and α-CD3-stimulated T cells were incubated with proteinase K following a binding reaction with the GM-CSF 5-AU riboprobe. Factors

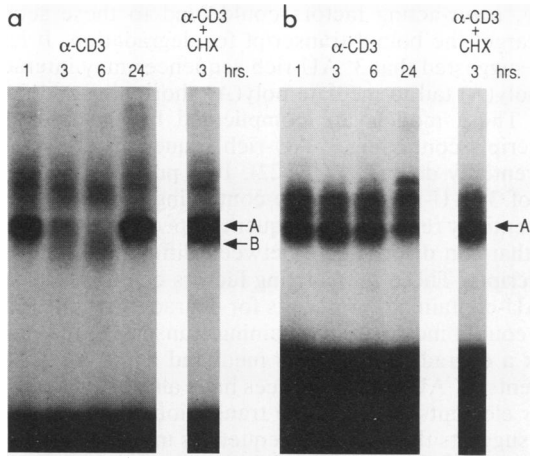

FIG. 5. Kinetics and dependence of new protein synthesis of factor B binding following α-CD3 stimulation. Cytoplasmic extracts were prepared from T cells that had been stimulated with α-CD3 antibody (α-CD3) for 1, 3, 6, or 24 h or from T cells that had been stimulated with an α-CD3 antibody for 3 h in the presence of 10 μg of cycloheximide per ml (α-CD3 + CHX). Protein (8 μg) from these extracts was incubated with 4 fmol of [^{32}P]UTP-labeled GM-CSF 5-AU riboprobe (a) or c-*myc* 3′ UTR riboprobe (b). Arrows A and B indicate the positions of migration of complexes A and B, respectively.

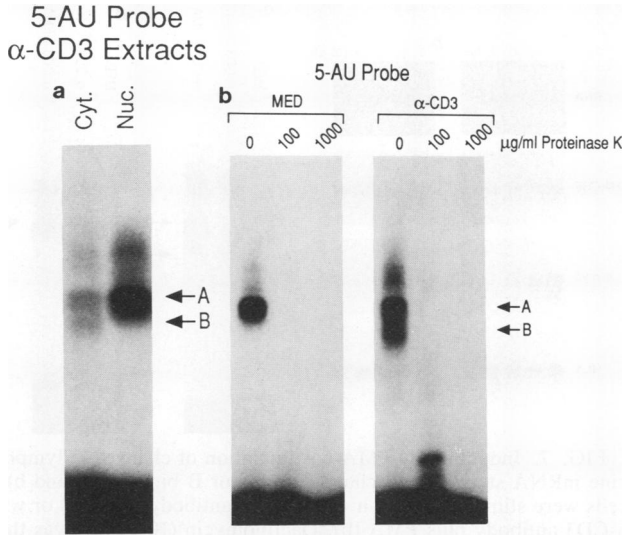

FIG. 6. Subcellular localization and composition of factors A and B. (a) Approximately 8 × 10^5 cell equivalents of cytoplasmic (Cyt.) extract (10 μg of protein) or nuclear (Nuc.) extract (0.5 μg of protein) from T cells that had been stimulated for 6 h with α-CD3 antibody were incubated with 4 fmol of [^{32}P]UTP-labeled GM-CSF 5-AU riboprobe. (b) Cytoplasmic extracts (8 μg of protein) from unstimulated (MED) T cells or from T cells that had been stimulated for 6 h with anti-CD3 antibody (α-CD3) were incubated with 4 fmol of [^{32}P]UTP-labeled GM-CSF 5-AU transcripts; the reaction mixtures were treated sequentially with RNase T_1 and heparan sulfate and then were incubated at 37°C for 15 min in the absence of proteinase K or in the presence of 100 or 1,000 μg of proteinase K per ml. Arrows A and B indicate the positions of migration of complexes A and B, respectively.

A and B are both eliminated by proteinase K treatment (Fig. 6b). This demonstrates that these factors are at least partially composed of protein but does not exclude the possibility that they also contain nonprotein components.

Costimulation of α-CD3-stimulated T cells with PMA induces changes in lymphokine mRNA degradation and changes in factor B binding. Lymphokine as well as c-*myc* transcripts are unstable following α-CD3 stimulation (16). However, Shaw and Kamen (23) have demonstrated that GM-CSF mRNA is stabilized upon stimulation of a T-cell line with PMA. Costimulation of α-CD3-stimulated T cells with PMA induced specific stabilization of lymphokine transcripts and had relatively little effect on c-*myc* mRNA stability (Fig. 7b). Therefore experiments were designed to determine whether PMA costimulation would induce changes in factor B binding. As seen in Fig. 7c, α-CD3 stimulation induced factor B binding to the GM-CSF 5-AU probe. Again, the appearance of factor B binding correlated with decreased factor A binding, as seen previously, and is likely to be due to competition between factors A and B for the GM-CSF 5-AU riboprobe. In contrast, costimulation with PMA resulted in decreased factor B binding to the GM-CSF 5-AU probe and increased factor A binding compared with α-CD3 stimulation alone. Factor A binding to the 3′ c-*myc* probe was approximately equivalent in extracts from α-CD3-stimulated and α-CD3-plus-PMA-stimulated T cells (Fig. 7d), demonstrating that the differences in binding to the GM-CSF 5-AU probe were not due to differences in the amount of factor A in the extracts. Thus it appears that PMA costimulation results in decreased factor B binding activity and increased lymphokine mRNA stability.

DISCUSSION

The observation that lymphokine mRNA degradation is regulated differentially from c-*myc* mRNA degradation (16, 22) suggested that cells have a mechanism to distinguish between these transcripts. In this work, we report the identification of a novel cytoplasmic RNA-binding factor (factor B) that binds specifically to AUUUA multimers present in the 3′ UTR of GM-CSF, IL-2, and TNF-α mRNAs but does not bind to AU-rich sequences present in the 3′ UTR of c-*myc* mRNA. Each of these lymphokine mRNAs contains at least three reiterated copies of the sequence AUUUA (Table 1). 3′ c-*myc* mRNA also contains several copies of this sequence, but these copies are separated from each other by at least 25 nucleotides. Factor B RNA-binding activity is not present in cytoplasmic extracts from unstimulated T cells but is induced by α-CD3 stimulation. The induction of factor B RNA-binding activity requires new transcription and translation, since it is blocked by dactinomycin or cycloheximide. Factor B activity is present by 3 h after α-CD3 stimulation, peaks at 6 h, and is gone by 24 h. These kinetics closely parallel the expression of several lymphokine mRNAs following α-CD3 stimulation and are consistent with factor B acting as a transiently expressed regulator of lymphokine gene expression.

An AU-binding protein present in cytoplasmic extracts from the T-cell line Jurkat was recently reported (17). This Jurkat AU-binding protein resembles the RNA-binding factor A identified in this report. Factor A is present in extracts from unstimulated and α-CD3-stimulated T cells and binds to AU-rich sequences present in the 3′ UTRs of TNF-α, IL-2, GM-CSF, and c-*myc* mRNAs. The finding that factor A binds to 3′ AU-rich sequences from lymphokine as well as proto-oncogene mRNAs suggests that this factor does not mediate the differential degradation of lymphokine and

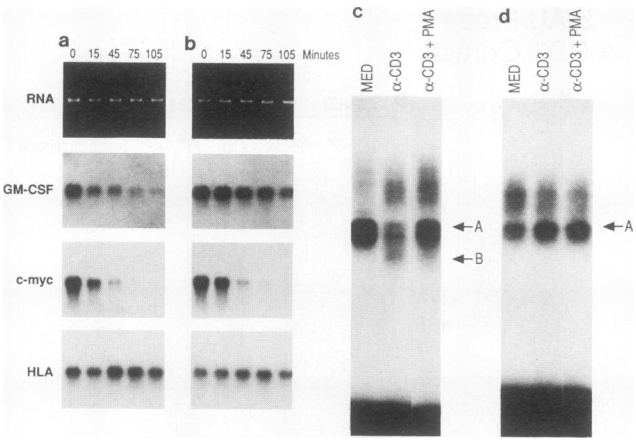

FIG. 7. Induction by PMA costimulation of changes in lymphokine mRNA stability and changes in factor B binding. (a and b) T cells were stimulated for 6 h with α-CD3 antibody alone (a) or with α-CD3 antibody plus PMA (b). Dactinomycin (10 μg/ml) was then added to the cultures, and cells were harvested at the times shown. Total cellular RNA was isolated and equalized for rRNA by ethidium bromide staining of aliquots separated on a 1% nondenaturing agarose gel (RNA panel). Northern blots prepared from equalized samples of RNA were hybridized sequentially with nick-translated GM-CSF, c-*myc*, and HLA probes. The exposures shown display equal hybridization intensity for the two culture conditions at the 0-min time point to facilitate the visual comparison of mRNA stability. α-CD3-plus-PMA stimulation actually augments GM-CSF mRNA expression fivefold over that seen with α-CD3 stimulation alone. (c and d) Cytoplasmic extracts (4 μg of protein) from unstimulated T cells (MED) or from T cells that had been stimulated for 6 h with α-CD3 antibody (α-CD3) or with α-CD3 antibody plus PMA (α-CD3 + PMA) were mixed with 4 fmol of [^{32}P]UTP-labeled GM-CSF 5-AU riboprobe (c) or c-*myc* 3′ UTR riboprobe (d). RNase T_1 was immediately added, and the mixtures were incubated for 10 min; heparan sulfate was added, and the mixtures were incubated for an additional 10 min. The reaction mixtures were then UV cross-linked and separated by electophoresis on 10% polyacrylamide gels under reducing conditions. Arrows A and B indicate the positions of migration of complexes A and B, respectively.

proto-oncogene transcripts. Factor A is more abundant in nuclear extracts than in cytoplasmic extracts from purified human T cells. Numerous single-stranded RNA-binding proteins identified as components of small nuclear ribonucleoprotein particles and heterogenous ribonucleoprotein particles are known to leak into cytoplasmic extracts during extraction procedures (13, 26). This suggests that factor A may be a nuclear factor that leaked out of the nucleus during the extraction procedure. Although factor A is abundant in the nucleus, it may also be present in the cytoplasm and play a role in cytoplasmic mRNA metabolism. Although Malter (17) has previously referred to factor A as AU-binding protein, it is now clear that cells can contain more than one RNA-binding factor with specificity for AU-rich sequences. In the future, we will refer to factor A as AU-A and to factor B as AU-B.

The AU-rich sequences present in the 3′ UTR of a variety of transiently expressed mRNAs, including lymphokine and proto-oncogene mRNAs, have been shown to function as instability elements (10, 23, 31), but the mechanism by which they function is not known. Several models have been proposed to explain the role of 3′ AU-rich sequences in mediating mRNA instability (2, 22, 31). AU-rich sequences could act directly as targets for endonucleases. Alternatively, *trans*-acting factors could bind to these sequences and target the bound transcript for degradation. It has also been suggested that 3′ AU-rich sequences may interact with the poly(A) tail to mediate poly(A) shortening and degradation. These models are complicated by the findings that transcripts containing 3′ AU-rich sequences are sometimes differentially degraded (16, 22). It is possible that degradation of 3′ AU-rich sequence-containing transcripts may be differentially regulated by sequence-specific *trans*-acting factors that can discriminate between different AU-containing transcripts. These *trans*-acting factors could selectively target AU-containing transcripts for degradation; alternatively, they could bind to AU-containing transcripts and passively block a degradation process mediated by 3′ AU instability elements. 3′ AU-rich sequences have also been shown to act as *cis* elements that regulate translational efficiency (4, 14). This suggests that AU-rich sequences may play dual roles in regulating mRNA metabolism or that translation and mRNA degradation are coupled. Thus, it is possible that regulatory events that influence translation could also influence mRNA degradation.

Since AU-B (factor B) is an inducible cytoplasmic factor that binds specifically to lymphokine AU-rich sequences, AU-B is likely to be a cytoplasmic regulator of lymphokine mRNA metabolism. The observation that AU-B displays binding kinetics that parallel the expression of lymphokine mRNA following TCR/CD3 stimulation suggests several possible functions for AU-B: (i) AU-B may facilitate translation of lymphokine mRNA; (ii) AU-B may transiently stabilize lymphokine mRNA, allowing it to be translated; or (iii) AU-B may target lymphokine mRNA for degradation, thus limiting the increase in lymphokine mRNA levels following α-CD3 stimulation and leading to a rapid fall in mRNA levels once peak levels are reached. Shaw and Kamen (23) showed that PMA stimulation increased GM-CSF mRNA stability in T-cell lines. We found that PMA also increased GM-CSF mRNA stability in α-CD3-stimulated purified human T cells, and this increase in GM-CSF mRNA stability correlated with decreased AU-B binding to the GM-CSF 5-AU RNA sequence. This correlation raises the possibility that AU-B may target lymphokine mRNA for degradation. However, since PMA stimulation has such diverse and incompletely characterized effects on T cells, no firm conclusion about the function of AU-B can yet be drawn. To further define the function of AU-B, we are now involved in purifying AU-B to determine whether it has effects on mRNA stability or translation in in vitro systems.

ACKNOWLEDGMENTS

We thank John Lowe, Jeffrey Leiden, Richard Hodes, Jeffrey Ross, Nahum Sonnenberg, and Richard Klausner for many stimulating discussions and for their thoughtful review of the manuscript.

P.R.B. is supported by a Lutheran Brotherhood M.D./Ph.D. scholarship from the Life and Health Insurance Medical Research Fund.

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