Identification of Sequences within the Murine Granulocyte-Macrophage Colony-stimulating Factor mRNA 3'-Untranslated Region That Mediate mRNA Stabilization Induced by Mitogen Treatment of EL-4 Thymoma Cells

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Phorbol esters (TPA) and concanavalin A (ConA) are known to induce granulocyte-macrophage colony-stimulating factor (GM-CSF) production in murine thymoma EL-4 cells by mRNA stabilization. The role of the 3'-untranslated region (3'-UTR) in GM-CSF mRNA stabilization induced by TPA and ConA in EL-4 cells was examined by transfection studies using chloramphenicol acetyltransferase (CAT) constructions. The GM-CSF 3'-UTR contains a 63-nucleotide region at its 3' end with repeating ATTTA motifs which is responsible for mRNA degradation in a variety of cell types (Shaw, G., and Kamen, R. (1986) Cell 46, 659–666). We produced constructs containing most of the GM-CSF 3'-UTR (303 nucleotides, pRSV-CATgm) or the 3'-terminal AT-rich region (116 nucleotides, pRSV-CATau) and measured CAT enzyme activity and CAT mRNA after transient transfection into EL-4 and NIH 3T3 cells. Low levels of CAT activity were seen in both cells with either plasmid compared with levels of CAT activity obtained with pRSV-CAT. TPA treatment caused an ~10-fold increase in CAT activity and mRNA in EL-4 cells transfected with pRSV-CATgm. No increases were seen in EL-4 cells transfected with pRSV-CATau or pRSV-CAT. No response to TPA was detected in transfected NIH 3T3 cells, indicating that the response to TPA is relatively cell-specific. There was no increase in CAT activity after ConA treatment in EL-4 or NIH 3T3 cells transfected with any of the constructs suggesting that the GM-CSF 3'-UTR lacks elements that can respond alone to ConA. Nuclear run-on and actinomycin D chase experiments in EL-4 cells showed that TPA induces CAT activity via mRNA stabilization. By linker-substitution mutagenesis we show that TPA inducibility depends on a 60-nucleotide region of the 3'-UTR whose 5' end is located 160 nucleotides upstream of the 5' end of the AU-rich region.

Granulocyte-macrophage colony-stimulating factor (GM-CSF), a member of the family of hematopoietic growth factors, is produced by a variety of cells including T and B cells (1, 2), monocytes (3), endothelial cells (4), and fibroblasts (5). A number of laboratories including ours have shown that antigens, lectins, and phorbol esters (TPA) induce GM-CSF mRNA expression in T cells along with other cytokines such as interleukins 2 and 3 and interferon-γ (1, 6, 7). In the case of the EL-4 murine thymoma cell line earlier studies show that TPA and concanavalin A (ConA) induction of GM-CSF mRNA is controlled primarily at the posttranscriptional level by mRNA stabilization (8). The response of EL-4 cells to ConA differs from its response to TPA, however, in that much lower levels of GM-CSF mRNA are produced and the prolongation of mRNA half-life is much less. Gene regulation by modulation of mRNA stability in a number of cell lines also has been demonstrated by other groups for GM-CSF (9) as well as for interleukin 3 and c-fos (10–12).

It is clear that adenosine-uridine (AU)-rich sequences in the 3'-untranslated region (UTR) of the mRNA of various genes play an important role in mediating rapid RNA degradation (13). The murine GM-CSF mRNA contains a 63-nucleotide region at its 3' end with eight copies of an AUUA motif. The human GM-CSF mRNA has a very similar structure. If the AU-rich region of the human GM-CSF mRNA is substituted for the 3'-UTR of a normally stable mRNA such as β-globin, the β-globin mRNA becomes unstable (13). There is very little information regarding possible mechanisms of mRNA stabilization. It is also unclear how diverse stimuli, such as ConA and TPA, cause differing degrees of mRNA stabilization. We have explored this problem using the EL-4 cell as a model system. We have transfected EL-4 cells and murine fibroblast NIH 3T3 cells (which do not produce GM-CSF) with plasmids containing different parts of the GM-CSF 3'-UTR linked to a chloramphenicol acetyltransferase (CAT) reporter gene and treated the cells with ConA and TPA. We confirmed that mRNA instability in untreated cells is mediated by AU-rich elements, and we showed that this phenomenon is not specific to the murine T cell. TPA causes mRNA stabilization in EL-4 cells, but not in NIH 3T3 cells, transfected with plasmids containing most of the 3'-UTR. No response to TPA was seen in EL-4 cells transfected with

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1 The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; CAT, chloramphenicol acetyltransferase; 3'-UTR, 3'-untranslated region; ConA, concanavalin A; ActD, actinomycin D; DMEM, Dulbecco's modified Eagle's medium; bp, base pairs; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid.

2 Y. Iwai, M. Bickel, R. Cohen, and D. H. Pluznik, submitted for publication.
plasmids containing only AU-rich elements. No response to ConA was seen in any of the transfections. By deletion-substitution mutagenesis of the 3'-UTR we showed that mRNA stabilization induced by TPA depends on a 60-base region of the GM-CSF mRNA located ~160 bases upstream of the AU-rich region.

MATERIALS AND METHODS

Reagents—The tumor-promoting phorbol ester, 12-O-tetradecanoylphorbolester (TPA), was purchased from Consolidated Midland Co. (Brewater, NY). Actinomycin D (ActD), ConA, G-418 (a neomycin analogue), chloramphenicol, and tetracyclin were purchased from Sigma. TPA was dissolved in dimethyl sulfoxide at a concentration of 20 mM and diluted in medium before addition to the cells (the final concentration of dimethyl sulfoxide is less than 0.001%). Fetal calf serum was purchased from Hyclone (Logan, UT). RPMI 1640 medium, Dulbecco's modified Eagle medium (DMEM), penicillin, and streptomycin were obtained from GIBCO.

cDNA and cRNA Probes—The plasmid containing the GM-CSF cDNA was a gift from Drs. N.M. Gough and A.W. Burgess (Ludwig Institute for Cancer Research, Melbourne, Australia) (14). The glyceraldehyde-3-phosphate dehydrogenase cDNA has been described previously (15). For RNA protection analysis of CAT mRNA, the coding region of the CAT gene was subcloned into the ribovector vectors (Promega, Madison, WI). The vector was digested with NcoI and transcribed with SP6 RNA polymerase to give a 267-nucleotide probe.

pRSV-CAT Plasmid Constructions and Deletion-substitution Mutagenesis—To prepare pRSV-CATgpm and pRSV-CATau we modified pRSV-CAT (16) by digestion with Hpal and BamHI to remove sequencing vector sequences. To ensure that the resulting plasmids contained only sequences upstream of the AU sequences within the 3'-UTR of GM-CSF, pRSV-CAT was digested with XhoI and gel purified. Extraction was performed by two rounds of cesium chloride gradient centrifugation or polyethylene glycol precipitation followed by p7525 column purification (5'-3', West Chester, PA).

DNA Transfection and Preparation of Cell Extracts for CAT Assays—The murine thymoma cell line EL-4 (19) and murine fibroblast cell line NIH 3T3 (20) were maintained in RPMI 1640 medium and DMEM, respectively, supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Ten μg of plasmid were transfected into 106 EL-4 and NIH 3T3 cells by electroporation (530 microfarads, 300 V) (21) using the Cell-Parator System (Bethesda Research Laboratories, Bethesda, MD). After electroporation, cells were cultured at 37 °C for 12–24 h. Transfected cells were then divided into three portions, one of which was left untreated while the other two were stimulated with TPA (20 nm) or ConA (5 μg/ml) for 24 h. Co-transfections with pRSV-luciferase were performed to control for transfection efficiency (22). Cells were collected and extracts were prepared by three cycles of freeze-thawing in 0.1 M Tris-HCl, pH 7.8. Each experiment was performed a minimum of three times with at least two separate batches of purified plasmid.

To generate permanently transfected cell lines containing pRSV-CAT, cells were transfected with pRSV-CATgpm and selection with G418 (600 μg/ml) for 2 weeks. Resistant clones were selected with G418 (600 μg/ml) over a 2-week period. They were then maintained in the absence of G-418 and have been found to be stable over a period of at least 3 months.

CAT Assay—CAT assays were performed using the scintillation fluid diffusion method described previously (24). Cell extracts (20 μg of protein) were mixed with 250 μl of reaction buffer (25 mM Tris-HCl, pH 7.8, 250 μM chloramphenicol, 0.25 μCi of 3H-labeled acetyl coenzyme A (200 mCi/mmol, Du Pont-New England Nuclear)) and then overlaid with 5 ml of a water-immiscible scintillation fluid and counted on a Beckman liquid scintillation counter. Thereafter, the vials were counted for 1 min by liquid scintillation.

RNA Protection Analysis for CAT mRNA—Twelve hours after transfection with pRSV-CAT or pRSV-CATgpm, cells were stimulated for an additional 6 h with TPA (20 nm) or left untreated. Total RNA was then prepared by a single-step method using guanidine thiocyanate (25). Fifty μg of total RNA were hybridized overnight with 2×106 cpm labeled CAT cRNA in a buffer containing 80% formamide, 40 mM Pipes, pH 6.7, 0.4 mM NaCl, and 1 mM EDTA at 40 °C. RNA hybrids were then digested with 80 units of ribonuclease T1 per sample, extracted with phenol/chloroform, and ethanol precipitated. Protected fragments were separated by electrophoresis of 8 M urea, 15% polyacrylamide sequencing gels which were dried and exposed for at least 1 week to Kodak XAR film at ~70 °C. These RNA samples were also analyzed by blot hybridization with glyceraldehyde-3-phosphate dehydrogenase to establish that analyzed RNA amounts were equivalent to separate 30 bp fragments.

Transcriptional Analysis—Nuclear run-on transcription assays were performed according to the method of Groudine et al. (26). Nuclei were prepared by lysing 106 cells in a buffer (10 mM Tris-HCl, pH 7.4, 3 mM CaCl2, 2 mM MgCl2) containing 0.2% Nonidet P-40. After pulse labeling with 3HUTP, radiolabeled RNA was prepared by sequential DNAase I digestion, organic extractions, ethanol precipitation, and trichloroacetic acid precipitation. Plasmids corresponding to the various cDNAs (5 μg/slot) which had been blotted onto Nytran membranes using a slot blot apparatus (Schleicher & Schuell) were hybridized for 48 h at 65 °C with the pulse-labeled RNA. After washes at high stringency, the membranes were exposed to x-ray film at ~70 °C for 5 days.

Actinomycin D Dose Experiment and Semiquantitative PCR Measurements of CAT mRNA—EL-4 cells permanently transfected with pRSV-CATgpm and pRSV-CATau were treated with 0–200 nM TPA (21). The cells were collected 4 h following ActD (5 μg/ml). Aliquots of cells were harvested at intervals of 0, 60, and 120 min. Total RNA was prepared and analyzed by a semiquantitative competitive PCR method (27, 28). Antisense primers (5'TTCGGAGACATGGGAAAGC and sense (CGAATATTGCGCGTTTACG) primers for CAT are complementary to positions 1492–1508 of the GM-CSF sequence). PCR reactions were ligated together overnight and then digested with BamHI and NcoI. Products of the correct length were gel purified and ligated to the pRSV-CATgpm backbone which had been prepared by digestion with BamHI and NcoI and gel purification. Nucleotide sequencing of all PCR-generated regions and extensive restriction enzyme analysis were performed to verify plasmid structure. Plasmids were prepared by two rounds of cesium chloride gradient centrifugation or polyethylene glycol precipitation followed by p7525 column purification (5'-3', West Chester, PA).

Verification of the Gene Splicing by Overlap Extension Method (18)—To generate pRSV-CATgpm and pRSV-CATau we modified pRSV-CAT (16) by digestion with Hpal and BamHI to remove sequences containing the SV40 polyadensylation signal. To ensure that the resulting plasmids contained only sequences upstream of the AU sequences within the 3'-UTR of GM-CSF, pRSV-CAT was digested with XhoI and gel purified. Extraction was performed by two rounds of cesium chloride gradient centrifugation or polyethylene glycol precipitation followed by p7525 column purification (5'-3', West Chester, PA).
RESULTS

Transfection Analysis of Hybrid RSV-CAT/GM-CSF Plasmid Constructs—Previous studies have shown that TPA and ConA induce GM-CSF gene expression in mouse EL-4 thymoma cells through increased stabilization of mRNA (8) which is intrinsically unstable owing to AU-rich sequences in the 3'-UTR (13). Further, we have shown that TPA causes a greater degree of GM-CSF mRNA stabilization than ConA and that this difference appears to account largely for the differences in GM-CSF production induced by the two stimuli. To localize the sequences responsible for mRNA stabilization and to explore the differences between TPA and ConA, we developed a transient transfection system in EL-4 cells. We first constructed a set of plasmids containing different portions of the GM-CSF cDNA or mRNA. These constructs were then mixed with serial dilutions of linearized (HindIII) GM-CSF cDNA and give rise to a product of 586-606 and 368-388, respectively, of the murine glyceraldehyde-3-phosphate dehydrogenase cDNA and give rise to a 238-bp product. As competitor we used a mutated pRSV-CAT plasmid containing a BamHI linker in the NcoI site. When this competitor plasmid is amplified with the CAT primers a 262-bp product is made which can be cleaved into 204- and 158-bp fragments by BamHI. The CAT detection primer hybridizes only to the 104-bp fragment, thus allowing the products derived from the mRNA and competitor plasmid to be distinguished easily. Ten µg of total RNA was reverse transcribed by 200 units of MMLV RT (BRL) at 37 °C for 1 h, using 2 µg each of antisense primers for CAT and glyceraldehyde-3-phosphate dehydrogenase, in a final volume of 50 µl. Reverse transcription products were then mixed with serial dilutions of linearized (HindIII) competitor plasmid spanning a range of concentrations from 0.003 to 100 pg of DNA. These mixtures were then amplified for 40 cycles (94 °C for 1.5 min, 55 °C for 1.5 min, and 72 °C for 4 min). Aliquots of amplified DNA were then digested with 10 units of HpaI alone (pRSV-CAT) or digestion with HpaI alone (pRSV-CATm). The percentage of CAT activity is expressed as the amount of CAT activity after stimulation with the test sample compared to the concentration of RNA in the test sample.

In control for RNA amounts, the same reverse transcribed samples were also amplified for 25 cycles using glyceraldehyde-3-phosphate dehydrogenase sense and antisense primers. Aliquots of amplified DNA were analyzed by gel electrophoresis, blotting, and hybridization to a glyceraldehyde-3-phosphate dehydrogenase detection oligonucleotide (GATG, 1108-1128) which had been "P-end-labeled to a specific activity of 5-10 × 10^6 cpm/µg. After washing at 55 °C, 0.1 x SSC, 0.1% sodium dodecyl sulfate, blots were exposed to Kodak XAR film at -70 °C. The concentration of competitor plasmid which gives rise to a band equivalent in intensity to the band produced by the test sample corresponds to the concentration of mRNA in the test sample. To control for RNA amounts, the same reverse transcribed samples were also amplified for 25 cycles using glyceraldehyde-3-phosphate dehydrogenase sense and antisense primers. Aliquots of amplified DNA were analyzed by gel electrophoresis, blotting, and hybridization to a glyceraldehyde-3-phosphate dehydrogenase detection oligonucleotide (GTATGCGTGAACACGAC, 473-493).

TABLE 1
Effects of TPA and ConA on inducibility of CAT activity in EL-4 transfected cells

|               | Unstimulated | TPA | ConA | TPA- ConA |
|---------------|--------------|-----|------|-----------|
| CAT activity  | 100.0        | 107.0±5.4 | 111.0±3.3 | 1.1±1.1   |
| (n = 7)       |              |       |      |           |
| pRSV-CAT-Tg   | 1.5±0.4      | 14.0±2.7 | 13.0±0.1 | 9.4±0.9  |
| (n = 7)       |              |       |      |           |
| pRSV-CAT-Tau  | 12.0±5.1     | 10.0±1.3 | 11.0±1.9 | 0.9±0.9  |
| (n = 5)       |              |       |      |           |
| pRSV-CAT-Tava | 97.0±5.2     | 101.0±7.6 | 116.0±15.0 | 1.0±1.2  |
| (n = 3)       |              |       |      |           |

pRSV-CAT-Tau also gives rise to low levels of enzyme activity (12% of wild type). Interestingly, CAT enzyme levels seen with this plasmid are consistently about 10 times higher than those seen with pRSV-CAT-Tg (12 versus 1.5%). Transfection...
with RSV-CATava gives wild type enzyme levels (97%). Transfection efficiency was controlled in each instance by co-transfection with RSV-luciferase (22), and no significant differences in luciferase activities were found among the different transfection experiments (data not shown). Thus, the observed differences in CAT activity levels are due to the inclusion of the GM-CSF 3'-UTR sequences.

The GM-CSF 3'-UTR Confers Responsiveness to TPA but Not to ConA—We next examined the effects of TPA and ConA which are known inducers of GM-CSF. We wanted to determine if cells transfected with plasmids containing portions of the GM-CSF 3'-UTR could respond to either stimulus. For each plasmid, cells were transfected, grown for 12-24 h, and then divided into three portions. One of these was left untreated while the other two were treated with ConA or TPA. As shown in Table I (under “CAT Activity, TPA”), TPA treatment of cells transfected with pRSV-CATava causes a small increase in CAT activity (107 versus 100%). On the other hand, treatment of cells transfected with pRSV-CATgm leads to an average 9.4-fold increase in CAT activity (14 versus 1.5%). TPA treatment of cells transfected with pRSV-CATau does not cause an increase in CAT activity (10 versus 12%). Finally, TPA treatment of cells transfected with pRSV-CATava has no effect (101 versus 97%). These data indicate that a TPA-responsive element is present in the GM-CSF 3'-UTR and that this element must involve sequences in addition to the AU boxes.

We then examined the effect of ConA on CAT activity. In contrast to the TPA experiments, ConA treatment of transfected cells did not change CAT activity (Table I, “CAT Activity, ConA”). Thus, the GM-CSF 3'-UTR can respond to TPA but not to ConA. The lack of response to ConA is not an artifact of the transfection procedure. When we analyzed these ConA-treated transfected cells for endogenous GM-CSF mRNA by Northern blotting we detected message in amounts comparable to those seen in untransfected ConA-treated cells (data not shown).

The AU Box Effect Is Not Cell-specific—To test whether the TPA response is cell-specific we transfected these same constructs into NIH 3T3 fibroblast cells. NIH 3T3 cells do not produce GM-CSF biological activity or mRNA when treated with ConA or TPA. As shown in Table II, pRSV-CATgm and pRSV-CATau transfections resulted in very low levels of CAT activity just as was seen in EL-4 cells. Hence, the ability to degrade mRNA containing AU sequences is shared by these two cell types. Stimulation of NIH 3T3 cells with either TPA or ConA had no effect on CAT activity levels. This lack of effect is not due to the NIH 3T3 cell's inability to respond to TPA because the cells do synthesize increased amounts of c-fos mRNA when treated with TPA. Thus, the response of the GM-CSF 3'-UTR to TPA appears to be cell-specific.

It is noteworthy that in NIH 3T3 cells, as was the case in EL-4 cells, the complete GM-CSF 3'-UTR appears to cause lower levels of CAT activity than a 3'-UTR containing only AU elements (compare pRSV-CATgm to pRSV-CATau, Table II). These data further reinforce the idea that sequences beyond the AU boxes may contribute to mRNA degradation.

Regulation of CAT Activity by TPA Occurs at the Level of mRNA and Involves mRNA Stabilization—It is likely that the increases in CAT activity are due to increases in CAT mRNA, but it is formally possible that regulation is occurring via increased translation or enhanced protein stability. To address these possibilities we analyzed CAT mRNA by RNase protection. Transfection conditions for EL-4 cells were optimized to obtain high levels of CAT activity and thus detectable mRNA levels. EL-4 cells transfected with pRSV-CAT or pRSV-CATgm were grown for 12 h and treated with TPA for 6 additional hours, followed by isolation of total RNA. As shown in Fig. 2 (lanes 1 and 2), TPA treatment of pRSV-CAT-transfected cells caused only a slight increase in CAT mRNA accumulation compared to the level in untreated cells. This small increase in mRNA parallels the CAT enzyme activity increase in Table I. The amount of CAT mRNA in cells transfected with pRSV-CATgm is barely detectable (lane 3). TPA causes a marked increase in CAT mRNA accumulation in pRSV-CATgm-transfected cells (lane 4). The increase in mRNA is comparable to the increase in CAT enzyme activity (approximately 10-fold by densitometry; compare with CAT result in Table I). Thus, the mRNA data indicate that changes in CAT activity are regulated by alterations in

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**TABLE II**

**Effects of TPA and ConA on inducibility of CAT activity in transfected NIH 3T3 cells**

See Table I for calculations. Mean values with wild type pRSV-CAT and mock transfected cells are 14,872 ± 5,151 and 883 ± 94 cpm, respectively.

| Plasmid       | TPA | ConA |
|---------------|-----|------|
| CAT activity  |     |      |
| Unstimulated  | 100.0 | 98.0 ± 3.8 |
| (n = 4)       | 104.0 ± 4.9 | 1.0 ± 1.0 |
| pRSV-CATgm    | 2.8 ± 0.8 | 1.8 ± 0.6 |
| (n = 4)       | 3.2 ± 0.5 | 0.6 ± 0.6 |
| pRSV-CATau    | 29.5 ± 2.2 | 38.0 ± 0.8 |
| (n = 2)       | ND*  | 1.3 ± 1.3 |

*ND, not done.

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**Fig. 2. RNase protection and Northern analysis of mRNA from transfected cells.** A. RNase protection analysis for CAT mRNA. Twelve hours after transfection with RSV-CAT or RSV-CATgm, cells were stimulated for an additional 6 h with TPA (20 nM) or left untreated. Total RNA was then prepared. Equal aliquots (50 μg) of RNA were analyzed using a 267-bp CAT antisense riboprobe labeled with [32P]UTP. A 72-h autoradiographic exposure is shown. B. Northern analysis for glyceraldehyde-3-phosphate dehydrogenase mRNA. Ten μg of RNA were resolved by electrophoresis on 1% agarose gels and hybridized to the glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

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*Y. Iwai, unpublished data.

*A. Larner, personal communication.*
the mRNA level rather than changes in mRNA translation or protein stability.

To determine the mechanism for the increase in CAT mRNA in pRSV-CATgm-transfected EL-4 cells we produced EL-4 cell lines permanently transfected with pRSV-CAT (EL-4cat) and pRSV-CATgm (EL-4gm). We first validated their responses to TPA and ConA and showed that their responses are identical to those seen in the transient assays (Table III). Very low levels of CAT activity are seen in cells transfected with pRSV-CATgm, and TPA treatment causes a 9.3-fold induction of CAT activity (Table III, “CAT Activity, TPA”) whereas ConA has no effect (Table III, “Induction, ConA”). TPA causes a slight increase in CAT activity in cells transfected with pRSV-CAT.

We used the permanent cell lines to study CAT gene transcription. After 5 h of incubation with TPA, nuclei were prepared and pulse-labeled in vitro with [*P]UTP. Purified radiolabeled RNA was hybridized to cDNA clones immobilized on Nylon membranes. As shown in Fig. 3, there is no significant difference in CAT mRNA transcription levels when untreated and TPA-treated cells are compared. The slots corresponding to glyceraldehyde-3-phosphate dehydrogenase and genomic DNA show that equal amounts of radiolabeled RNA are being compared. The slot corresponding to the c-myc cDNA shows that cells are responding to TPA with a decrease the amount of c-myc transcription after 5 h of treatment, which is a consistent observation in TPA-treated EL-4 cells. Each lane also shows some hybridization to pUC8 DNA. This particular plasmid usually is employed as a background control, but in this experiment a signal is expected because the permanent cell lines contain sequences derived from pUC8. In summary, the data indicate that TPA does not have a significant effect on CAT gene transcription in EL-4cat or EL-4gm.

Because TPA did not cause a change in gene transcription we next examined whether changes in CAT mRNA half-life were occurring. We used a competitive PCR method (see “Materials and Methods”) to measure reliably CAT mRNA, because it was generally quite difficult to detect CAT mRNA in untreated EL-4gm cells using alternative methods such as RNase protection or Northern analysis (see, for example, Fig. 2, lane 3). We treated EL-4gm cells with TPA (20 nM) for 6 h, added ActD to block further transcription (5 µg/ml), and harvested cells at intervals of 1 h and prepared total RNA. Aliquots (1 µg) of total RNA from each of the time points were reverse transcribed and mixed with serial dilutions of a mutated competitor plasmid (see “Materials and Methods”). The amount of CAT mRNA at each time point therefore was measured in a separate set of PCR reactions. The results are shown in Fig. 4. With increasing exposure to ActD the amounts of CAT mRNA decrease. The rate of decrease is clearly less in cells treated with TPA. During the experiment we estimate that the amount of CAT mRNA shifts by two to three lanes (going from approximately 2 pg at 0 h to 0.1 pg at

**TABLE III**

Effects of TPA and ConA on inducibility of CAT activity in permanently transfected EL-4 cell lines

See footnote of Table I for calculations. Mean values with unstimulated EL-4cat cells and with mock transfected cells are 44,166 ± 8,076 and 637 ± 57 cpm, respectively.

| CAT activity | Unstimulated | TPA | ConA | TPA | ConA |
|--------------|--------------|-----|------|-----|------|
| % old        | -fold        |-----|------|-----|------|
| EL-4cat (n = 5) | 100.0 | 110.0 ± 5.4 | 94.6 ± 7.9 | 1.1 | 0.9  |
| EL-4gm (n = 5) | 0.9 ± 0.3 | 8.3 ± 3.1 | 0.7 ± 0.2 | 9.3 | 0.8  |

**Fig. 3.** Nuclear transcriptional analysis of the permanently transfected cell lines, EL-4cat and EL-4gm. Nuclei were isolated from the permanently transfected cell lines, EL-4cat and EL-4gm treated with or without TPA (20 nM) for 5 h and then pulse-labeled with [*P]UTP. Equal amounts of radiolabeled RNA were hybridized with a variety of cDNAs including CAT which had been immobilized on Nytran membranes.

**Fig. 4.** Measurement of CAT mRNA half-life. A, semiquantitative PCR analysis for CAT mRNA in EL-4gm. EL-4gm cells were left untreated or treated with TPA (20 nM) for 6 h. ActD (5 µg/ml) was then added and cells harvested at the indicated intervals. Total RNA was prepared and 1-µg aliquots reverse transcribed with CAT and glyceraldehyde-3-phosphate dehydrogenase antisense primers. Equal aliquots of reverse transcription products (unknown) were then mixed with serial dilutions of a mutated cDNA (known) and amplified by PCR (40 cycles) in the presence of common primers. Products derived from mRNA (unknown) and from mutated cDNA (known) are resolved on the basis of size by digestion with BamHI, agarose gel electrophoresis and blot hybridization. mRNA concentrations were estimated by finding the concentration of mutated cDNA which gives rise to a band equivalent in intensity to that derived from mRNA. B, PCR analysis for glyceraldehyde-3-phosphate dehydrogenase mRNA. The same reversed transcribed samples were also amplified in the presence of glyceraldehyde-3-phosphate dehydrogenase primers by PCR (25 cycles), then resolved by electrophoresis and hybridized to a glyceraldehyde-3-phosphate dehydrogenase detection oligonucleotide.
2 h) in untreated EL-4 gm cells, whereas it shifts only one lane (10–3 pg) in treated cells. Using these data we estimate that the half-life of CAT mRNA in untreated cells is about 30 min, whereas in TPA-treated cells it is about 70 min. Together with the RNase protection and nuclear run-on transcription experiments, these data indicate that TPA induces the accumulation of CAT mRNA in EL-4 through RNA stabilization, rather than through effects on gene transcription.

A TPA Response Element Maps to a 60-bp Region within the 3'-UTR, Whose 5' End Is Located ~160 Bases Upstream of the 5' End of the AU Boxes—To determine more precisely which sequences are responsible for the TPA response we produced five 30-bp deletion-substitution mutations of the GM-CSF 3' UTR within the pRSV-CATgm plasmid (Fig. 1C) and transfected these constructs into EL-4 cells. These mutations extend from nucleotides 474–623 of the mRNA up to the region with the AU-rich sequences. They therefore cover all but 27 nucleotides of the upstream portion of the 3'-UTR contained in pRSV-CATgm (see Fig. 1). It is also important to emphasize that by using a deletion-substitution strategy we ensure that mRNA lengths produced from each of the constructs will be identical to each other and to the parental mRNA from pRSV-CATgm. As shown in Table IV, CAT enzyme activity in untreated EL-4 cells transfected with each mutation was at a very low level similar to that in untreated cells transfected with pRSV-CATgm. TPA treatment induced a 6–11-fold increase of CAT activity in cells transfected with pRSV-CAT mutations 3, 4, and 5. However, little or no increase in CAT activity was seen in cells transfected with pRSV-CATgm mutations 1 and 2. The same result was obtained with permanently transfected EL-4 cell lines (data not shown). These results indicate that the region defined by mutations 1 and 2 (474 to 533) can account completely for TPA inducibility in our transfection experiments.

**DISCUSSION**

We studied EL-4 cells as a model system for the examination of the molecular basis of GM-CSF mRNA stabilization induced by TPA and ConA. We also have begun to dissect elements within the GM-CSF mRNA that respond to different stimuli such as TPA. In the studies presented here we saw a marked decrease in CAT activity levels in both EL-4 and NIH 3T3 cells when we compared cells transfected with RSV-CATgm to those transfected with RSV-CAT. The most likely explanation of this difference was a decrease in mRNA stability caused by the insertion of the complete GM-CSF 3'-UTR into the RSV-CAT vector. The semiquantitative PCR/RNA folding (30) of the entire muGM-CSF mRNA or the 3'-UTR (wild type and mutations) does not reveal any notably stable stem-and-loop structures of the type that have been seen in the ferritin and transferrin mRNA iron response elements. Computer-assisted analysis of the ferritin mRNAs containing AU clusters (31) indicates that the mRNA destabilization linked to AU clusters is large and that this effect cannot be completely abolished by AU-rich RNA folding (30) of the entire mGM-CSF mRNA or the 3'-UTR (wild type and mutations) does not reveal any notably stable stem-and-loop structures of the type that have been seen in the ferritin and transferrin mRNA iron response elements. However, in unstimulated EL-4 cells, this result is consistent with the observations of Shaw and Kamen (13) who showed that the GM-CSF AU boxes inserted into the 3'-UTR of a normally stable rabbit β-globin mRNA destabilize the resulting mRNA. It was also not surprising that the pRSV-CATau construct gave rise to very low levels of CAT activity because mRNA produced from this plasmid must also contain AU sequences. Although the major influence on mRNA instability appears to be the AU boxes, it was interesting to note in our experiments that elements upstream from the AU boxes may contribute somewhat to decreased mRNA stability. As shown in Tables I and II, for example, CAT levels seen with pRSV-CATgm were always lower than the levels seen with pRSV-CATau. These differences cannot be explained by variable transfection efficiencies because the pRSV-luciferase transfection controls never varied by more than 2-fold (data not shown). Further, the differences have been seen in at least five consecutive experiments with three separate batches of plasmid DNA.

In the NIH 3T3 cell lines, we detected low levels of CAT activity in cells transfected with pRSV-CATgm and pRSV-CATau. These levels were as low as those levels seen in comparable transfections of EL-4 cells. These results indicate that the mechanism of RNA degradation linked to AU boxes is probably not cell-specific. Perhaps message degradation mediated by AU boxes is an universal "default" mechanism, and cell-specific or inducible proteins are required to override it.

What is most striking in these experiments is that the only construct that responded in a significant manner to TPA was pRSV-CATgm which contains almost the entire GM-CSF 3'-UTR. This result indicates that at least one TPA response element is located in the 3'-UTR. The observation that cells transfected with pRSV-CATau did not respond to TPA indicates that mRNA stabilization by TPA must be more complicated than a simple reversal of the effect conferred by the AU boxes. If that had been the case cells transfected with pRSV-CATau should have been able to respond to TPA. mRNA stabilization after TPA treatment must therefore involve a complex interplay of stabilizing and destabilizing elements. Analogous observations have been made showing that GM-CSF AU elements alone cannot respond to interleukin 1 in fibroblasts (29).

Our data from a mutation analysis of the 3'-UTR indicate that a 60-bp region (474 to 533) whose 5' end is located ~160 bp upstream from the 5' end of the AU-rich region, contains at least one of the elements required for mRNA stabilization induced by TPA (Fig. 1C and Table IV). It is formally possible that the 27 bp of the 3'-UTR in pRSV-CATgm that we have not mutagenized may also contribute to the TPA response, but we think this is unlikely since mutations 1 and 2 completely ablate TPA inducibility. The region we have defined is large and efforts to fine map it with five additional 12-bp deletion-substitution mutations across the region defined by mutations 1 and 2 have not succeeded. These smaller mutations are all induced 6–10-fold by TPA similar to pRSV-CATgm. Data of this type suggest that the TPA response element(s) may prove to be quite large and complex, perhaps involving other portions of the mRNA. Computer-assisted RNA folding (30) of the entire muGM-CSF mRNA or the 3'-UTR (wild type and mutations) does not reveal any notably stable stem-and-loop structures of the type that have been seen in the ferritin and transferrin mRNA iron response elements.

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TABLE IV

| CAT activity | Induction | TPA |
|--------------|-----------|-----|
| Unstimulated | %         | -fold |
| pRSV-CAT     | 100.0     | 1.2 |
| pRSV-CATgm   | 1.1 ± 0.2 | 10.5 ± 3.4 | 9.4 |
| pRSV-CATgm mut 1* | 0.8 ± 0.3 | 1.0 ± 0.3 | 1.2 |
| pRSV-CATgm mut 2 | 1.1 ± 0.5 | 1.6 ± 0.7 | 1.4 |
| pRSV-CATgm mut 3 | 0.9 ± 0.2 | 10.3 ± 3.6 | 11.4 |
| pRSV-CATgm mut 4 | 1.2 ± 0.3 | 11.9 ± 3.6 | 9.4 |
| pRSV-CATgm mut 5 | 1.1 ± 0.3 | 7.6 ± 3.8 | 6.9 |

* mut = mutation.

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5. Y. Iwai and R. B. Cohen, unpublished observations.
elements, for example (31). This includes the region defined by mutations 1 and 2. The lack of TPA induction seen in cells transfected with pRSV-CATgm mutations 1 and 2 is due to low transfection efficiency, because CAT activity levels in untreated cells transfected with these constructs were comparable to those seen in untreated cells transfected with pRSV-CATgm mutations 3–5 or parental pRSV-CATgm (Table IV). As an additional control, we transfected each of these constructs (and the parent pRSV-CATgm) into human U293 (an adenovirus-transformed embryonic kidney cell that expresses Ad5 E1A) cells in which we see extremely high CAT mRNA and protein levels that are unregulated despite the presence of AU elements. This effect is presumably the result of very high levels of gene transcription mediated by E1A protein. The CAT activities seen with all of the mutated constructs were identical, indicating that each is competent to produce CAT mRNA and protein.

Our data suggest that different stimuli (e.g., lectins, phorbols, cytokines) may mediate their effects on mRNA stability through different mRNA elements. Cells transfected with pRSV-CATgm, for example, respond to TPA but not to ConA. One possibility is that there are no ConA response elements in the 3′-UTR or that they reside in the region from 434 to 447 which we left out of our constructs. Other equally likely possibilities are that the ConA response elements are located in the 5′-UTR or within the coding region or that some combination of 5′ and 3′ elements may be required.

The results from the nuclear run-on transcription and ActD chase experiments in the permanent cell lines showed that TPA had no significant effects on CAT gene transcription rate and that the predominant effect of TPA was to prolong CAT mRNA half-life (Figs. 3 and 4). These results also establish that the low levels of CAT activity seen in untreated cells transfected with pRSV-CATgm are due to message instability rather than to an ineffective transcription or a translational defect. We note that the TPA-driven increases in CAT protein and mRNA conferred by the presence of the GM-CSF 3′-UTR are considerable in this model system (almost 10-fold) but that the response of the endogenous GM-CSF gene to TPA in EL-4 cells is at least 10 to 100 times greater. It is also clear from the data in Tables I and III that TPA treatment of cells transfected with pRSV-CATgm does not raise CAT levels to those seen with pRSV-CAT. Further, our data show that TPA prolongs the half-life of pRSV-CATgm mRNA to 70 min (Fig. 3), whereas the half-life of endogenous GM-CSF mRNA is prolonged by TPA to more than 3 h (8). It is therefore likely that other portions of the GM-CSF mRNA contribute to the increased GM-CSF mRNA levels induced by TPA. These other regions may also contribute to mRNA stability because our previous work has demonstrated no significant effects of TPA on GM-CSF gene transcription or GM-CSF mRNA translation (8). Multiple elements which contribute to the regulation of mRNA stability are not without precedent. They have been found in c-fos, for example, in which elements within the 3′-UTR and coding region independently modulate mRNA instability (12). Data from Bagby and co-workers, showing that the AU-rich region of GM-CSF alone cannot respond to interleukin 1 in fibroblasts (29) and the data presented here regarding the lack of response to ConA also point to the possibility that there are important regulatory elements outside the 3′-UTR. These postulated elements may work independently as was shown in the case of c-fos or they may interact with the 3′-UTR and form larger structures.

Our studies have shown that upstream portions of the GM-CSF 3′-UTR are necessary for GM-CSF mRNA stabilization induced by TPA. Current studies will concentrate on mapping with greater precision the TPA-responsive element(s) within the 3′-UTR with the goal of determining whether cytoplasmic proteins exist whose activity is modulated by TPA.

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REFERENCES
1. Granselli-Piperno, A., Inaba, K., and Steinman, R. M. (1984) J. Exp. Med. 160, 1792-1802
2. Bickel, M., Anastad, P., Tsuda, H., Sulis, C., Asoskary, S., Mengenangen, S. E., and Pluznik, D. H. (1987) J. Immunol. 138, 2984-2988
3. Clark, S. C., and Kamen, R. (1985) Science 236, 1229-1237
4. Queensland, P. J., and Gimbrelle, M. A., Jr., (1988) Blood 65, 1060-1067
5. Guez, M., and Sachs, L. (1975) FEBS Lett. 37, 134-135
6. Bickel, M., Wahl, S. M., Mengenangen, S. E., and Pluznik, D. H. (1988) Exp. Hematol. 16, 691-695
7. Bickel, M., Tsuda, H., Anastad, P., Evequez, V., Mengenangen, S. E., Wahl, S. M., and Pluznik, D. H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 3274-3277
8. Bickel, M., Cohen, R. B., and Pluznik, D. H. (1990) J. Immunol. 145, 840-845
9. Nimer, S. D., Gates, M. J., Koeffer, H. P., and Gassen, J. C. (1989) J. Immunol. 143, 2574-2577
10. Wodnaw-Filipowicz, A., and Moroni, C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 777-781
11. Shyu, A.-B., Greenberg, M. E., and Belasco, J. G. (1989) Gene Dev. 3, 6072
12. Ryan, R., Milton, S. E., Lopez, A. P., Bandy P. G., Vadaz M. A., and Shannon, M. F. (1991) Blood 77, 1185-1202
13. Shaw, G., and Kamen, R. (1986) Cell 46, 659-667
14. Gough, N. M., Metcalf, D., Gough, J., Grail, D., and Dunn, A. R. (1985) EMBO J. 4, 645-653
15. Piechaczyk, M., Blanchard, J. M., Marty, L., Dani, Ch., Panabière, F., El Sabouaty, S., Fort, Ph., and Jeanteur, Ph. (1984) Nucleic Acids Res. 12, 6961-6963
16. Gorman, C. M., Merloino, G. T., Willingham, M. C., Pastan, I., and Howard, B. H. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6777-6781
17. Saki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, T., Mullin, K. B., and Erlich, H. A. (1988) Science 239, 487-491
18. Horton, R. M., Cai, Z., Ho, S. N., and Pease, L. R. (1990) Biotechniques 8, 528-529
19. Farrar, J. J., Fuller-Farrar, J., Simon, F. L., Hilfiker, M. L., Stauder, B. M., and Farrar, W. L. (1980) J. Immunol. 125, 2555-2559
20. Jainchill, J. L., Aaronson, S. A., and Todaro, G. J. (1969) J. Virol. 4, 549-553
21. Neumann, E., Schafer-Ridder, M., Wang, Y., and Hofschneider, P. H. (1982) EMBO J. 1, 841-845
22. de Wett, J. R., Wood, K. U., DeLucia, M., Heinisler, D. R., and Subramani, S. (1987) Mol. Cell. Biol. 71, 725-737
23. Southern, P. J., and Berg, P. (1982) J. Mol. Biol. Genet. 1, 327-341
24. Neuman, J., Morency, C. A., and Russian, K. O. (1987) Biotechniques 5, 444-448
25. Czeczuga, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159
26. Goudine, M., Peretz, M., and Weintrob, H. (1981) Mol. Cell. Biol. 1, 281-287
27. Gilliland, G., Perrin, S., Blanchard, K., and Bunn, H. F. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2725-2729
28. Akahane, K., Cohen, R., Bickel, M., and Pluznik, D. H. (1991) J. Immunol. 146, 4190-4190
29. Band, L., Shaw, G., and Bagby, C. G. (1990) Exp. Hematol. 18, 623 (abstr)
30. Stieger, P., Carbon, P., Megerle, E., Ebel, J.-P., and Ehrenmann, C. (1981) Nucleic Acids Res. 9, 2153-2172
31. Hentze, M. W., Bouh, T. A., Harford, J. B., and Klausner, R. D. (1989) Science 244, 367-379

* R. B. Cohen and Y. Iwai, unpublished data.