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Binding of sequence-specific proteins to the adenosine- plus uridine-rich sequences of the murine granulocyte/macrophage colony-stimulating factor mRNA

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ABSTRACT **Adenosine + uridine (AU)-rich sequences in the 3' untranslated region (3'UTR) of the mRNA of many cytokines and oncogenes play an important role in mediating RNA degradation. Among the cytokines containing such AU-rich sequences in their 3'UTR is the hematopoietic growth factor granulocyte/macrophage colony-stimulating factor (GM-CSF). GM-CSF gene expression in T cells is regulated by modulation of mRNA half-life. Transfection studies using murine EL-4 thymoma cells have demonstrated that degradation depends on the presence of specific elements in the 3'UTR, including the AU-rich sequences. A number of AU-binding factors have recently been discovered, suggesting that specific regulation may occur through specific protein–mRNA interaction(s). We present evidence from gel-shift analyses and label-transfer experiments that murine cells contain proteins that bind specifically to AU-rich sequences. Three major proteins of 33, 39.5, and 42 kDa are detected. Phorbol ester treatment of cells does not alter the abundance or apparent binding affinity of the proteins. The 33-kDa protein is present in the cytoplasm of murine and human cells, whereas the 39.5- and 42-kDa proteins are present in murine extracts only. Constitutively expressed AU-binding proteins of the type that we describe may function by directing mRNA degradation in the absence of a stimulus to the contrary.**

An important control point in gene expression is regulation of the steady-state level of mRNA. For certain mRNAs, achievement of high steady-state levels depends on mRNA stabilization (for reviews see refs. 1 and 2). It is clear that adenosine + uridine (AU)-rich sequences in the 3' untranslated regions (3'UTRs) of many cytokine and oncogene mRNAs are responsible for their rapid degradation in the cytoplasm (3, 4). Addition of these sequences to normally stable messages such as β-globin renders them unstable, and deletion of these sequences from oncogene mRNAs such as c-*fos* makes that mRNA stable (5). A role for AU-rich sequences in controlling mRNA decay is not restricted to cytokines and oncogenes. mRNAs of the vasoconstrictor preproendothelin-1 (6) and rat liver cholesterol 7 αhydroxylase (7) genes contain several such AUUUA motifs and are relatively unstable (6, 7).

A number of observations suggest that genetic alterations of AU-rich sequences may play a role in various diseases. For example, mast cells show enhanced tumorigenicity when transfected interleukin-3 genes lacking the normal AU-rich 3'UTR are overexpressed (8). When AU motifs are removed from the protooncogene c-*fos* mRNA 3'UTR, there is a correlation with increased oncogenicity (5). A 3' truncation of the c-*myc* gene that eliminates AT-rich sequences appears to be responsible for increased mRNA stability in a human T-cell leukemia cell line (9). In a related set of observations, Ross *et al.* (10) reported that in a variety of tumor cells the AU-rich sequences in the 3'UTR of granulocyte/macrophage colony-stimulating factor (GM-CSF) mRNA do not have the same destabilizing effect as in normal cells. These authors postulated that another region of the cytokine mRNA is involved in determining mRNA stability in tumor cells. Alternatively, the AU-rich sequences may not function properly in the tumor cell because of missing or altered proteins.

How these AU-rich sequences mediate their biological actions is not known. One possibility is that AU-specific endoribonucleases degrade those transcripts that contain reiterated AUUUA sequences (11). Recently, a number of investigations have shown the existence of one or more nuclear and cytoplasmic proteins that can bind to AU-rich RNA. Malter and co-workers demonstrated the presence of low molecular mass (15- to 19-kDa) AU-binding factors (AUBF) in the cytoplasm of human peripheral blood mononuclear cells (12) and in Jurkat cell lines (13). They proposed that AUBF play a primary role in the control of mRNA stability in T cells and were up-regulated by phorbol esters and calcium ionophores through a mechanism involving redox switches and phosphorylation (14). Vakalopoulou *et al.* (15) also identified a 32-kDa nuclear protein in human cells that binds to AU-rich sequences, and Bohjanen *et al.* (16) showed that human T cells contain an inducible 30-kDa factor that binds to various lymphokine mRNA 3'UTRs but not to c-*myc* mRNA 3'UTRs and contain a 34-kDa protein that binds to both 3'UTR species. This latter constitutively expressed protein resembles that described by Vakalopoulou *et al.* (15). Thus, it is clear that a spectrum of constitutive and inducible proteins that interact with AU-rich regions exists in the nucleus and cytoplasm.

In studying murine GM-CSF gene expression in EL-4 thymoma cells as a model system for the regulation of mRNA stability, we and others have shown that GM-CSF expression is regulated primarily by modulation of mRNA half-life (10, 17, 18). By means of transient transfections of EL-4 cells using hybrid constructs containing portions of the GM-CSF 3'UTR linked to a reporter gene (chloramphenicol acetyltransferase, CAT) we have shown that the AU-rich sequence is the key element in controlling mRNA instability (19). Furthermore, the function of the AU-rich elements was not cell type-specific; these elements were as effective at destabilizing mRNA in EL-4 thymoma cells as they were in NIH3T3 fibroblasts.

To further explore the mechanism of mRNA degradation mediated by AU-rich sequences we characterized proteins capable of binding to these regions. Gel mobility-shift and label-transfer experiments detected at least three proteins

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Abbreviations: GM-CSF, granulocyte/macrophage colony-stimulating factor; 3'UTR, 3' untranslated region; TPA, phorbol 12-tetradecanoate 13-acetate (12-*O*-tetradecanoylphorbol 13-acetate); CAT, chloramphenicol acetyltransferase; nt, nucleotide(s).

that specifically interact with the 3'UTR of the mouse GM-CSF mRNA. We mapped the binding site of these proteins and showed that it is limited to the AU-rich sequences. Finally, the proteins are expressed in a variety of cell types from two species and the levels of these proteins do not change with phorbol ester treatment, suggesting that regulation occurs at the level of enzyme activity.

MATERIALS AND METHODS

Reagents. Reagents were purchased as follows: phorbol 12-tetradecanoate 13-acetate (12-*O*-tetradecanoylphorbol 13-acetate; TPA), Consolidated Midland (Brewster, NY); RNasin, Promega; RNase T1 and RNase A, GIBCO/BRL; micrococcal nuclease, Boehringer Mannheim; all other reagents were from Sigma.

Cell Culture and Lysate Preparation. Murine thymoma EL-4 cells (20), murine NIH3T3 fibroblasts, and human embryonic kidney U293 fibroblasts were maintained as described (21). Cytoplasmic extracts were prepared as described (22). Nuclear extracts were dialyzed against 100 mM KCl and spun briefly at $15,000 \times g$ to remove debris. Protein concentrations were measured by the Bradford assay (Bio-Rad) and lysates were frozen at −70°C in small aliquots.

RNA Probes. The GM-CSF 3'UTR [nucleotides (nt) +447 to +750] was synthesized by PCR (19, 23). After digestion with *Bam*HI and gel purification the insert was cloned in pGEM3Z (Promega) and digested with *Sma* I and *Bam*HI. Linearization with *Xba* I produces a 310-nt RNA that includes almost the entire GM-CSF 3'UTR. Linearization with *Mbo* II produces a transcript lacking the AU-rich sequences (3'UTR-*Mbo*II) (Fig. 1*A*). For competition experiments a plasmid (3'UTR-mut) was used with a 30-base-pair (bp) mutated region (nt +504 to +533, Fig. 2*A*; for details see ref. 19) and was transcribed after linearization with *Pst* I. The CAT gene (Pharmacia) was cloned in forward orientation in the pSP64 poly(A) vector (Promega). Linearization with *Pst* I produces the 805-nt RNA transcript used as nonspecific competitor (CAT-A_n). Plasmids were linearized and transcribed *in vitro* and the RNA probes were isolated by standard protocols (Promega). RNA concentrations were determined by UV spectroscopy at 260 nm and by denaturing agarose gel electrophoresis and UV fluorescence in the presence of ethidium bromide.

Binding Reactions and Band-Shift Assay Procedures. Protein extracts (25–100 μg) were incubated for 30 min at room temperature with *in vitro* transcribed [^{32}P]UTP-labeled RNA probes (20,000 cpm per reaction, specific activity 3×10^{12} Bq/mg) in a binding buffer [10 mM Hepes, pH 7.6/3 mM $MgCl_2$, 5% (vol/vol) glycerol/1 mM dithiothreitol] containing poly(I) (5 μg per reaction; Pharmacia), heparin sulfate (100 μg per reaction; Fisher Scientific), or both, and RNasin (1 unit per reaction). Binding mixtures were then treated with RNase T1 (25–100 units per reaction as indicated for each experiment) for 10 min at room temperature and loaded onto nondenaturing 4% polyacrylamide gels (acrylamide/bisacrylamide = 60:1) containing 5% glycerol in 0.5× TBE (1× TBE = 0.09 M Tris–borate, pH 8.3/2 mM EDTA) buffer. After electrophoresis at 125 V at 4°C, gels were dried on Whatman 3MM paper and exposed to Kodak XAR film at −70°C.

UV Crosslinking of Binding Reaction Products. Reaction mixtures in 1.5-ml Eppendorf tubes were exposed to UV light while on ice at 180 to 1440 mJ/cm^2 (Stratalinker 2400; Stratagene). After digestion with RNase A (10 μg per tube) at 37°C for 15 min samples were boiled for 2 min in SDS sample buffer and electrophoresed on 12% polyacrylamide/SDS gels. Dried gels were exposed to XAR film overnight at −70°C.

RESULTS

Detection of Specific Protein–RNA Complexes. To determine if the GM-CSF 3'UTR could interact with cytoplasmic proteins from EL-4 cells, we used a uniformly ^{32}P-labeled RNA consisting of the terminal 303 nt of the GM-CSF mRNA [Fig. 1*A*; linearization of the plasmid with *Xba* I (3'UTR) before transcription].

As shown in Fig. 1*B*, a strong band of very slow mobility is visible at the top of the gel (arrowhead, lane 1) when the binding reactions are done with poly(I) as nonspecific competitor. A second fainter band is visible further down. To demonstrate that these bands result from a sequence-specific interaction, competition experiments were performed with unlabeled transcripts corresponding to the radiolabeled probe and unrelated sequences. Lanes 2–5 show that the bands disappear as specific competitor (3'UTR) increases. Fragments containing GM-CSF 3'UTR sequences lacking the AU-rich sequences (3'UTR-*Mbo*II) do not compete (lanes 6–9), nor do unrelated RNA transcripts [CAT-A_n, CAT including a 30-nt poly(A) tail; lanes 10–13]. We have mapped a TPA-response element to a 60-nt region located 69 nt upstream from the *Mbo* II site by means of transfection studies (19). To analyze whether these sequences influence protein binding, we used a transcript containing an extensive mutation of this region as a competitor (3'UTR-mut). As shown in Fig. 2*B*, no differences were observed between

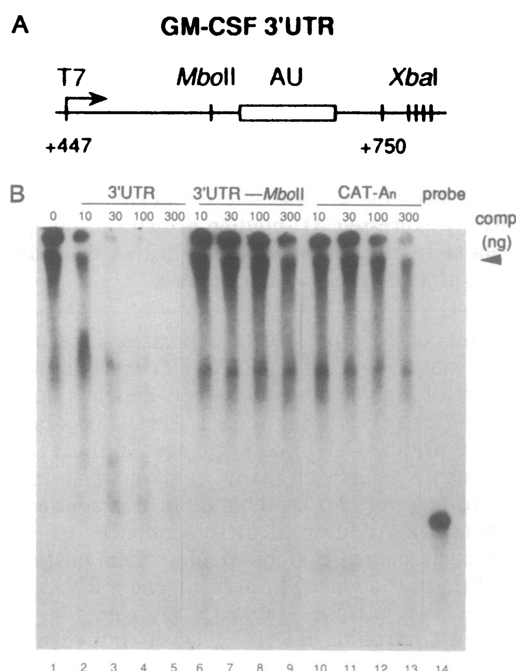

FIG. 1. Detection of specific RNA–protein complexes by band-shift analysis with poly(I) as nonspecific competitor. (*A*) Schematic drawing of the GM-CSF 3'UTR plasmid construct that was used to generate radiolabeled RNA or unlabeled competitor. The plasmid was cut with either *Xba* I or *Mbo* II to produce 3'UTR or 3'UTR-*Mbo*II RNA as indicated in each experiment. (*B*) Cytoplasmic proteins from EL-4 cells (30 μg per reaction) were mixed with a radiolabeled 310-nt GM-CSF 3'UTR mRNA (10,000 cpm per reaction) in the presence of 5 μg of poly(I) (250 μg/ml). After 20 min, reaction mixtures were digested with RNase T1 for 10 min, and RNA–protein complexes were resolved on a nondenaturing 4% polyacrylamide gel. Competitions were performed with increasing amounts (ng) of specific competitor (3'UTR, lanes 2–5), competitor lacking AU-rich sequences (3'UTR-*Mbo*II, lanes 6–9), and unrelated competitor (CAT-A_n, lanes 10–13). Shifted complexes are denoted with an arrow at the right. Lane 14 shows the mobility of the probe alone.

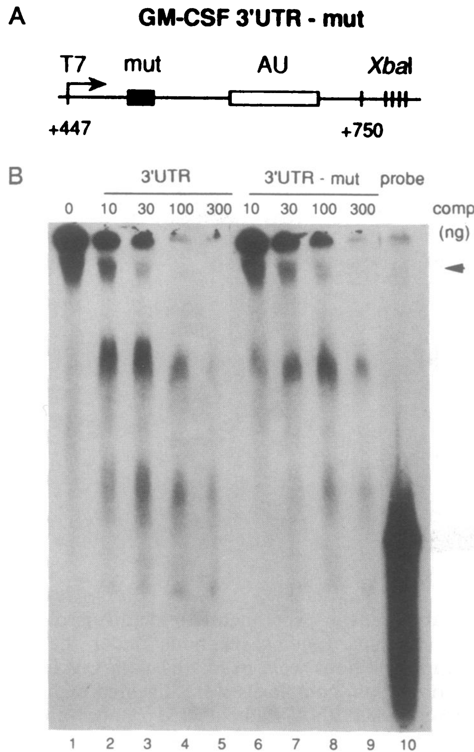

FIG. 2. Detection of specific RNA–protein complexes by band-shift analysis with mutated and nonmutated GM-CSF 3′UTR as competitors. (*A*) Schematic drawing of the GM-CSF 3′UTR plasmid construct with location of the deletion-substitution mutation (mut). (*B*) See legend to Fig. 1*B*. Competitions were performed with increasing amounts of 3′UTR (lanes 2–5) and 3′UTR-mut (lanes 6–9). Shifted complexes are denoted with an arrowhead at right. Lane 10 shows the mobility of the probe alone.

wild-type (3′UTR, lanes 2–5) and mutated competitor (3′UTR-mut, lanes 6–9).

When heparin sulfate was used as a nonspecific competitor in addition to poly(I) the mobility of the shifted bands was greater (Fig. 3). These bands corresponded to the faint bands visible in Fig. 1*B*, lanes 2–5. These complexes also reflect sequence-specific RNA–protein interactions, since they disappear in the presence of specific competitor (3′UTR, lanes 2–5) but not nonspecific competitors (3′UTR-*Mbo*II and CAT-A_n, lanes 6–13). On the bottom of this gel there are bands that increase in density with increasing specific competitor (lanes 2–5). These bands are RNase T1-resistant probe fragments 63 nt in length (see below). In Fig. 1*B* these bands were allowed to run off the gel. Thus, at least two different types of RNA–protein interactions take place: under one set of conditions a very-low-mobility complex is formed, while under a slightly different set of conditions a more rapidly migrating complex is formed.

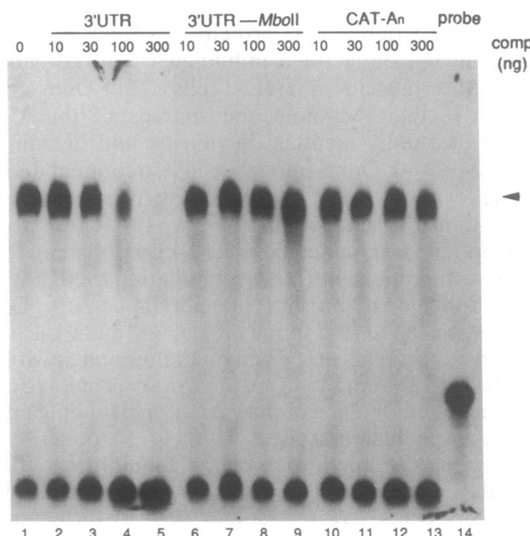

FIG. 3. Detection of specific RNA–protein complexes by band-shift analysis with poly(I) and heparin as nonspecific competitor. See legend to Fig. 1*B*. Binding reactions were performed in the presence of 5 μg of poly(I) (250 μg/ml) and 100 μg of heparin sulfate (5 mg/ml).

Localization of the Binding Site in the Two Complexes. The competition data suggest that the shifted bands result from interactions with AU-rich sequences because RNA transcripts lacking the AU-rich sequences fail to compete (Fig. 1*B* and Fig. 3, lanes 2–5). To more precisely define the binding site, RNase T1 mapping was performed on the RNA fragments in the shifted bands. RNA-shift gels were run under the poly(I) or poly(I)/heparin conditions. After a brief autoradiographic exposure shifted and free complexes were cut out of the gels and RNA was further purified by electroelution and organic extractions. Subsequent digestion with RNase T1 was performed and the products of these reactions were separated on 8% sequencing gels along with markers consisting of undigested and RNase T1-digested probe. As shown in Fig. 4*A*, lanes 1 and 3) the eluted RNA fragments from complexes formed under the poly(I) or poly(I)/heparin conditions are the same size, 63 nt, and do not change after RNase T1 digestion (lanes 2 and 4). It is clear that the protected region in each instance corresponds to the stretch of AU sequences, a region that lacks the G residues that RNase T1 recognizes (lane 6). Interpretations of these results are that the binding site subtends the entire AU-rich sequence or that these fragments are due to the absence of G residues. To distinguish these we repeated the experiment, using an endonuclease that is less sequence specific (micrococcal nuclease) to digest the formed complexes before nondenaturing gel electrophoresis and electroelution. The results (Fig. 4*B*) indicate that the sizes of the protected RNA fragments are substantially the same as when RNase T1 was used, if not somewhat larger after micrococcal nuclease treatment (lane 1). Thus, the binding site appears to include the entire AU-rich sequences of the GM-CSF 3′UTR. This discrepancy in the size of the protected fragments (compare lane 3 of Fig. 4*A* with lane 1 of Fig. 4*B*) might be due to protection of a larger fragment from micrococcal nuclease digestion.

Three Proteins Are Complexed with 3′ RNA. To determine the molecular mass of the proteins, RNA–protein complexes were labeled by UV crosslinking. Preliminary experiments explored energies from 180 to 1440 mJ/cm^2 and showed that more than 720 mJ/cm^2 was necessary for efficient crosslinking. Noncrosslinked RNA was removed with RNase T1 or RNase A. The products were resolved on 12% polyacrylamide/SDS gels. Fig. 5*A* shows an autoradiograph of a gel with samples from a binding performed under the poly(I) condition. A major band with a mass of 33 kDa and two minor bands of 39.5 and 42 kDa are seen (lane 1). All three bands compete specifically with unlabeled specific competitor (lane 2) but are unchanged with unrelated RNA (lane 3). Similarly, when reaction mixtures created under the poly(I)/heparin condition (Fig. 5*B*) were crosslinked and analyzed by SDS/PAGE, two major bands of 39.5 and 42 kDa were seen that were specifically competed with by unlabeled specific RNA (lanes 2–4) but not by nonspecific RNA (lanes 5–7). These findings indicate that at least three proteins exist that specifically bind to AU sequences. None of the bands were seen without UV light (lane 8).

Murine GM-CSF 3′UTR-Binding Proteins Are Not Regulated by TPA. We have shown that TPA raises GM-CSF

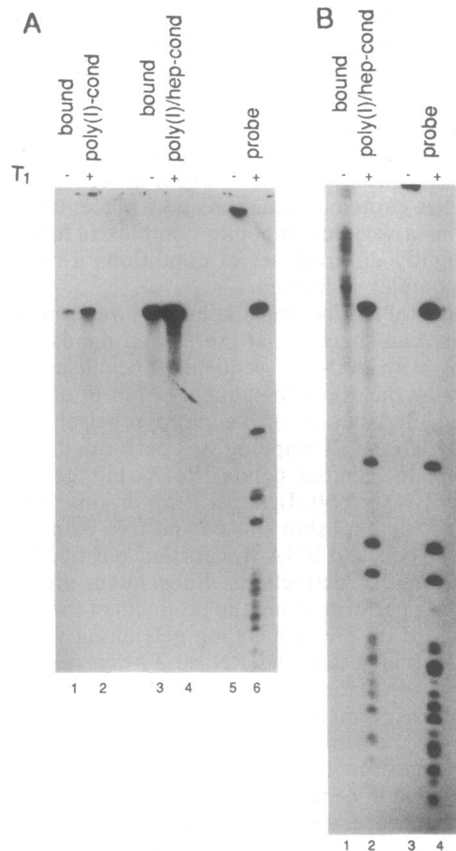

FIG. 4. Localization of the binding site in the two complexes. Binding reactions were performed as described in legends to Figs. 1 and 3. Complexes were digested with either RNase T1 (*A*) or micrococcal nuclease (*B*) prior to nondenaturating shift-gel electrophoresis. After wet autoradiography, shifted complexes and free RNA species were localized and cut out, and the RNA was electroeluted. Purified RNA was then loaded directly onto 8% polyacrylamide sequencing gels or further digested with RNase T1 prior to denaturing gel electrophoresis. (*A*) Autoradiograph of an 8% sequencing gel containing RNA isolated from RNA–protein complexes under the poly(I) and the poly(I)/heparin conditions (lanes 1 and 3) and the same RNA digested with RNase T1 (lanes 2 and 4). Lanes 5 and 6 show the mobility of probe alone and probe digested with RNase T1, respectively. (*B*) Analogous experiment from complexes established under the poly(I)/heparin condition, except that the complexes were first digested with micrococcal nuclease. Lane descriptions in *B* (lanes 1–4) correspond to those in *A* (lanes 3–6).

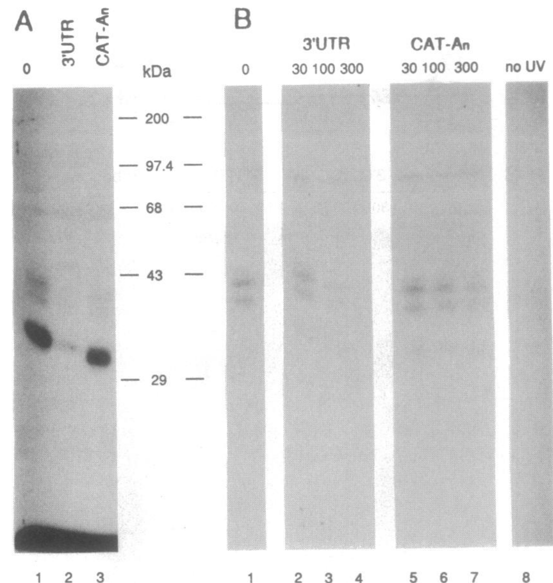

FIG. 5. Label transfer experiments to identify proteins interacting with AU regions. Binding reactions under the poly(I) and poly(I)/heparin conditions were irradiated with UV light (720 mJ/cm²). After irradiation, complexes were digested with RNase A to remove noncrosslinked RNA. Radiolabeled probes were resolved on 12% polyacrylamide/SDS gels. Competition experiments analogous to those in Figs. 1 and 3 were performed with specific (3′UTR, 300 ng) and nonspecific (CAT-A_n, 300 ng) competitor RNA. (*A*) Autoradiograph of a gel with samples established under the poly(I) condition without competitor (lane 1), with specific competitor (lane 2), or with nonspecific competitor (lane 3). (*B*) Similar experiment performed under the poly(I)/heparin condition, using three different concentrations (ng) of competitors. When UV light is omitted no complexes are detectable (lane 8).

mRNA levels in EL-4 cells by means of mRNA stabilization (17), an effect that is mediated mainly by RNA sequences located just upstream from the AU boxes (19). We therefore analyzed whether any of the proteins identified by label transfer are regulated by TPA in the EL-4 thymoma cell line and whether they are present in extracts from NIH3T3 fibroblasts (in which the GM-CSF gene does not respond to TPA) or a human fibroblast cell line (U293). As shown in Fig. 6, TPA treatment does not affect the intensity or mobility of the radiolabeled bands under the poly(I) (*A*, lanes 1 and 2) or poly(I)/heparin (*B*, lanes 1 and 2) conditions. This agrees with the gel-shift data, which also showed no changes in shift band intensity after TPA treatment (not shown). Fig. 6*A* also shows that the prominent 33-kDa band is clearly present in extracts from a variety of cell lines, including EL-4 (lanes 1 and 2), NIH3T3 (lane 3), and U293 (lane 4). The higher molecular mass 39.5- and 42-kDa proteins are present only in murine cell extracts (lanes 1–3), whereas two distinct bands of slightly lower molecular mass are present in extracts from human fibroblasts (lane 4).

DISCUSSION

We have identified three murine proteins of 33–42 kDa that bind to AU-rich sequences of the GM-CSF 3′UTR. These proteins form complexes with the GM-CSF 3′UTR that can be visualized by band-shift analyses. The three proteins are detected in the cytoplasm of murine EL-4 thymoma cells and NIH3T3 fibroblasts as well as in human U293 fibroblasts. All three proteins are also detectable in nuclear extracts (data not shown).

It is likely that the 33-kDa protein described here corresponds to the 34-kDa factor A in human T cells (16) and the human 32-kDa protein in HeLa cells (15). One striking observation is that the molecular masses of the 33-kDa proteins are virtually identical in murine and human cells (Fig. 6*A*, lanes 2–4). Our finding that TPA treatment does not change the level or size of this protein is in agreement with the observations of Vakalopoulou *et al.* (15). These investigators identified and characterized a 32-kDa polypeptide that correlates with reduced mRNA accumulation though it does not appear to have any intrinsic nucleolytic activity. On the other hand, we have not found in our extracts the lower molecular mass proteins described by Malter and co-workers (12, 13). Different methods of extraction or proteolytic artifacts may account for some of the variation in binding protein size among various laboratories.

After varying the conditions of the binding reactions at least two different types of complexes were detected by the gel-shift assay (Figs. 1*B* and 3) and this, in turn, made it possible for us to identify at least two additional AU-binding proteins in label-transfer studies. With poly(I) alone as nonspecific competitor, a very-slow-moving predominant complex was detected by gel-shift assay, and a corresponding complex set of specific binding proteins on SDS gels was also

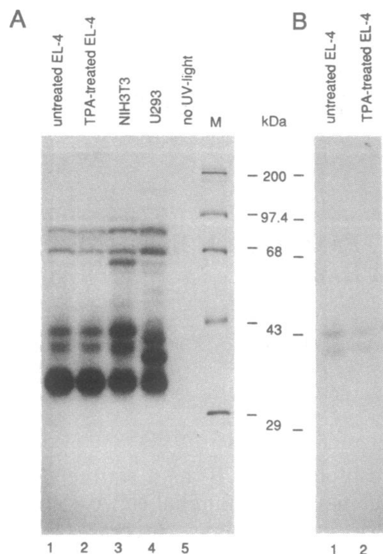

FIG. 6. Presence of binding proteins in untreated and TPA-stimulated EL-4, NIH3T3, and U293 cells. Using the experimental conditions described for Figs. 1 and 3 and the label transfer method described for Fig. 5, we set up binding reactions with extracts from untreated or TPA-treated EL-4 cells. Extracts were also made from NIH3T3 and U293 fibroblasts. Equal amounts of protein were used in each lane. (*A* and *B*) Results of experiments performed under the poly(I) and poly(I)/heparin conditions, respectively. Lane M, molecular mass markers.

detected after label transfer. The addition of heparin sulfate, as a nonspecific competitor, made the shifted complex migrate more rapidly and simplified the pattern of proteins on SDS gels. Heparin also increased the intensity of the faint band observed under the poly(I) condition (Fig. 1*B*), and this band showed essentially the same mobility as the complexes formed under the poly(I)/heparin condition (Fig. 3). The more rapid mobility of the shift band with heparin and poly(I) may be due to loss of participation of the 33-kDa protein in the complex. The fainter band that appears below the strong band in the gel shown in Fig. 1*B* may be the same protein seen under the poly(I)/heparin condition shown in Fig. 3. The sensitivity of the binding of the 33-kDa protein to heparin suggests an electrostatic component in its interaction with mRNA.

The existence of multiple proteins capable of binding to the AU-rich region has been recently proposed by others (15, 16). Our data hint at the potential complexity of regulation mediated by proteins binding to these AU-rich sequences. In contrast to other reports, in this report we have directly mapped the binding sites of the proteins in the shift complexes instead of inferring the identity of the binding site from competition or differential labeling experiments. Using two different endonucleases, we have shown that the binding site apparently includes the entire 63-nt AU-rich sequences. This could be due to protection by a single large protein (which is very unlikely) or by binding of several proteins to each of the reiterated AUUUA motifs.

The AU-rich region appears to behave independently as a binding site and does not seem to be influenced by adjacent sequences. For example, our competition experiments show that simultaneous or prior incubation with unlabeled competitors containing only upstream portions of the 3′UTR had no influence on the strength of protein binding to AU sequences (Figs. 1*B* and 3). Furthermore, competitors containing physiologically meaningful mutations of sequences upstream from the AU-rich sequences (19) did not affect binding to the AU sequences (Fig. 2*B*). These experiments do not exclude potential contributions from the coding region or 5′UTR.

The lack of regulation of these proteins in our system is not surprising in light of functional data from transient assays (19). Those results indicate that AU-rich sequences by themselves play a role in mRNA degradation but cannot alone mediate mRNA stability induced by TPA. Other portions of the 3′UTR are required for induction by TPA. These results together with those of others (4, 24, 25) imply that mRNA degradation and stabilization may proceed through separate pathways and need to be studied independently. AU-binding proteins of the type that we describe in this report, which are constitutively expressed, may function in a "default" mode, directing mRNA degradation in the absence of an alternative stimulus. In such a regulatory scheme proteins binding to other regions of the mRNA or binding directly to the AU-binding proteins would be required to block mRNA degradation. We have been unable to find proteins that can bind to regions just upstream of the AU-rich sequences. Another possibility is that AU-binding proteins are directly influenced by interactions with mRNA sequences upstream of the AU-rich sequences. Functional TPA response elements of the type that we have detected in a 60-nt region in the upstream part of the GM-CSF 3′UTR (19) would in this case interact with and directly activate proteins constitutively bound to AU-rich sequences.

One reasonable strategy for characterizing this system further is to purify the constitutively expressed AU-binding factors, with the idea that they cooperate with other inducible proteins that modulate their activity through a protein–protein interaction.

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