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Posttranscriptional Regulation of IL-10 Gene Expression Through Sequences in the 3′-Untranslated Region[1]

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IL-10 is an 18-kDa immunoregulatory cytokine the transcription of which is controlled by the ubiquitously expressed transcription factors Sp1 and Sp3. Although many cell types express IL-10 mRNA, not all make detectable amounts of protein, and levels of protein expression vary enormously. We show here that much of this variation can be accounted for by posttranscriptional mechanisms. Multiple copies of potential mRNA destabilizing motifs AUUUA and related sequences can be found to the 3′-untranslated region (UTR) of IL-10 mRNA distributed through three potential regulatory regions. Evidence of RNA-destabilizing activities in all three regions was deduced from luciferase reporter assays. The half-life of RNA containing the 3′-UTR of IL-10 mRNA was quite short in both nonstimulated ($t_{1/2}$ = 1 h), and PMA-stimulated EL-4 cell ($t_{1/2}$ = 3 h). In contrast, the half-life of RNA lacking the 3′-UTR was much longer ($t_{1/2}$ = >12 h) whether cells were stimulated or not. This suggests that many cells are poised to secrete IL-10 and will do so if they receive appropriate posttranscriptional signals. ***The Journal of Immunology*****, 2000, 165: 292–296.**

Interleukin-10 is a potent antiinflammatory and immunosuppressive cytokine, initially described as "cytokine synthesis inhibitory factor" because of its ability to suppress production of IFN-γ, IL-2, and proinflammatory cytokines (1, 2).

IL-10 can be expressed by a wide range of cell types including T cells, B cells, monocytes, macrophages, and keratinocytes, and many tumor cells (1, 3–8). Recently, we have shown that IL-10 transcription is regulated by the transcription factors Sp1 and Sp3 which are known to be constitutively and ubiquitously expressed (9). We have also shown that the low levels of IL-10 mRNA in resting T cell and macrophage cell lines as well as bone marrow dendritic cells could be up-regulated by activation (9). Weak PMA response elements were identified between −802 and −305 of the IL-10 promoter (9). However, the weak activity of the response elements could not account for the large increase in IL-10 mRNA accumulation upon PMA stimulation. We wondered whether IL-10 production is regulated by other transcriptional regulatory elements such as enhancers and/or posttranscriptional control mechanisms. The latter possibility had been implied by the finding that a T cell clone that was actively transcribing IL-10 did not yield detectable mature IL-10 mRNA (10). Consistent with this, we observed multiple copies of potential mRNA-destabilizing motifs AUUUA and AU-rich elements (ARE)[3] (11) in the 3′-untranslated region (UTR) of mouse IL-10 mRNA (12).

Our own observations on posttranscriptional control of this gene began with the finding that although comparable promoter activity could be measured in resting and stimulated EL4 (T cell) and RAW 264 (macrophage) cell lines, the levels of IL-10 mRNA varied between the lines after activation (9). In this article, we have been able to show directly that the 3′-UTR of IL-10 mRNA is indeed destabilizing for IL-10 mRNA. We have identified the sequences concerned using transient and stable transfectants. The results of this and the IL-10 promoter analysis described in the accompanying paper (9) led us to conclude that many cell types might be constitutively transcribing IL-10 mRNA but that much of the regulation is determined posttranscriptionally. This might ensure that the immune system can react rapidly in moderating an inflammatory stimulus.

Materials and Methods

Plasmids

Luciferase reporter plasmids were constructed using the pGL3-Control Vector (Promega, Madison, WI) carrying a SV40 promoter/luciferase expression unit. Four fragments, AU1 (+727 to +818), AU2 (+807 to +936), AU3 (+1157 to +1239), and AU4 (+727 to +1239), containing the 3′-UTR sequences of IL-10 cDNA were amplified by PCR and cloned into an *Xba*I site (+1934) located between the luciferase gene and the poly(A) signal in the pGL3-Control Vector. The pGL3-Basic Vector was used as a negative control plasmid for a luciferase assay.

A fusion gene of mouse IL-10 (+11 to +609) (12) and the human Ig (Ig) heavy chain constant region (+427 to +1132) (13) was amplified and assembled by PCR. This fragment was cloned downstream of the EF-1α promoter in the pMTF expression vector carrying a neomycin resistance gene. The resulting plasmid (IL-10/Ig "no-UTR") was used to construct plasmid IL-10/Ig "plus-UTR." A fragment containing a 3′-UTR sequence (+610 to +1295) of IL-10 cDNA was amplified and cloned downstream of the IL-10/Ig fragment in plasmid IL-10/Ig no-UTR.

Cell culture and transfection

EL-4 and RAW 264 cells were cultured in IMDM with 5% FCS. For the luciferase reporter assay, 2×10^7 cells were electroporated with 10 μg luciferase reporter plasmids and 1 μg pRL-TK (Promega) as an internal control plasmid. Transfected cells were cultured in 10 ml IMDM-10% FCS. If required, PMA (50 ng/ml) or LPS (20 μg/ml) was added 6 h postelectroporation. Cells were harvested 48 h postelectroporation, and luciferase activities were analyzed by the Dual-Luciferase Reporter Assay System (Promega). These assays were repeated more than three times, and the activities were normalized to *Renilla* luciferase activities.

To generate stable transfectants, 2×10^6 EL-4 cells were electroporated with 20 μg of plasmids IL-10/Ig plus-UTR or no-UTR. Stable transfectants were selected by G418 (1 mg/ml).

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[3] Abbreviations used in this paper: ARE, AU-rich element; UTR, untranslated region.

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Enzyme-linked immunosorbent assay

EL-4 transfectants were cultured with or without PMA (50 ng/ml) for 24 h. Culture supernatants were harvested and diluted (1/1 to 1/512). IL-10/Ig fusion protein in culture supernatants was analyzed by ELISA using anti-human IgG (The Jackson Laboratory, Bar Harbor, ME) as the capture Ab and biotin conjugated anti-mouse IL-10 (SXC10, PharMingen, San Diego, CA) as the detection Ab.

Analysis of RNA half-life

EL-4 transfectants generated using plasmids IL-10/Ig no-UTR or plus-UTR were cultured for 6 h with or without PMA (50 ng/ml). Actinomycin D was added (10 μg/ml), and cells were harvested at different time points (0, 0.5, 1, 1.5, 2, 3, and 4 h). RNA were isolated, and IL-10/Ig RNA was analyzed by Northern blot hybridization using a human Ig constant-region cDNA as a probe. As a control, HPRT mRNA was also analyzed. To measure the half-life of IL-10/Ig RNA, the intensity of IL-10/Ig and HPRT bands was analyzed by phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

Results

Identification and characterization of mRNA destabilizing activities in the 3′-UTR of IL-10 mRNA

Potential mRNA destabilizing motifs AUUUA and related sequences (AUUUUA, AUUUUUA) are located in the 3′-UTR of IL-10 mRNA. The relative positions of these sequences are indicated in Fig. 1*A*. We have classified these AUUUA and related sequences into three potential regulatory regions by both location and surrounding sequences. The first consists of three nonclustered AUUUAs located between +741 and +806, and with surrounding sequences that are not AU-rich (Fig. 1, *A* and *B*, fragment AU1). The second consists of clustered AUUUAs located between +827 and +895. Here, the surrounding sequences are of the typical AU-rich type (Fig. 1, *A* and *B*, fragment AU2) which are similar to those previously identified as mRNA-destabilizing elements (11). The third is located near the 3′-end of IL-10 mRNA (between +1183 and +1225). It contains nonclustered AUUUA and AUUUUUA sequences with surrounding sequences that are not AU-rich (Fig. 1, *A* and *B*, fragment AU3). To investigate whether these sequences regulate stability of IL-10 mRNA, a luciferase reporter assay was performed. Four fragments containing AUUUA and related sequences (AU1, +727 to +818; AU2, +807 to +936; AU3, +1157 to +1239 ; AU4, +727 to +1239) from the 3′-UTR of IL-10 cDNA (Fig. 1, *A* and *B*) were cloned between the luciferase cDNA and the poly(A) additional signal in the pGL3-Control Vector carrying a SV40 promoter/luciferase expression unit. Transcripts from the resulting plasmids would therefore contain the luciferase-coding region plus a part of the IL-10 mRNA 3′-UTR sequence. Luciferase activities in transfected cells using these plasmids were compared with that using a control plasmid (no insert), pGL3-Control Vector. If the inserted fragments from the IL-10 3′-UTR contain RNA-destabilizing activity, the luciferase activity generated will be less than that of the control plasmid. In non-stimulated EL-4 cells, reporter activity fell by ~50% when any of the AU1, AU2, or AU3 sequences in the 3′-UTR were used (Fig. 1*C*) and by 80% with plasmid AU4, which carries the longest 3′-UTR sequence (containing AU1, AU2, and AU3 sequences) (Fig. 1*C*). However, the results in PMA-stimulated EL-4 cells were different. We observed a reduction of reporter activity with plasmids AU1, AU3, and AU4, but not with plasmid AU2. This suggests that some part of mRNA destabilizing activity may have been overridden by PMA stimulation, whereas some still remained functional. Similar results were obtained using macrophage cell line RAW 264 cells except that the strong mRNA-destabilizing activity of fragment AU3 was sustained in LPS-stimulated RAW 264 cells, implicating the region between +1157and +1239 in this process. These results suggest that the stability of IL-10 mRNA is regulated by heterogeneous mRNA-destabilizing elements that may be dependent on cell type and nature of activation signals located in the 3′-UTR of IL-10 mRNA.

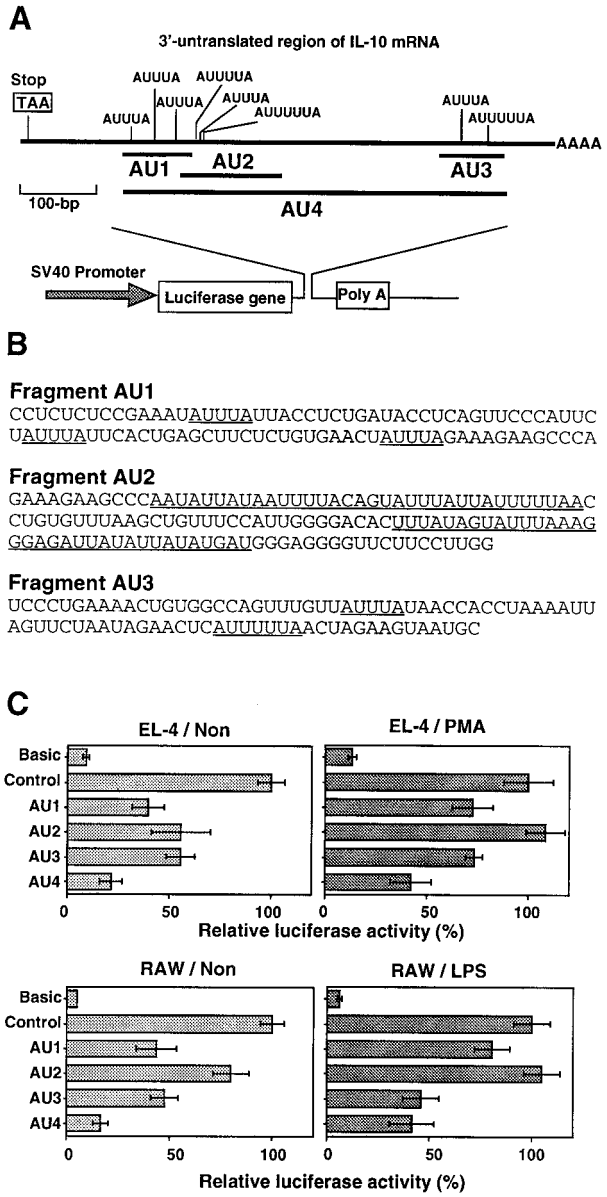

FIGURE 1. Identification of RNA destabilizing activities in the 3′-UTR of IL-10 mRNA. *A*, Relative positions of AUUUA, AUUUUA, and AUUUUUA sequences in 3′-UTR of IL-10 mRNA are indicated. The structures of luciferase reporter plasmid AU1 to AU4 are shown below the map. The positions of inserted fragments (AU1 to AU4) in the reporter plasmids are indicated by solid lines. *B*, Nucleotide sequences of fragments AU1 to AU4 are shown. AUUUA, AUUUUA, AUUUUUA, and AU-rich sequences are underlined. *C*, Luciferase activities generated using pGL3-Basic Vector (Basic) (negative control, no promoter), Plasmid AU1, AU2, AU3, and AU4 in EL-4 and RAW 264 cells were compared with that using pGL3-Control Vector (Control) (no insert). If required, PMA (50 ng/ml) or LPS (20 μg/ml)was added 6 h postelectroporation. Luciferase assays were repeated more than three times, and the activities were normalized using *Renilla* luciferase activity.

IL-10 production is negatively regulated by its 3′-UTR in nonstimulated EL-4 cells

To further investigate IL-10 mRNA stability, we generated stable EL-4 transfectants using IL-10 transgenes possessing or lacking

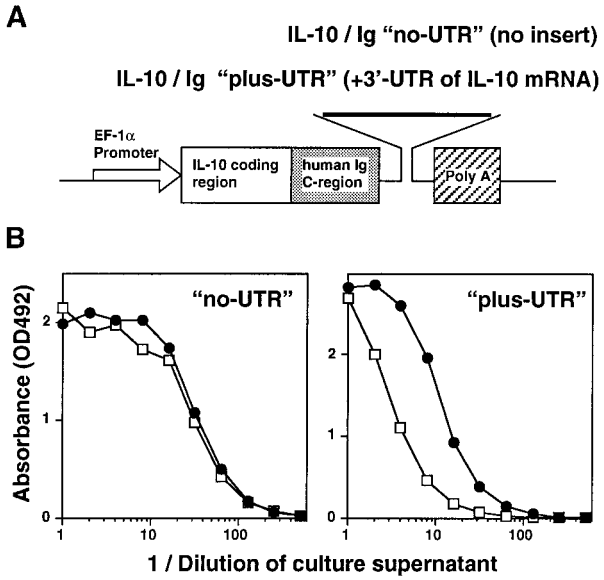

FIGURE 2. Impact of the 3′-UTR of IL-10 mRNA on translation of the protein. *A*, Stable transfectants were generated using plasmids IL-10/Ig "no-UTR" and "plus-UTR". The structure of the expression plasmids is shown. IL-10/Ig "plus-UTR" contains the 3′-UTR of IL-10 mRNA (+610 to +1295) indicated by a solid line. *B*, Culture supernatants of nonstimulated (open square) and PMA-stimulated (closed circle) transfectants IL-10/Ig "no-UTR" and "plus-UTR" were titrated, and IL-10/Ig protein was detected by ELISA using anti-human IgG as a capture Ab and anti-mouse IL-10 as a detection Ab.

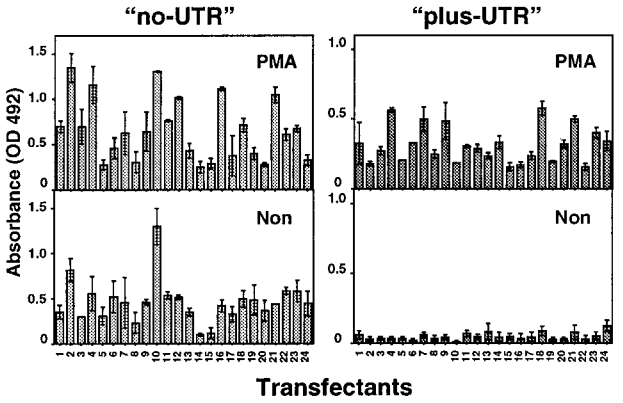

FIGURE 3. Measurement of the impact of the 3′-UTR on the expression of an IL-10/Ig fusion protein in stable transfectants. EL-4 cells were transfected using plasmid IL-10/Ig "no-UTR" or "plus-UTR". Twenty-four independent transfectants were randomly selected and cultured for 24 h without (Non) or with (PMA) PMA (50 ng/ml). IL-10/Ig protein in the culture supernatants was detected by ELISA.

the 3′-UTR sequence. To distinguish the transgene product from endogenous IL-10, we used an IL-10/human Ig constant-region fusion protein gene (IL-10/Ig), which was cloned into an expression vector carrying the constitutive EF-1α promoter to ensure strong expression, and a neomycin resistance gene for selection (Fig. 2*A*). The resulting plasmids IL-10/Ig "no-UTR" (lacking the 3′-UTR of IL-10 cDNA) or IL-10/Ig "plus-UTR" (possessing the 3′-UTR) were transfected into EL-4 cells, and clones of stable transfectants were selected. The ELISA assay, which measured the Ig component of the IL-10/Ig construct, was performed using culture supernatants diluted appropriately so as to provide the most sensitive readout. We observed no difference in the amount of IL-10/Ig protein that accumulated in the culture supernatants of nonstimulated and PMA-stimulated "no-UTR" transfectants (Fig. 2*B*). However, the results with "plus-UTR" transfectants were different. PMA stimulation raised the level of protein some 4- to 5-fold almost up to the level of the nonstimulated groups (Fig. 2*B*). To exclude positional effects of the transgenes, 24 independent transfectants were analyzed. EL-4 cells were transfected with the test plasmids by electroporation and immediately inoculated at limiting dilution into 96-well plates. Twenty-four transfectants were randomly selected and subcultured. One set of subcultures was stimulated with PMA for 24 h, whereas the other was not. Culture supernatants from 48 clones were diluted 1/50 for the ELISA assay. The reaction was stopped before the no-UTR group achieved saturation. Significant amounts of IL-10/Ig protein was detected in almost all culture supernatants of the "no-UTR" transfectants (22 of 24 nonstimulated and 24 of 24 PMA-stimulated cells) (Fig. 3). On the other hand, IL-10/Ig protein was detected only in the culture supernatants of all PMA-stimulated "plus-UTR" transfectants, but not from the nonstimulated counterparts (Fig. 3). These results suggest that IL-10 production was negatively regulated by the 3′-UTR of IL-10 mRNA in nonstimulated EL-4 cells, although PMA stimulation is capable of partially overriding the effect.

The IL-10/Ig mRNA half-life increases significantly after PMA stimulation

We have shown that IL-10 production is regulated through posttranscriptional mechanisms. We estimated the extent of IL-10 mRNA stability in nonstimulated and PMA-stimulated EL-4 cells by measuring the half-life of IL-10/Ig mRNA. EL-4 transfectants were cultured with or without PMA for 6 h and then treated with actinomycin D to block transcription. RNAs were isolated at different time points and analyzed by Northern blot hybridization using human Ig and mouse HPRT cDNAs as probes (Fig. 4). The "no-UTR" RNA was quite stable in both nonstimulated and PMA-stimulated EL-4 cells (half-life, >12 h) (Fig. 4); in contrast, the half-life of IL-10/Ig "plus-UTR" RNA was 1 h in nonstimulated cells increasing to 3 h in the PMA-stimulated transfectants. Within the 6 h PMA-stimulated groups, the "no-UTR" RNA was more stable than "plus-UTR" RNA, suggesting that some mRNA destabilizing signals in the 3′-UTR of IL-10 mRNA were still active.

Discussion

In the accompanying paper (9), we have shown that the transcription of IL-10 gene is regulated by the factors Sp1 and Sp3 and that IL-10 mRNA could be up-regulated by PMA activation. We were also able to detect weak PMA response elements in the IL-10 promoter (9). However, because constitutively expressed Sp1 is a key transcription factor in IL-10 expression, it is difficult to explain the up-regulation of IL-10 mRNA by promoter activity alone. Here, we show that IL-10 expression is controlled by posttranscriptional regulation. Constitutively expressed IL-10 mRNA was unstable in nonstimulated cells, and therefore poorly translated. Activation by PMA was able to restabilize the mRNA, thus leading to protein production. We infer that there must be equivalent signals in normal cells that can also increase IL-10 mRNA stability.

We have classified AUUUA motifs and the related sequences into three potential regulatory regions by both location and surrounding sequences. These regions identified in the mouse IL-10 mRNA sequence (12) are also conserved in the human sequence (14). AUUUA motifs between +807 and +936 (Fig. 1, *A* and *B*, fragment AU2) of the 3′-UTR are present in AU-rich sequences.

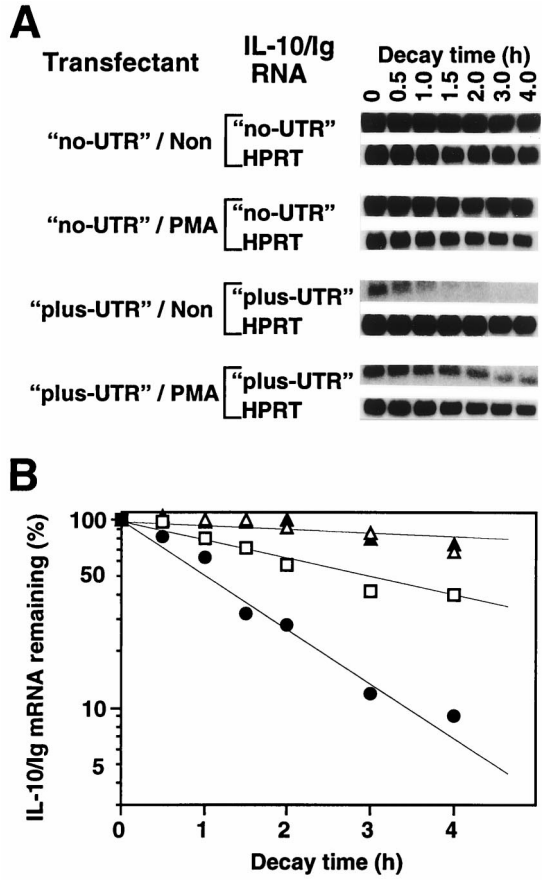

FIGURE 4. RNA stability is regulated by posttranscriptional control through 3′-UTR of IL-10 mRNA. *A*, RNAs were isolated at different time points (0, 0.5, 1, 1.5, 2, 3, and 4 h) post-actinomycin D treatment from nonstimulated (Non) and PMA-stimulated (PMA) transfectants IL-10/Ig "no-UTR" and "plus-UTR". The same transfectants were previously used in Fig. 2. IL-10/Ig RNA was analyzed by Northern blot hybridization using human IgG constant region cDNA as a probe. As a control, hypoxanthine phosphoribosyltransferase (HPRT) RNA was also measured. *B*, To determine RNA half-life, the intensities of IL-10/Ig RNAs from nonstimulated IL-10/Ig no-UTR (▲), 6-h PMA-stimulated IL-10/Ig no-UTR (△), nonstimulated IL-10/Ig plus-UTR (●), and 6 h PMA-stimulated IL-10/Ig plus-UTR (□) transfectants were assessed by phosphorimaging.

mRNA-destabilizing activity in this particular region was not observed in PMA-stimulated EL-4 cells and LPS-stimulated RAW 264 cells. However, mRNA-destabilizing activity remained in the other two regions (AU1 and AU3) in both cells. Particularly, strong mRNA-destabilizing activity in the fragment AU3 was observed in LPS-stimulated RAW 264 cells. Stabilization of IL-10 mRNA may be dependent on cell type and/or stimuli. We have also confirmed these data with stable transfectants.

Regulatory region AU2 in the 3′-UTR of IL-10 mRNA contains clustered AUUUA and related sequences, i.e., long AREs (Fig. 1*B*, fragment AU2) which is similar to other mRNA-destabilizing sequences observed in 3′-UTR of many cytokine genes and oncogenes. Recently, several proteins binding to AREs (15–19) and proteasomes recognizing AREs (20) have been identified, suggesting that these proteins and proteasomes might be involved in mRNA destabilization. IL-10 mRNA stability might be regulated by these factors and proteasomes through region AU2. mRNA-destabilizing activity in AU2 seems only to function in nonstimulated EL-4 cells. In contrast, in stimulated EL-4 cells, mRNA stability was no different to the control. This suggests that any putative destabilizing factor(s) may not be expressed and/or be unstable in stimulated cells. Alternatively, it may be that AU2 contains a PMA-responsive element that mediates mRNA stabilization. This may be overridden by the destabilization activities of AU1 and AU3 in full length sequence. In nonstimulated RAW 264 cells, the luciferase activity produced by the AU2 plasmid was only slightly less than that of the control plasmid. Because this reduction of luciferase activity is not large, we cannot conclude the presence of mRNA-destabilizing activity. However, in LPS-stimulated RAW 264 cells, we did not observe any reduction in luciferase activity with the AU2 plasmid. This suggests that destabilizing activity was lacking in stimulated cells. Because RAW 264 is a macrophage cell line, RAW 264 cells might have been partially stimulated (through "danger") by DNA transfection. Under these circumstances, RNA-destabilizing activity might have been partially inhibited.

Although regulatory region AU1 and AU3 contain AUUUA and AUUUUUA sequences, these sequences are not clustered, and the surrounding sequences are not AU rich. Therefore, these regions are not similar to typical AREs previously identified as mRNA-destabilizing sequences. Lagnado et al. (21) also proposed that a minimal mRNA destabilizing sequence is not AUUUA but may be UUAUUUA(U/A)(U/A). The IL-10 3′-UTR contains similar sequences, AUAUUUAUU and CUAUUUAUU in fragment AU1 and the identical sequence UUAUUUAUA in fragment AU3. These sequences might function as mRNA-destabilizing sequences. A protein binding to the short AU-rich sequence UUAUUUAUU has also been identified (19). Such a factor may regulate IL-10 mRNA stability through the region AU1 and AU3.

Comparison of the luciferase activities produced using AU1 and AU3 plasmids with that using control plasmid in PMA-stimulated EL-4 cells resulted in a small reduction of luciferase activity (Fig. 1, AU1 and AU3 in EL-4/PMA) (given the inherent variability of the luciferase assay, we could not conclude that these reductions were significant). However, the same plasmids in nonstimulated EL-4 cells produced large reductions in luciferase activity. We conclude that the putative RNA-destabilizing factors binding to AU1 and AU3 may express poorly in PMA-stimulated cells but also that these factors may be more stable than those binding to AU2, resulting in the outcome of no apparent destabilizing activity on AU2 and partial destabilizing activity on AU1 and AU3. We need additional experiments to resolve this issue (e.g., identification and characterization of factors binding to AU1, AU2, and AU3).

mRNA-destabilizing activity in the longest fragment (AU4) is stronger than the others. We speculate that this might be caused by gathering of destabilizing activity in each fragment. However, it is possible that these regions might cooperate with each other to regulate RNA stability.

In LPS-stimulated RAW 264 cells, similar RNA-destabilizing activities in fragments AU3 and AU4 (fragment AU4 contains the sequence in fragment AU3) were detected. IL-10 mRNA in LPS-stimulated RAW 264 cells seems to be destabilized mainly through the sequence in fragment AU3. The likelihood is, then, that the different RNA destabilizing elements described here might depend on distinct destabilizing signals. The corollary is that cells capable of producing high levels of IL-10 (Tr1 cells, Th2 cells, and certain tumors) may fail to destabilize mRNA.

The results of this and previous (9) papers lead us to the "regulatory" model of IL-10 expression. Transcription of IL-10 is ubiquitously and constitutively regulated by Sp1 and Sp3. In nonstimulated cells, the constitutively expressed IL-10 mRNA is kept at low levels through mRNA-destabilizing signals. Upon activation,

IL-10 transcription is up-regulated through PMA response elements located in IL-10 promoter, and then IL-10 mRNA levels are further increased through RNA stabilization controlled by regulatory regions located in the 3′-UTR. Variable expression levels of IL-10 may be determined by the heterogeneity of mRNA-destabilizing signals.

This combination of ubiquitous transcriptional and posttranscriptional mechanisms for IL-10 expression might be critical to the role of this cytokine. Low concentrations of IL-10 might maintain the background tone of the immune system, thus controlling proinflammatory cytokine expression. When appropriate activation signals arise, IL-10 production could be rapidly up-regulated at sites of inflammation to limit proinflammatory cytokine cascades and restore immune homeostasis via negative feedback.

Acknowledgments

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