IL-10 Gene Expression Is Controlled by the Transcription Factors Sp1 and Sp3

Masahide Tone, Mark J. Powell, Yukiko Tone, Sara A. J. Thompson and Herman Waldmann

J Immunol 2000; 165:286-291; doi: 10.4049/jimmunol.165.1.286

http://www.jimmunol.org/content/165/1/286

References

This article cites 41 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/165/1/286.full#ref-list-1

Subscription

Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
IL-10 Gene Expression Is Controlled by the Transcription Factors Sp1 and Sp3

Masahide Tone, Mark J. Powell, Yukiko Tone, Sara A. J. Thompson, and Herman Waldmann

IL-10 is an 18-kDa cytokine with a key role in homeostatic control of inflammatory and immune responses. We have investigated how transcription of the IL-10 gene is regulated, so as to be able to understand the circumstances of IL-10 expression in both health and disease. In the mouse, IL-10 gene expression is regulated by a TATA-type promoter with a critical cis-acting element containing GGA repeats located at −89 to −77. Its complementary sequence is similar to the cis-acting elements (TCC repeats) in the promoters of genes encoding epidermal growth factor receptor and CD58. All these elements comprise a common CCTCCT sequence with less conserved C + T-rich sequences. Eliminating this CCTCCT sequence results in a marked reduction in promoter activity, suggesting a necessary role in IL-10 gene expression. Despite its dissimilarity to the G + C-rich Sp1 consensus sequence (GC box), Sp1 and Sp3 transcription factors could be shown to bind to this motif. The requirement for Sp1 and Sp3 in transcription of IL-10 was confirmed using Drosophila SL2 cells, which lack endogenous Sp factors. These results suggest that the transcription of IL-10 is positively regulated by both Sp1 and Sp3. The Journal of Immunology, 2000, 165: 286–291.

Interleukin-10 is an 18-kDa glycoprotein (1, 2), initially identified as a product of Th2 cells that has subsequently been shown to be produced by a wide range of cell types, including Th1 cells, Tr1 cells, B cells, monocytes, macrophages, keratinocytes, and many tumor cells (2–8). It behaves like a potent antiinflammatory and immunosuppressive molecule, inhibiting the production and release of IL-2 and IFN-γ and a range of proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-12 (2, 9).

Despite its crucial role in immunoregulation, the factors regulating expression of IL-10 gene are poorly understood at both the transcriptional and posttranscriptional levels. Control at the posttranscriptional level may permit a rapid homeostatic response to inflammation.

Materials and Methods

Reverse transcriptase-PCR

IL-10 mRNA levels were determined by RT-PCR. First strand cDNA was prepared from 1 µg total RNA using an oligo(dT) primer. This reaction mixture (20 µl) was diluted with 80 or 180 µl water, and then 0.5, 5, 10, or 20 µl of the cDNA solution were utilized for PCR amplification using sense and antisense primers (IL-10; 25 cycles, hypoxanthine phosphoribosyltransferase (HPRT); 17 cycles). The primer sequences used were: IL-10 sense primer, CCAGTTTTACCTGTTAGAAGGTGATG; IL-10 antisense primer, TGTCAGCTCGTGCAAGCAGACTC; HPRT sense primer, ACAGCCCAAAAATGTTGAAG; and HPRT antisense primer, TCTCGGAGGGCGAGCTCGAGCTC. The amplified cDNAs were detected by Southern blot hybridization using cDNA probes.

Mapping of transcription start sites

To determine transcription start sites, we performed the rapid amplification of cDNA ends procedure (RACE) as described previously (16). cDNA for IL-10 was amplified using a poly(C) primer and an antisense primer, TCTGGGGACGCAGCAACTGAC. The amplified cDNAs were cloned, and the DNA sequences were determined.

Assessment of promoter activity using the luciferase assay

Luciferase reporter plasmids were constructed using deletion mutants of the 5′-flanking region of the IL-10 gene and pGL3-Basic Vector (Promega, Madison, WI). This 5′-flanking fragment was subcloned from a λ IL-10 genomic clone, a kind gift from Dr. K. Moore (DNAX Research Institute, Palo Alto, CA). These deletion mutants (D1 to D11) were generated by the exonuclease III/mung bean nuclease procedure or by PCR. The structures of these deletion mutants are indicated in Fig. 2A. The resulting deletion mutants, Km/ISacI fragments, were cloned upstream of the luciferase gene in pGL3-Basic Vector. The SacI site (3′-end of the deletion mutants) is located at +63 in the IL-10 gene.

To construct plasmid D1 CCT KO and D6 CCT KO, the CCTCCT sequences (−85 to −80) in plasmid D1 and D6 were replaced by GAATTc (EcoRI site) by PCR.
EL-4 and RAW 264 cells (2 × 10^7) were transfected by electroporation using 10 μg of luciferase reporter plasmids with 0.1 μg pRL-SV40 or pRL-TK (Promega) as an internal control plasmid. If required, cells were stimulated with PMA (50 ng/ml)/ionomycin (1 μM) or LPS (20 μg/ml) 7 h postelectroporation. Cells were harvested 48 h postelectroporation, and promoter 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.

*Drosophila* SL2 cells (2 × 10^6) were transfected using 0.5 μg of the luciferase reporter plasmid containing the IL-10 promoter (D6 ‐ 305 to +63) with pPac, pPacSp1, and pPacUSp3. Scheider SL2 cells were obtained from American Type Culture Collection (Manassas, VA). The expression plasmids were kind gifts from Dr. G. Suske (Philipps-Universitat, Marburg, Germany). Transfection was performed using Ptx-20 transfection reagent (Promega). Luciferase activities were analyzed 48 h posttransfection. These assays were repeated more than three times.

**Gel mobility band shift assays**

A nuclear extract from EL-4 cells was prepared using methods described by Dignam et al. (17). Gel mobility band shift assay was performed as described previously (18). To perform super gel mobility band shift assay, the reaction mixture was incubated with anti-Sp1 (Santa Cruz, PEP2) and/or anti-Sp3 (Santa Cruz D-20) Abs.

**DNase I footprinting**

A DNase I footprinting assay was performed according to the method described by Dynan and Tjian (19). A DNA fragment of the promoter region was amplified by PCR using a 32P-labeled primer (CTGAAGGCTCAGTGGGGCCCTTCC) and an unlabeled primer (CCAGTTCTTTAGGCCTTA CAATGC). The PCR product was isolated and incubated with 0–50 μg of a nuclear extract in 20 mM HEPES (pH 7.9), 2 mM MgCl2, 50 mM NaCl, 1 mM DTT, 20% glycerol, and 4 μg poly[d(I-C)]-poly[d(I-C)]. The mixtures were treated with DNase I at 25°C for 1 min. Purified DNAs from these mixtures were analyzed by sequencing gel with sequence ladders generated using the same labeled primer.

**FIGURE 1.** Expression of IL-10 mRNA in different type cells. IL-10 mRNA levels in indicated cell lines and cells were analyzed by RT-PCR. cDNAs were prepared using RNAs from indicated cells. A 20-μl sample of the cDNA solution was diluted with 180 μl of water. For PCR amplification, 0 μl (lane 0), 5 μl (lane 1), 10 μl (lane 2), and 20 μl (lane 3) of the diluted cDNA solution were used. To detect low levels of IL-10 mRNA, 20 μl of the cDNA reaction mixture were diluted with 80 μl water and then 0 μl (lane 0), 5 μl (lane 4), 10 μl (lane 5), and 20 μl (lane 6) of this diluted cDNA solution were used for PCR amplification. Amplified cDNA fragments were detected by Southern blot hybridization using IL-10 and HPRT cDNA probes.

**FIGURE 2.** Promoter activity of the IL-10 gene. A, Luciferase reporter plasmids were constructed using pGL3-Basic Vector (Promega) and 5′-flanking fragments of the IL-10 gene. The position of TATA box, the relative lengths and positions of the fragments (solid lines), and the position of the 5′-ends of these fragments (D1 to D11) are indicated. B, Luciferase activities generated using the reporter plasmids (D1 to D11) were compared with that using the negative control plasmid (no insert) pGL3-Basic vector (Basic) in EL-4, PMA/ionomycin-stimulated EL-4, RAW 264, and LPS-stimulated RAW 264 cells. Luciferase assays were repeated more than three times, and the activities were normalized using Renilla luciferase activity.

**Results**

**cis-Acting elements for IL-10 gene expression**

IL-10 production has been demonstrated in diverse cell types (2–8). We have analyzed IL-10 mRNA levels by RT-PCR using RNAs from a fibroblast cell line, L929, a T cell line, EL-4, a macrophage cell line, RAW 264, and a B cell line, A20, as well as bone marrow-derived dendritic cells (bmDC). IL-10 mRNA was detected in all these cell types, albeit at different levels (Fig. 1). High level expression of IL-10 mRNA was observed in PMA/ionomycin-stimulated EL-4, A20, PMA-stimulated A20, and LPS-stimulated bmDC (Fig. 1). We have investigated IL-10 transcription in two of these populations, namely, EL4 (lymphoid) and RAW 264 (nonlymphoid) cells.

A transcription start site for the IL-10 gene was determined by 5′-RACE using RNA from PMA/ionomycin-stimulated EL-4, PMA-stimulated A20 cells, and LPS-stimulated bmDC. A total of 26 of 42 5′-RACE clones contained the same 5′-end (EL-4, 7 of 11; A20, 11 of 20; bmDC, 8 of 11), and this position was mapped 30 bp downstream of a typical TATA box sequence (i.e., 67 bp upstream of the first ATG), suggesting that IL-10 transcription might be regulated by a TATA-type promoter. The major transcription start site is then defined as position +1. The other 5′-ends of 12 of 42 clones (EL-4, 3 of 11; A20, 6 of 11; bmDC, 3 of 11)
Promoter activity of the 5'-flanking region was analyzed by luciferase reporter assays using a series of deletion mutants in stimulated and nonstimulated EL-4 and RAW 264 cells. The structures of the luciferase reporter plasmids are shown in Fig. 2A. Luciferase activities generated in these cells using the reporter plasmids were compared with that using a negative control plasmid (no insert, pGL3-Basic Vector) (Fig. 2B). The negative control plasmid exhibited high background luciferase activity in PMA/ionomycin-stimulated and nonstimulated EL-4 cells (often 4-fold above that in nonstimulated cells). This meant that the calculated relative luciferase activities in these cells was low, thus precluding useful comparison between stimulated and nonstimulated cells. IL-10 promoter activity was observed in all tested cells. Surprisingly, no significant reduction of promoter activity was observed by the 673-bp deletion from position −140 to −56 (Fig. 2B, lane 2) compared with that using a negative control plasmid (no insert, lane 0). The 55-bp deletion at position −92 to −71 (Fig. 2B, lane 3) was also observed in all tested cells. Protection from DNase I by binding of NFs was detected at position −92 to −71 (Fig. 4). This region overlapped with the NF-binding region detected by the gel mobility band shift assay using probe P4 (−95 to −71). To determine a more accurate location for the cis-acting element, seven mutant oligonucleotide probes (M1 to M7) were designed, synthesized, and annealed. The DNA sequence of the mutant probes are shown in Fig. 5B. The complexes C1, C2, and C3 were detected with wild-type probe P4 and mutant probes M1 and M7 but not with probes M2 to M6 (Fig. 5A), indicating that 13-bp sequence AGGGAGGAGGAGC is required for NF binding (Fig. 5B, CONSENSUS).

**IL-10 gene expression is regulated by a CCTCCT motif**

We could find no potential NF recognition sequence in this 13-bp region using the transcription factor database (20). However, transcription of the human epidermal growth factor receptor (EGFR) (21) and CD58 (22) genes is known to be regulated by cis-acting elements containing TCC repeats. We noticed the presence of TCC repeats in the complementary sequence (GCTCCTCCCTCC) of the 13-bp region. This 13-bp sequence (antisense) plus the boundary sequence was, therefore, aligned with sequences of the four TCC repeating cis-acting elements in the EGFR promoter, the cis-acting element in CD58 promoter, and corresponding region in the human IL-10 promoter (Fig. 6A). A CCTCCT sequence was found...
in all aligned sequences and was followed by C + T-rich sequences. This CCTCCT sequence is also found at the corresponding region in the human IL-10 promoter (Fig. 6A). We investigated whether this CCTCCT sequence could provide the core sequence of the cis-acting element for IL-10 transcription by changing the CCTCCT sequence to GAATTC (EcoRI site) in two luciferase reporter plasmids D1 and D6 (Fig. 2A, D1, D6; Fig. 6B, D1, CCT KO; D6, CCT KO). Promoter activity was then assessed in EL-4 cells (Fig. 6C). This change produced a large reduction of promoter activity with both D1 and D6 plasmids, indicating that the CCTCCT sequence was required for IL-10 gene expression. This loss of promoter activity was accompanied by the disappearance of all three complexes (C1, C2, and C3) observed in the gel mobility band shift assay using probe P4 (Fig. 5, M4). These results suggest that the CCTCCT sequence is the core of this cis-acting element, is required for binding of these NFs, and can therefore be defined as the “CCTCCT motif.”

In PMA/ionomycin-stimulated EL-4 cells, weak promoter activity was still detected using plasmid D1 CCT KO (Fig. 6, D1 CCT KO). Significant reduction of this activity was observed by the 497-bp deletion from −802 to −305 (Fig. 6, D1 CCT KO to D6 CCT KO). This was not observed in nonstimulated cells, suggesting that PMA/ionomycin response elements are located between positions −802 and −305.

IL-10 gene expression is regulated by the transcription factors Sp1 and Sp3

Because we could not find any transcription factor recognition sequences using the transcription factor database (20), we could not implicate any particular NFs for binding to this CCTCCT motif. However, previous reports have described the binding of the zinc finger protein WT1 (a product of the Wilms tumor suppressor gene) to TCC repeats in the EGFR promoter (23) and to a cis-acting element (not a CCTCCT motif) in c-myc promoter (24).

Furthermore, the transcription factors Sp1 and Egr-1 also bind to this c-myc element (24). We wondered whether the three complexes (C1, C2, and C3) involving the CCTCCT motif in the IL-10 promoter were formed with any of these transcription factors. To investigate this possibility, a super gel mobility band shift assay was performed using Abs that bind to WT1, Sp factors (Sp1, Sp2, Sp3, and Sp4) (25–27), and Egr factors (Egr-1, Egr-2, and Egr-3) (28–31). We were indeed able to show binding of anti-Sp1 and anti-Sp3 Abs to these complexes. Complex C1 disappeared with an anti-Sp1 Ab (Fig. 7, lanes 2 and 4), revealing a further shifted complex (Fig. 7, lanes 2 and 4, Sp1 + Ab). Complexes C2 and C3 also disappeared with an anti-Sp3 Ab (Fig. 7, lanes 3 and 4) with a new shifted complex appearing (Fig. 7, lanes 3 and 4, Sp3 + Ab). Two complexes disappeared with the anti-Sp3 Ab, suggesting that this Ab binds to two different sized Sp3 as described previously (32). However, only one further shifted band was observed (Fig. 7, lane 3, Sp3 + Ab). It is conceivable that a further shifted band is located, and thus not detected, in the same position as complex C1.
expression is positively regulated by both transcription factors Sp1 and Sp3.

**Discussion**

We have determined a transcription start site for IL-10 gene expression. A typical TATA box, TATAAAA is located at −30 to −24, indicating that transcription of IL-10 is regulated by a TATA-type promoter as are many other cytokine genes, although not all (e.g., IL-7 (33), IL-12 p35 (34, 35), and IL-18 (36) genes). We have also demonstrated that IL-10 gene expression is regulated by transcription factors Sp1 and Sp3, which are known to be constitutively expressed and to bind to the G + C-rich consensus sequence (GC box). These transcription factors, therefore, can regulate constitutive expression of many housekeeping genes controlled by G + C-rich promoters. Ubiquitous IL-10 expression might be regulated in a manner similar to that of other housekeeping genes. However, the binding sequence of these transcription factors in IL-10 promoter is not the GC box but a cis-acting element containing CCTCCT sequence as a core. One previous report shows Sp1 binding to the CTCTCCT sequence in the WT1 promoter (37) which contains the CCTCCT core. It seems, therefore, that Sp1 and Sp3 recognize this “CCTCCT motif” as a second consensus sequence.

We speculate that the Sp1 and Sp3 are also the major positive regulators of human IL-10 transcription. The CCTCCT motif is conserved in the human IL-10 promoter region, and when replaced by a GAATTC sequence, promoter activity is substantially reduced (M. Tone et al., unpublished data).

Sp3 has in other circumstances been known to cause repression of Sp1-mediated transcriptional activation (32). However, this inhibitory activity was not observed for IL-10 transcription as shown in *Drosophila* SL2 cells transfected with both pPacSp1 and pPacUSp3 (data not shown). Inhibition of Sp1-mediated transcriptional activation has also been observed with WT1. WT1 is known to bind a CTCTCCT motif in the EGFR promoter (23) and can down-regulate expression of that gene (38). Although we did not detect binding of WT1 to the IL-10 CCTCCT motif using the nuclear extract from EL-4 cells, we could not completely rule out a role for it as a negative regulator of IL-10 gene expression.

By use of a luciferase assay using promoter constructs D1 CCT KO and D6 CCT KO (Fig. 6), we have found that PMA/ionomycin response elements might be located between positions −802 and −305. However, we could not detect significant reduction of luciferase activity using a series of deletion mutants (Fig. 2). Presumably, the weak activity of this response element is hidden by the strong Sp1 and Sp3 activities. A further set of luciferase reporter plasmids (e.g., plasmids constructed using deletion mutants that do not contain the Sp1 and Sp3 recognition sequence) will be required to identify these particular response elements.

We have shown strong IL-10 promoter activity in nonstimulated EL-4 cells. The pattern of the gel mobility shift assay using nuclear extracts from nonstimulated EL-4 cells with probe P4 was also identical with that using a nuclear extract from PMA/ionomycin-stimulated EL-4 cells (data not shown). However, the IL-10 mRNA level in nonstimulated EL-4 cells was much lower than that in stimulated cells (Fig. 1). The amount of IL-10 mRNA expressed in nonstimulated EL-4 cells seems to be maintained at a low level through posttranscriptional mechanisms. Six AUUUA mRNA destabilization sequences with A + U-rich sequences are located in the 3′-untranslated region of IL-10 mRNA, and in the accompanying paper (13), we show that they are responsible for posttranscriptional regulation of IL-10 expression through these RNA destabilizing signals. We do not rule out the possibility of other
transcriptional regulatory elements not included in the promoter fragment (e.g., enhancers).

IL-10 seems to be a major homeostatic regulator of inflammation, immune responses, and involved in prevention of autoimmunity (39). Why then should one find that the transcriptional control of the IL-10 gene is so dependent on these ubiquitously and constitutively expressed transcription factors, Sp1 and Sp3? It may be that the immune system requires rapid availability of homeostatic regulators such as IL-10 and achieves this by ensuring that the gene is actively transcribing in a variety of cell types, but determines protein availability through posttranscriptional mechanisms.

Acknowledgments

We thank Dr. Paul Fairchild for providing bmDC and Mark Frewin for technical assistance.

Addendum

Since submission of our manuscript, Brightbill et al. (40) have published on transcriptional control of IL-10 gene expression. They too have established a role for Sp1 in the transcriptional control of the IL-10 gene, but only in stimulated RAW 264 cells. Our conclusions differ insofar as we detect promoter activity in both stimulated and nonstimulated RAW 264 cells, perhaps as a result of the use of luciferase vectors in our study (41). In the accompanying paper (13), we explain the effects of stimulation on IL-10 expression, as a consequence of posttranscriptional control.

References


Downloaded from http://www.jimmunol.org/ by guest on July 27, 2017