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Interleukin-11 mRNA Stabilization in Phorbol Ester-Stimulated Primate Bone Marrow Stromal Cells

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12-*O*-Tetradecanoylphorbol-13-acetate (TPA) stimulation of PU-34 cells, a primate bone marrow stromal cell line, resulted in a prolonged elevation of interleukin-11 (IL-11) mRNA, which can be inhibited by protein synthesis inhibitors. Nuclear run-on assays and actinomycin D experiments demonstrated that the up-regulation of IL-11 gene expression is mainly controlled at the posttranscriptional level through the protein kinase C (PKC) pathway. Inhibition of PKC activity by calphostin C generated an IL-11 mRNA degradation intermediate in TPA-stimulated PU-34 cells. This intermediate retains the 5′ untranslated region (5′UTR) and coding region of the IL-11 mRNA but has lost the poly(A) tail and the 3′UTR. The mechanisms underlying IL-11 mRNA stabilization were further investigated by transfections with a variety of chimeric IL-11 constructs and deletion mutants. Two important observations were made from these transient expression experiments: (i) the same 3′UTR of IL-11 mRNA shown to confer instability in one chimeric transcript may not function as a destabilizer in another chimeric RNA, and (ii) the 5′UTR, coding region, and 3′UTR all contribute to IL-11 mRNA decay, and labile IL-11 deletion transcripts are not necessarily stabilized by TPA stimulation. Our study suggests that multiple regions within the IL-11 mRNA are involved in TPA-stimulated IL-11 mRNA stabilization, possibly through a unique RNA folding conformation involving interactions of various RNA sequences within the IL-11 mRNA molecule.

Interleukin-11 (IL-11) was originally cloned from a primate bone marrow-derived stromal fibroblast cell line, PU-34, on the basis of its ability to stimulate the proliferation of an IL-6-dependent mouse plasmacytoma cell line, T1165 (32). The human IL-11 gene has since been sequenced and mapped to chromosome 19 by in situ hybridization (29). In PU-34 cells, the IL-11 gene gives rise to two mRNA transcripts (1.5 and 2.5 kb in size) which differ at their 3′ polyadenylation sites yet encode the same functional protein. Expression of the IL-11 gene has been detected in a variety of cells, including human lung fibroblast cell lines MRC-5 (32) and CCL-202 (12); a human stromal cell line, KM-102 (21); a human trophoblast cell line, TPA30-1 (32); human chondrocytes and synoviocytes (27); and human fibroblasts and endothelial cells (41). Many studies have established that IL-11 is a multifunctional cytokine affecting hematopoiesis and other biological processes such as adipogenesis and neuronal differentiation (reviewed in reference 47). Recently it was demonstrated that IL-11 shares certain signal transduction pathways with IL-6, leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor but utilizes a different receptor complex (17, 22, 48).

Even though the expression of IL-11 can be induced by many agents, such as IL-1α, transforming growth factor β, the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and the calcium ionophore A23187, the detailed mechanisms underlying IL-11 induction by these different agents are not well understood. Expression of the IL-11 gene appears to be controlled by different mechanisms in different cell types. Recently we have demonstrated that IL-1α stimulation of PU-34 cells transiently stabilizes IL-11 mRNA through activation of genistein-sensitive tyrosine kinase(s) without affecting transcription of the IL-11 gene (46). The present study was initiated when we observed that exposure of PU-34 cells to TPA resulted in a prolonged elevation of IL-11 mRNA, whereas IL-1α induction only resulted in a transient increase in the IL-11 mRNA level. Here we report that activation of protein kinase C (PKC) by the phorbol ester TPA is responsible for the increased steady-state level of IL-11 mRNA. The up-regulation of IL-11 expression in PU-34 cells is mainly a posttranscriptional event involving stabilization of IL-11 mRNA through PKC-facilitated protection of the IL-11 transcripts. Different RNA regions required for degradation and TPA-induced stabilization were further investigated with a variety of IL-11 chimeric constructs and deletion mutants.

MATERIALS AND METHODS

Cell culture and reagents. PU-34 cells, a primate bone marrow-derived stromal cell line, were cultured in Dulbecco's modified Eagle medium with 10% fetal calf serum as previously described (33). TPA was purchased from Sigma. Actinomycin D (Sigma) was used at 10 μg/ml for inhibition of gene transcription. Cycloheximide (Sigma) and puromycin (Clontech) were both used at 10 μg/ml for inhibition of ongoing protein synthesis in PU-34 cells. Stock solutions of genistein (GIBCO) and H7 (Seikagaku America, Inc.) were prepared in dimethyl sulfoxide and H_2O, respectively. The specific PKC inhibitor calphostin C was obtained from Calbiochem.

Northern (RNA) blot analysis and nuclear run-on assay. Total RNA was isolated from PU-34 cells by a guanidinium thiocyanate method (8). Poly(A)$^+$ mRNA was prepared with a Mini-Ribosep mRNA isolation kit (Collaborative Research, Inc.) according to the manufacturer's instructions. For Northern blot analysis, 10 μg of total RNA or 0.5 μg of poly(A)$^+$ mRNA was separated through a 1% formaldehyde-agarose gel by electrophoresis and transferred onto a nitrocellulose membrane (Schleicher & Schuell) for hybridization with the IL-11 probe (a 0.8-kb *Xho*I fragment of the primate IL-11 cDNA). The same membrane was stripped and rehybridized with a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe to ensure equal loading of total RNA into each lane. The following DNA fragments were used in the detection of transiently expressed exogenous transcripts: a 1.5-kb *Xba*I fragment from pXP2 (31) as the probe for luciferase message, a 0.5-kb *Xho*I-*Bam*HI fragment from pPyER-RBG (46) as the probe for rabbit beta globin (RBG) message, a

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3.4-kb *Bgl*II fragment from pPyER-RBG as the probe for histidinol dehydrogenase message, a 0.8-kb *Eco*RI-*Sma*I fragment from pCMV-β-gal (Clontech) as the cytomegalovirus (CMV) probe for CMV-IL-11 transcripts, and a 3.4-kb *Not*I fragment from pCMV-β-gal as the probe for β-galactosidase message. The nuclear run-on assay was performed as previously described (14) with 3×10^7 PU-34 nuclei collected after different treatments. The labeled run-on transcripts were then hybridized to nitrocellulose membranes slot blotted with single-stranded antisense primate IL-11, sense IL-11 (as a negative control), and denatured plasmid containing human GAPDH cDNA. After being washed until there was no background hybridization to the negative controls, the filters were exposed to X-ray films for 48 h at −70°C.

S1 nuclease protection assay. The S1 nuclease protection assay was performed as described previously (36). Twenty micrograms of total RNA from TPA (5 ng/ml)-treated cells or from cells treated with TPA (5 ng/ml) followed by calphostin C (2 μM) was ethanol precipitated together with 10^5 cpm of the S1 probe. The S1 probe was a 0.35-kb double-stranded DNA fragment which contains DNA sequence complementary to the entire 5′ untranslated region (5′UTR) and part of the coding region (positions 1 to 243) of the full-length IL-11 mRNA. The samples were resuspended in 20 μl of formamide hybridization buffer, denatured at 85°C for 10 min, annealed at 45°C overnight, and treated with 10 volumes of S1 nuclease mapping buffer (400 U/ml [Bethesda Research Laboratories]) at 30°C for 1 h. The reaction was then terminated by addition of 3 volumes of EDTA stop buffer, extracted with phenol-chloroform-isoamyl alcohol, and precipitated with ethanol. The S1 nuclease-resistant products were separated on a 6% polyacrylamide denaturing gel along with DNA sequencing ladders.

Plasmid construction and transfection of PU-34 cells. For reporter plasmid construction, a 0.79-kb *Pvu*II fragment (−730 to +60 relative to the transcription initiation site of the IL-11 gene) was cloned into the *Sma*I site within the polylinker of the firefly luciferase vector pXP2 (31) to generate plasmid pIL-Luc. The effect of the 3′UTR of the IL-11 mRNA on luciferase mRNA stability was tested by cloning the 3′UTR (nucleotides 767 to 1202) of the 1.5-kb IL-11 mRNA into the *Xba*I-*Sty*I sites within the 3′ end of the luciferase gene in plasmid pIL-Luc to generate plasmid pIL-Luc+3′UTR (see Fig. 6A). The same 3′UTR was also cloned into the *Xba*I-*Sal*I sites within the 3′ end of the RBG gene in plasmid pPyER-RBG^{GC} to generate plasmid pPyER-RBG$^{3'UTR}$ (Fig. 6A). The full-length IL-11 cDNA (1 to 1202) was cloned into the *Not*I sites of the pCMV-β-gal vector (Clontech) in place of the β-galactosidase gene to generate plasmid pCMV-IL-11$^{1-1202}$. A series of deletions of the IL-11 cDNA (shown in Fig. 7A) were carried out with pCMV-IL-11$^{1-1202}$ as the template DNA to generate additional truncation transcripts. Plasmid DNAs were introduced into PU-34 cells by the standard calcium phosphate coprecipitation method (36) (8 μg of chimeric IL-11 plasmid plus 2 μg of pCMV-β-gal DNA per 60-mm-diameter dish). After 5 h of incubation, the cells were shocked with 15% glycerol, incubated in Dulbecco's modified Eagle medium for 18 h, and then stimulated with TPA (5 ng/ml) for 5 h.

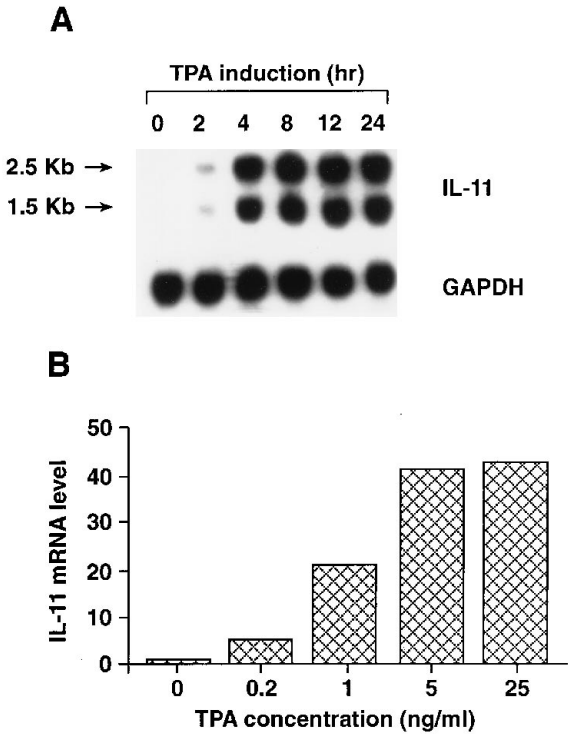


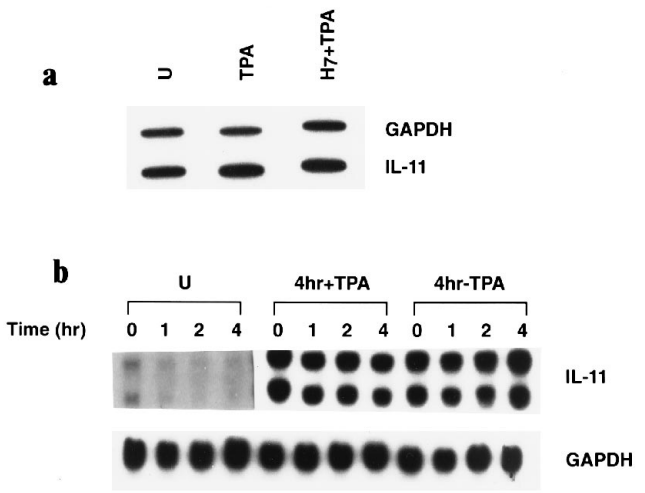
FIG. 1. Kinetics and dose dependence of TPA stimulation. (A) Confluent PU-34 cells were stimulated with TPA (5 ng/ml) for the indicated time periods. Ten micrograms of total RNA per lane was analyzed by hybridization with the primate IL-11 cDNA probe. The membrane was stripped and rehybridized with a human GAPDH cDNA probe. (B) Cells were stimulated with different doses of TPA for 4 h. IL-11 mRNA signals were then analyzed by Northern blotting.

RESULTS

IL-11 mRNA is stabilized by TPA stimulation. To determine the kinetics of IL-11 gene expression after TPA stimulation, PU-34 cells were treated with TPA (5 ng/ml) and analyzed at different time points after stimulation. As shown in Fig. 1A, the steady-state level of the IL-11 mRNA was very low in unstimulated cells. Addition of TPA to the cells steadily increased the IL-11 mRNA level, which reached the maximum after 4 h and was maintained at the same level for up to 24 h. TPA stimulation of IL-11 expression showed a dose-dependent behavior, with 5 ng/ml producing the plateau level of stimulation (Fig. 1B).

To determine whether the increase in IL-11 mRNA after TPA stimulation occurred at the transcriptional or posttranscriptional level, nuclear run-on assays were performed to measure the rates of IL-11 gene transcription. As shown in Fig. 2a, TPA stimulation of PU-34 cells resulted in a slight increase in the rate of IL-11 gene transcription. As a positive control for nuclear run-on assays, JunB gene transcription in TPA-stimulated PU-34 cells was measured and shown to increase more than 10-fold over the basal level (data not shown). It appears that the moderate increase in IL-11 gene transcription was not sufficient to account for the drastic change in the IL-11 mRNA level in TPA-stimulated cells. Thus, the half-lives of IL-11 mRNAs before and after TPA stimulation were measured in the presence of actinomycin D, which inhibits the ongoing gene transcription. As shown in Fig. 2b, the IL-11 mRNA level in TPA-stimulated cells was not affected by actinomycin D during the 4-h testing period, even in the absence of TPA (no significant decrease in IL-11 mRNA level was observed after 8 h), whereas IL-11 mRNA in unstimulated cells was rapidly degraded. By densitometric scanning (Fig. 2c), the half-life of IL-11 mRNA in unstimulated cells was estimated to be less than 1 h, and the half-life of IL-11 mRNA in TPA-stimulated cells was extrapolated to be more than 12 h. Thus, the increase in the IL-11 mRNA level after TPA stimulation is a result of the combination of the modest transcriptional activation of the IL-11 gene and the drastic change in the stabilization of IL-11 mRNA.

To test the effects of translation inhibitors on IL-11 mRNA stabilization, cycloheximide and puromycin were used in our study. Previous studies have demonstrated that these two agents inhibit protein synthesis by different mechanisms (18). Cycloheximide blocks translation elongation through direct interaction with the 60S subunit of the ribosomes and results in polysome aggregation, whereas puromycin is an aminoacyl transfer RNA analog leading to polysome dissociation. Cycloheximide treatment of cells had been widely reported to prolong the half-life of labile mRNAs (11, 45). As shown in Fig. 3, in unstimulated cells, cycloheximide treatment indeed increased the IL-11 mRNA level, while puromycin had no effect (lanes 1 to 3). It is surprising that puromycin treatment did not cause stabilization of IL-11 mRNA in unstimulated cells. In TPA-stimulated cells, however, both cycloheximide and puromycin showed inhibition instead of superinduction of IL-11 mRNA (lanes 4 to 6), suggesting TPA-induced IL-11 mRNA stabilization is indeed coupled with protein synthesis.



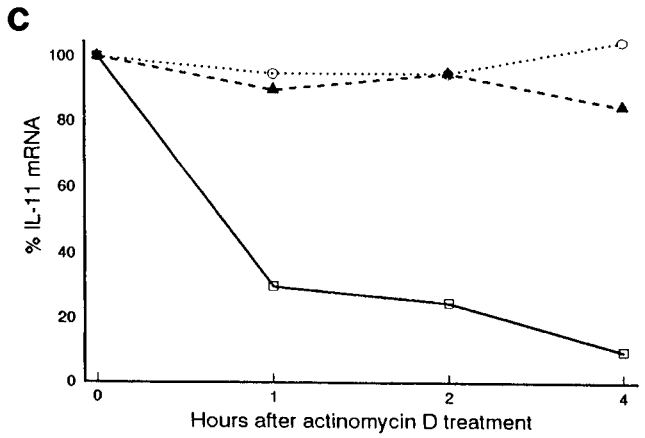


FIG. 2. Stabilization of IL-11 mRNA by TPA stimulation. (a) The transcription rate of the IL-11 gene was measured with nuclei from unstimulated PU-34 cells (U), cells stimulated with 5 ng of TPA per ml for 4 h (TPA), and cells treated with 5 ng of TPA per ml plus 10 μM H7 (H_7+TPA). (b) Actinomycin D (10 μg/ml) was added to unstimulated cells (U) or to cells stimulated with TPA (5 ng/ml) for 4 h. IL-11 mRNA was then analyzed 0, 1, 2, or 4 h after actinomycin D addition in the presence (4hr+TPA) or absence (4hr−TPA) of TPA. (c) IL-11 mRNA decay was normalized by densitometric scanning according to the signals associated with GAPDH mRNA. □, unstimulated cells; ▲, TPA-stimulated cells; ○, cells withdrawn from TPA.

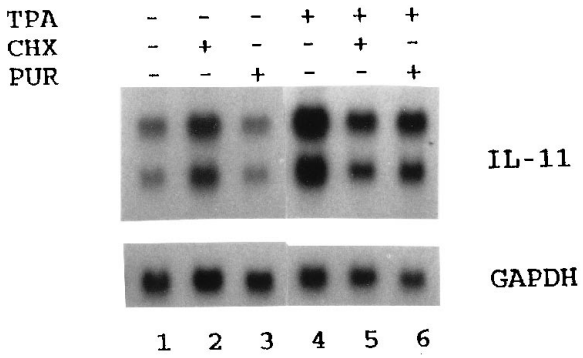


FIG. 3. Effects of protein synthesis inhibitors on IL-11 mRNA. The protein synthesis inhibitor cycloheximide (CHX [10 μg/ml]) or puromycin (PUR [10 μg/ml]) was added to cells in the absence (lanes 1, 2, and 3) or presence (lanes 4, 5, and 6) of TPA stimulation. After 5 h, IL-11 mRNA was analyzed by Northern blotting.

Stabilization of IL-11 mRNA by TPA is mediated through the PKC pathway. We have previously shown (46) that IL-1α-induced expression of the IL-11 gene in PU-34 cells is most likely mediated by activation of genistein-sensitive protein tyrosine kinase(s) but not H7-sensitive serine/threonine kinase(s). To investigate the involvement of protein phosphorylation in the up-regulation of IL-11 mRNA by TPA, these two kinase inhibitors were tested in TPA-treated PU-34 cells. As shown in Fig. 4A, at a concentration previously shown to effectively abolish IL-1α-induced stabilization in PU-34 cells, genistein (a tyrosine kinase inhibitor) (1) was much less effective than H7 (a serine/threonine kinase inhibitor) in the inhibition of TPA-stimulated IL-11 expression. These results indicated that different kinase systems may be involved in TPA-mediated IL-11 gene expression. Nuclear run-on assays showed that inhibition of the IL-11 mRNA level in TPA-stimulated cells by H7 is most likely to be posttranscriptional, since there were no appreciable changes in IL-11 gene transcription in H7-treated cells (Fig. 2A). Previously H7 has been shown to inhibit a variety of serine/threonine kinases by competing with the ATP binding sites within the catalytic domains of these kinases (20). To show that PKC activity is required for the expression of the IL-11 gene, a more specific PKC inhibitor, calphostin C, was used to block TPA stimulation. Calphostin C interacts irreversibly with the regulatory domain of PKC and does not inhibit cAMP-dependent protein kinase or other kinases, even at a concentration as high as 50 μM (42). As shown in Fig. 4B, calphostin C blocked TPA induction of IL-11 mRNA in a dose-dependent manner, and addition of calphostin C to TPA-stimulated PU-34 cells resulted in a decrease in the IL-11 mRNA level (Fig. 4C). Calphostin C treatment also decreased the GAPDH mRNA levels in these experiments; thus, 28S rRNA was used as the control for gel loading. Interestingly, an extra RNA band (about 0.8 kb in size) was detected in calphostin C-treated cells, suggesting the presence of a specific intermediate during IL-11 mRNA degradation.

The identity of this degradation intermediate was further examined by probes corresponding to different regions of the IL-11 mRNA. As shown in Fig. 5A, the intermediate could be detected by probes corresponding to the 5′UTR (1 to 156) and open reading frame (ORF) (243 to 753) of the IL-11 mRNA (lanes 2 and 4) but not by a probe (797 to 1202) corresponding to the 3′UTR of IL-11 mRNA (lane 6). In addition, the intermediate could be detected in the poly(A)$^-$ RNA instead of the poly(A)$^+$ mRNA fraction (Fig. 5B), indicating the lack of a poly(A) tail in this degradation intermediate. To determine whether the 5′UTR of the degradation intermediate is truncated after calphostin C treatment, an S1 nuclease protection assay was performed with a 5′-end-labeled probe, part of which is complementary to the IL-11 mRNA sequence 1 to 243. As shown in Fig. 5C, two major S1 nuclease-resistant products (242 and 243 bp) were detected which correspond to the transcription initiation sites previously determined by the primer extension experiment (46), suggesting that the degradation intermediate is not truncated within the 5′UTR. By probing with anti-sense oligonucleotides, the 3′ end of the degradation intermediate was localized near base 790, a site about 40 bp downstream of the translation stop codon UGA (data not shown). It is not clear at present whether this intermediate is the result of a specific endonuclease cleavage or whether it is the product of progressive 3′-to-5′ exonuclease degradation.

Multiple regions within IL-11 mRNA are required for TPA-induced mRNA stabilization. AU-rich motifs in the 3′UTR of many cytokine mRNAs have been well documented as being involved in selective mRNA degradation (38). Recent studies

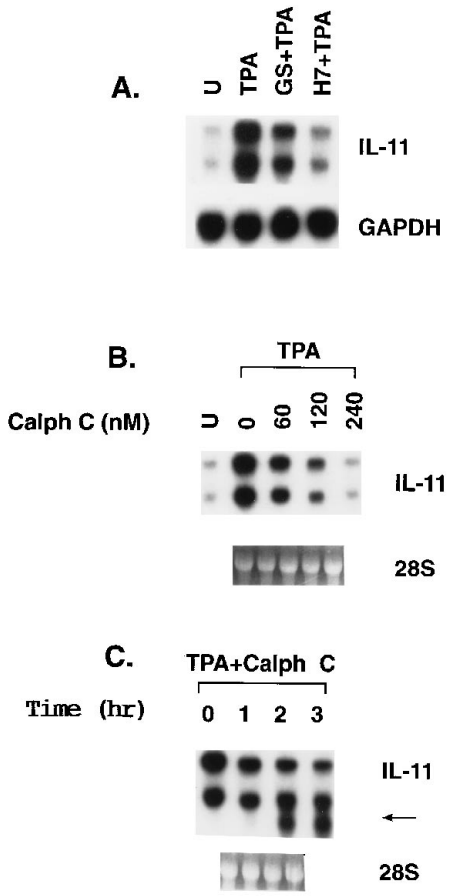
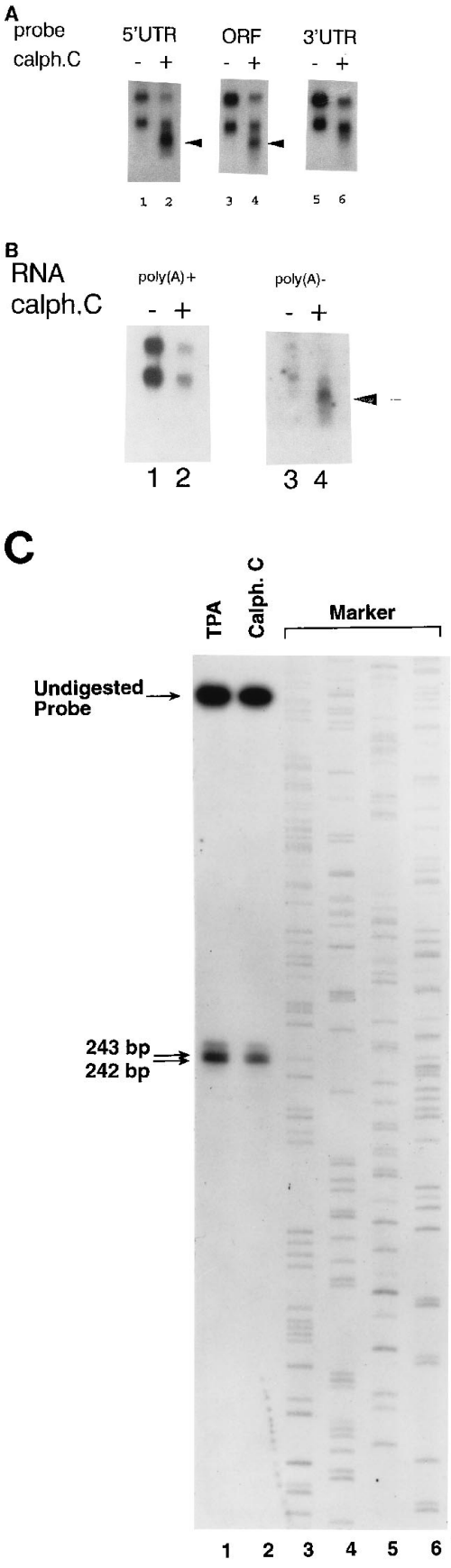


FIG. 4. Involvement of PKC in IL-11 mRNA stabilization. (A) PU-34 cells were preincubated with H7 (10 μM) or genistein (GS [20 μg/ml]) for 30 min, stimulated with TPA (5 ng/ml) for 4 h, and then analyzed by Northern blotting. (B) PU-34 cells were preincubated with different concentrations of calphostin C (Calph C) under fluorescent light for 10 min and then stimulated with 5 ng of TPA per ml for 4 h. (C) After 4 h of TPA stimulation, the cells were treated with calphostin C (2 μM) and further incubated under fluorescent light for 1, 2, or 3 h.

indicated that distinct mRNA decay pathways may be responsible for the degradation of chimeric RBG transcripts bearing AU-rich motifs from c-*fos* and granulocyte-macrophage colony-stimulating factor mRNAs (7). It has also been proposed that the nonamer UUAUUUAUU, instead of the pentamer AUUUA, is the key AU-rich sequence that mediates mRNA degradation (24, 50). Three copies of AUUUA repeats (including one copy of the nonamer UUAUUUAUU at 797 to 818) have been shown to be present in the 3′UTR of IL-11 mRNA (29). In an attempt to localize the RNA *cis*-elements responsible for TPA-induced IL-11 mRNA stabilization in PU-34 cells, a variety of plasmid constructs were generated for the expression of different chimeric IL-11 genes in PU-34 cells. The rationale behind these experiments is that if two constructs have similar structures and the expression of both constructs is driven by the same promoter, the different steady-state levels of the resultant chimeric RNAs inside the cells are likely to reflect corresponding changes in RNA stability. As shown in Fig. 6A, a 0.73-kb IL-11 promoter sequence was cloned into the polylinker region upstream of the firefly luciferase gene to generate pIL-Luc, and then the entire 3′UTR sequence (767 to 1202) of the 1.5-kb IL-11 mRNA was subcloned into pIL-Luc downstream of the luciferase reporter gene to generate pIL-Luc+3′UTR. Both plasmids were then transfected into PU-34 cells along with plasmid pCMV-β-gal as an internal control. As shown in Fig. 6B, luciferase gene expression driven by the IL-11 promoter was detected in cells transfected with pIL-Luc, and IL-11 promoter activity was not affected by TPA stimulation (lanes 1 and 2). Insertion of the 3′UTR from IL-11 mRNA into the 3′ end of the luciferase gene generated a message slightly larger than its parental mRNA. However, the steady-state level of this message was not affected by the presence of the 3′UTR from IL-11 mRNA, nor was the expression further induced by TPA stimulation (lanes 3 and 4). When the same 3′UTR sequence from the IL-11 mRNA (767 to 1202) was cloned into the 3′ noncoding region of the RBG gene (Fig. 6A), the steady-state level of the resultant chimeric $RBG^{3'UTR}$ message (generated from pPyER-$RBG^{3'UTR}$ [Fig. 6C, lane 3]) was as low as that of RBG^{AT} (generated from pPyER-RBG^{AT} [Fig. 6C, lane 1]), which contains the well-known destabilizing AU-rich motif from granulocyte-macrophage colony-stimulating factor mRNA (38) at the 3′ noncoding region of the RBG gene. In pPyER-RBG constructs, expression from the HisD gene cassette is not significantly affected by TPA and can be used as an internal control for transfection as well as a selection marker for stable transfectants. Like transfection with pIL-Luc+3′UTR, TPA stimulation of PU-34 cells did not further stimulate the expression of the chimeric pPyER-$RBG^{3'UTR}$ constructs (compare lanes 3 and 6). These results suggested that the AU-rich motifs from the 3′UTR of IL-11 mRNA neither conferred TPA responsiveness nor did they guarantee destabilization of different chimeric IL-11 messages. On the basis of these observations, we concluded that the destabilizing function of the AU-rich motif was largely dependent on the reporter gene into which it was inserted.

In order to mimic TPA-induced stabilization of the IL-11 mRNA in PU-34 cells, plasmid pCMV-IL-11^{1-1202} (Fig. 7A) was constructed for transient expression of the full-length IL-11 cDNA with minimal introduction of foreign DNA sequence. The resultant CMV-IL-11^{1-1202} transcript contains a short CMV sequence at the 5′ end of the full-length IL-11 mRNA sequence. Since the CMV promoter activity was not significantly affected by TPA treatment, as indicated by the cotransfection of pCMV-β-gal, the increased steady-state level of CMV-IL-11^{1-1202} after TPA treatment may suggest a prolonged RNA half-life. As shown in Fig. 7B, the CMV-IL-11^{1-1202} message by itself was unstable but could be stabilized by TPA stimulation along with the endogenous 1.5- and 2.5-kb IL-11 mRNA transcripts (lanes 1 and 2). To further dissect the RNA sequences controlling IL-11 mRNA stability and TPA responsiveness, IL-11 constructs containing a deletion of the entire 5′UTR (pCMV-IL-$11^{157-1202}$), deletions of various portions of the coding region (pCMV-IL-$11^{243-1202}$, pCMV-IL-$11^{444-1202}$, and pCMV-IL-$11^{570-1202}$), and deletions of the entire 5′UTR and coding region (pCMV-IL-$11^{767-1202}$) were generated for expression in PU-34 cells. After transfection, these exogenous CMV-IL-11 transcripts were examined by probing the tagged CMV sequence. As shown in Fig. 7B (lanes 3 through 12), deletions of the 5′UTR or coding region sequences of IL-11 mRNA affected the TPA-stimulated stabilization of these truncated CMV-IL-11 transcripts. All of these transcripts were unstable in PU-34 cells, except for CMV-IL-$11^{570-1202}$ (lanes 9 and 10), suggesting the region between 444 and 569 and the region between 570 and 767 had opposite effects on IL-11 mRNA stability. To localize the AU-rich motif that may contribute to IL-11 mRNA destabilization, three additional constructs (pCMV-IL-11^{-1AT}, pCMV-IL-11^{-2AT}, and pCMV-IL-11^{-3AT} [Fig. 7A]) containing deletions of one, two, or three copies of AU-rich sequences in the 3′UTR of the IL-11 mRNA



were tested. As shown in Fig. 7B (lanes 13 through 16), removal of the first one or two copies of AU-rich sequences from the IL-11 sequence did not significantly affect the stability and TPA responsiveness of the deletion transcripts CMV-IL-11^{-1AT} and CMV-IL-11^{-2AT} compared with that of the full-length CMV-IL-11^{1-1202}. When the remaining copy of AU-rich sequence (1056 to 1202) was deleted, however, the resultant CMV-IL-11^{-3AT} was relatively stable in PU-34 cells and no longer responded to TPA stimulation (Fig. 7B, lanes 17 and 18). To test the effects of coding region sequence on IL-11 RNA stability, a major portion of the coding region (247 to 818) was deleted in pCMV-IL-11^{-ORF} to generate the CMV-IL-11^{-ORF} transcript, which was unstable in PU-34 cells and not inducible by TPA (Fig. 7B, lanes 19 and 20). The steady-state level of each deletion transcript was quantitated by densitometric scanning, and the results are summarized in Fig. 7C. Taken together, these results indicated that sequences from the 5′UTR, coding region, and 3′UTR of the IL-11 mRNA are all important to TPA-induced IL-11 mRNA stabilization.

DISCUSSION

In the present study, we have shown that the phorbol ester TPA could stimulate IL-11 gene expression in PU-34 cells. In contrast to the modest increase in transcription of the IL-11 gene, TPA stimulation of PU-34 cells greatly stabilized IL-11 mRNA. We have also observed that one protein synthesis inhibitor (cycloheximide) could stabilize IL-11 mRNA in unstimulated cells, whereas another inhibitor (puromycin) could not. We speculate that IL-11 mRNA stabilization by cycloheximide in unstimulated PU-34 cells somehow correlates with polysome aggregation, which may also explain the failure of puromycin to stabilize IL-11 mRNA, since puromycin treatment of cells reportedly leads to polysome dissociation (18). In TPA-treated cells, IL-11 mRNA stabilization apparently utilizes a distinct mechanism which is inhibited by either cycloheximide or puromycin. A plausible explanation for the discrepancy is that TPA-induced IL-11 mRNA stabilization requires de novo synthesis of protein factor(s); cycloheximide and puromycin both block protein synthesis and therefore are capable of blocking TPA-induced IL-11 mRNA stabilization.

The effects of the protein kinase inhibitors genistein and H7 on the inhibition of IL-1α or TPA-stimulated IL-11 expression were also compared in our study. It is clear that different mechanisms and signaling events are involved in IL-11 mRNA stabilization by IL-1α and by TPA. H7-sensitive serine/threonine kinase(s), instead of genistein-sensitive tyrosine kinase(s), appears to play a major role in TPA-stimulated IL-11 expression. The involvement of PKC in the stabilization of the IL-11 mRNA was further demonstrated by the use of a specific PKC inhibitor, calphostin C, which was able to block the TPA in-

FIG. 5. Characterization of the degradation intermediate. After 4 h of TPA stimulation, cells were left untreated or treated with calphostin C (calph. C) 2 μM]) under fluorescent light for 3 h and then lysed for RNA isolation. (A) For lanes 1 to 6, total RNA was separated by electrophoresis and transferred to a nitrocellulose membrane. Identical nitrocellulose strips were then hybridized separately with IL-11 probes corresponding to the 5′UTR (1 to 156), the ORF (243 to 753), and the 3′UTR (797 to 1202). (B) Poly(A)$^+$ mRNA (lanes 1 and 2) was isolated from total RNA with an oligo(dT) cellulose column, and the corresponding flowthrough was designated as the poly(A)$^-$ RNA fraction (lanes 3 and 4). The blots were then hybridized with an IL-11 probe (243 to 1003). The degradation intermediate is indicated by an arrow. (C) The S1 nuclease protection assay was carried out with total RNA from TPA-stimulated cells (lane 1) or from cells stimulated with TPA and then treated with calphostin C (lane 2). The two major S1 nuclease-resistant products are indicated by arrows. Lanes 3 to 6, DNA ladders generated by determining the sequence of a control DNA.

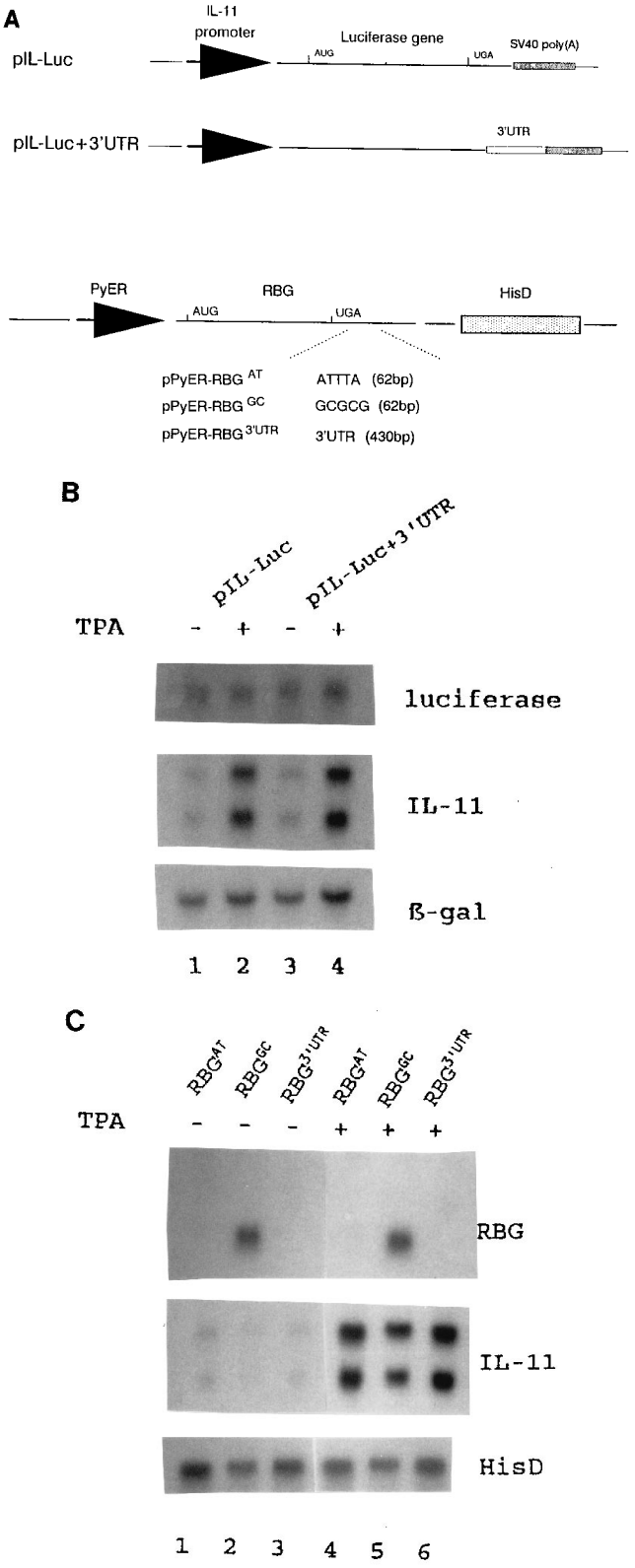


FIG. 6. Northern analysis of chimeric IL-11 messages. (A) Structures of the chimeric luciferase and RBG constructs. SV40 poly(A), simian virus 40 polyadenylation signal; PyER, polyomavirus early promoter; RBG, RBG gene; ATTTA, a 62-bp AU-rich sequence from the 3′UTR of granulocyte-macrophage colony-stimulating factor mRNA; GCGCG, a 62-bp sequence with AT-to-GC mutations within the AU-rich motifs; 3′UTR, the 430-bp 3′UTR from IL-11 mRNA (767 to 1202); HisD, histidinol dehydrogenase gene cassette. (B) Northern analysis of the chimeric luciferase messages. The same filter was reprobed to show the inducible expression of IL-11 mRNA by TPA. The β-galactosidase mRNA was generated from cotransfection of pCMV-β-gal and used as an internal control. (C) Northern analysis of the chimeric RBG messages. HisD signal was used as an internal control.

duction and to accelerate the degradation of the IL-11 mRNA. Inhibition of PKC activity by calphostin C generated a degradation intermediate in TPA-stimulated PU-34 cells. This is an interesting observation, since it has been widely speculated that one of the rate-limiting steps of eukaryotic mRNA degradation is the deadenylation of the poly(A) tail, and RNA messages without poly(A) tails are extremely unstable (9, 25). Our findings demonstrate that an mRNA degradation intermediate may be relatively stable inside the cells under certain conditions. Even though this degradation intermediate was shown to have lost the poly(A) tail and the 3′UTR, further studies are needed to determine whether this intermediate is the product of 3′-to -5′ exonucleolytic degradation (2) or specific endonuclease cleavage (10).

The expression of many cytokines has been shown to be regulated at the posttranscriptional level through mRNA stabilization (26, 35). Cytokine mRNA stability may vary among different cell types (13), and cytokine mRNA degradation may be regulated differently from oncogene mRNA within the same cell type, despite the fact that both are labile mRNAs and share similar AU-rich motifs in their 3′ UTRs (37). The mechanisms governing posttranscriptional regulation of cytokine mRNAs are complicated by the fact that a labile cytokine mRNA can be greatly stabilized by a variety of extracellular stimuli. In some noncytokine systems, the mechanisms underlying mRNA degradation have been well documented. The stability of β-tubulin mRNA is determined by the first 13 nucleotides of the coding sequence in a translation-dependent manner (43). Transferrin receptor mRNA stabilization is determined by the iron-responsive elements located within its 3′UTR (23). c-*fos* mRNA degradation can be monitored through the shortening of the poly(A) tail of the c-*fos* 3′UTR fused to the 3′ end of the RBG gene (5). The degradation of certain yeast mRNAs has been shown to be mediated by a deadenylation-independent pathway (30). Previous attempts to mimic cytokine mRNA induction, however, were largely unsuccessful when the 3′UTR of the cytokine mRNA was used in the chimeric constructs (19, 34). Although extensive studies have been carried out to characterize the *cis*-RNA elements (38, 40) and *trans*-RNA binding factors (4, 16, 28, 44, 49), little is known about the structural requirements for cytokine mRNA stabilization.

Our results demonstrate that IL-11 mRNA stabilization is a complex event which cannot be simply interpreted by the presence of a single RNA *cis*-element. In agreement with a recent report (6), we have shown that AU-rich motifs cannot act as a universal RNA destabilizer, since AU-rich motifs from IL-11 mRNA did not always render instability to all of the chimeric IL-11 transcripts studied so far. The localization of RNA *cis*-elements controlling IL-11 mRNA stability was carried out by various deletions of the CMV-IL-11$^{1-1202}$ transcript, which was shown to mimic the endogenous IL-11 mRNA stabilization in response to TPA stimulation. Each of the deletion transcripts, with the exception of CMV-IL-11$^{767-1202}$, contains an ORF either by utilizing the original translation start codon or by using an internal AUG codon. It needs to be pointed out here that the interpretation of the experimental data obtained by our deletional analysis may be oversimplified, since it does not take into the account the fact that deletion of the normal

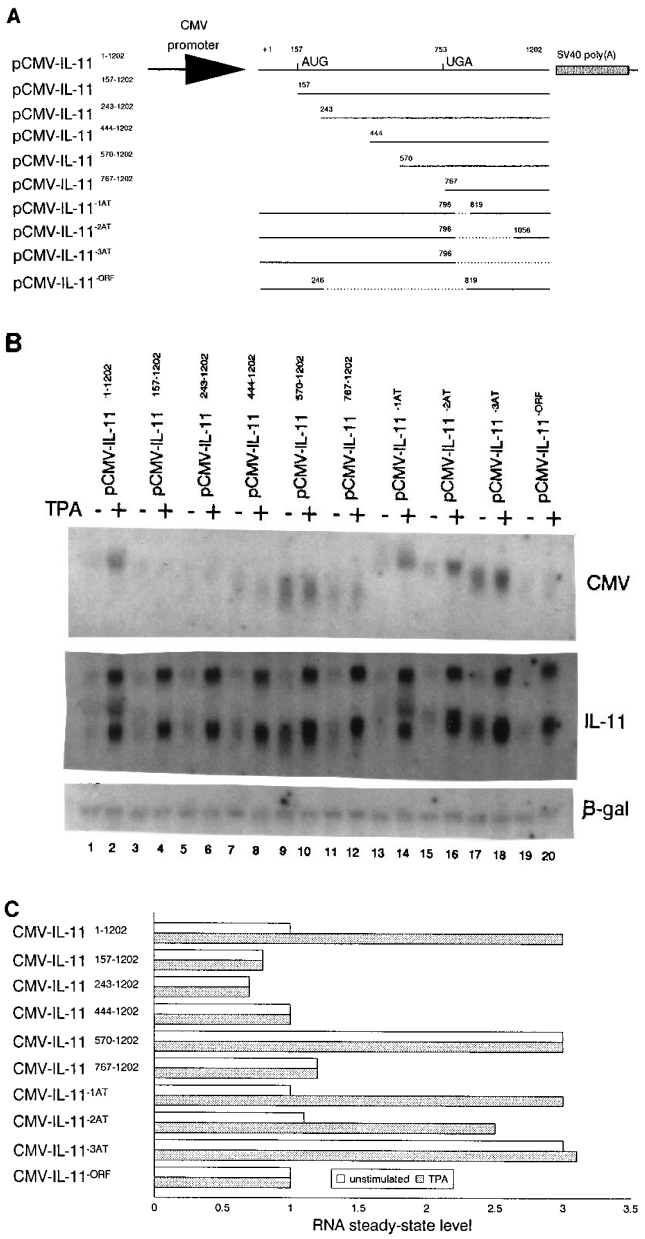


FIG. 7. Northern analysis of CMV-IL-11 deletion transcripts. (A) Structures of CMV-IL-11 deletion constructs. The number at the end of each construct represents the position of the deletion. Regions of internal deletions within the 3′UTR or coding region of the IL-11 mRNA are indicated as dotted lines. (B) Northern analysis of CMV-IL-11 deletion transcripts. CMV-specific probe was used to detect the CMV-tagged exogenous transcripts. The same membrane was also probed with IL-11 DNA to show TPA-inducible expression of the IL-11 gene. β-Galactosidase mRNA was generated from cotransfection of pCMV-β-gal and used as an internal control. (C) The steady-state level of each CMV-IL-11 deletion transcript was normalized according to the signal of the β-galactosidase control.

translation initiation codon, alteration of the normal reading frame, and premature termination of translation may affect mRNA stability. Even though further mapping analysis is required to determine the exact sequences involved, the results from our deletion experiments (Fig. 7) suggested that IL-11 mRNA degradation requires the participation of RNA sequences from the coding region of IL-11 mRNA in conjunction with sequences from the 3′UTR. Previously, it was reported that c-*fos* and c-*myc* mRNAs contain stability determinants in their coding regions (3, 39) which are distinct from stability determinants in their 3′UTRs, and the unstable yeast STE3 mRNA contains sequences both in its coding region and 3′UTR which can stimulate mRNA decay (15). To explain the different levels of RNA stability possessed by various IL-11 deletion transcripts, we hypothesize that IL-11 mRNA stability is controlled by a buffering element present between 570 and 767, flanked by two destabilizing elements between 444 and 569 and 1056 and 1202. The presence of the buffering element may override only one of the two destabilizing elements to confer stability to the deletion transcripts such as CMV-IL-$11^{570–1202}$ and CMV-IL-11^{-3AT}. It should be emphasized that although a number of deletion transcripts are unstable in PU-34 cells, these transcripts are not necessarily stabilized by TPA stimulation. In PU-34 cells, TPA-induced IL-11 mRNA stabilization requires, in addition to the coding region and the 3′UTR, the sequences from the 5′UTR of the IL-11 mRNA. We speculate that the multiple region requirement in TPA-induced IL-11 mRNA stabilization may be an indication of the involvement of RNA folding: proper RNA folding may not only determine the stability of a given CMV-IL-11 deletion transcript, it may also determine the TPA responsiveness of the same RNA molecule. Even though a number of CMV-IL-11 deletion transcripts may assume folding patterns susceptible to nuclease degradation, only a unique folding pattern (requiring the presence of IL-11 mRNA sequences from the 5′UTR, coding region, and 3′UTR for its proper conformation) is intrinsically unstable and is capable of undergoing stabilization after TPA stimulation.

Our conclusions about TPA-induced IL-11 mRNA stabilization should be applicable to IL-1α induction of IL-11 mRNA. Although IL-1α and TPA induce IL-11 mRNA through activation of different protein kinase systems in PU-34 cells, their effects on different chimeric IL-11 transcripts appear to be the same (reference 46 and unpublished data). A likely scenario is that protein tyrosine kinase signaling (activated by IL-1α) and PKC signaling (activated by TPA) converge on certain common factors, such as sequence-specific RNA-binding proteins and endo- or exonucleases, which may act in concert with multiple RNA *cis*-elements to regulate IL-11 mRNA stability in bone marrow stromal cells.

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