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GATA-3 Directly Remodels the *IL-10* Locus Independently of IL-4 in CD4$^+$ T Cells

John Shoemaker, Margarida Saraiva, and Anne O'Garra[1]

IL-10 is a major regulator in inflammatory responses. Although various transcription factors were defined to enhance IL-10, the molecular mechanism for the initiation of *Il-10* transcription, remains unknown. mRNA profiling of six distinct primary CD4$^+$ T cell populations showed differential expression of the transcription factor GATA-3 correlated with levels of IL-10 expression. We showed that ectopic expression of GATA-3 in naive primary CD4$^+$ T cells enhanced expression of IL-10 by these cells and uncovered a possible mechanism for this effect. We found that GATA-3 induced changes of the chromatin structure at the *Il-10* locus and that these changes occur even in the absence of IL-4. Furthermore we found that in the presence of GATA-3 the histones at the *Il-10* locus become acetylated. Despite being recruited in vivo to two locations on the *Il-10* locus, GATA-3 did not transactivate the IL-10 promoter. We therefore suggest a key role of GATA-3 in instructing *Il-10* gene expression in primary CD4$^+$ T cells, possibly by switching and stabilizing the *Il-10* locus into a transcriptionally competent status. ***The Journal of Immunology,*** **2006, 176: 3470–3479.**

Interleukin-10 is a cytokine that plays a critical role in the control of inflammation by directly regulating macrophage and dendritic cell function and thus the development of effector CD4$^+$ T cell responses (1, 2). IL-10 is produced by a variety of cell types, including activated macrophages, dendritic cells, B cells, and mast cells. IL-10 is also secreted by T cells, in particular by the effector subset of Th type 2 cells (Th2), and by some types of regulatory T (T_{reg})[2] cells (2). Deletion of the *Il-10* gene in mice is associated with spontaneous development of inflammatory bowel disease (3), increased susceptibility to pathology caused by infection with parasites or bacteria, and with endotoxin-induced shock (reviewed in Refs. 1, 2). T cell-specific inactivation of the *Il-10* gene in mice results in dysregulation of T cell responses to *Toxoplasma gondii* but has no effect on innate responses to LPS or skin irritants (4). The finding that various viral genomes encode a homolog of the *Il-10* gene, which manipulates the host immune response, further supports the crucial role of IL-10 in the regulation of the immune response (1, 5).

The molecular mechanisms that regulate expression of the *Il-10* gene are poorly understood. Recent studies have described changes in the chromatin structure at the *Il-10* locus that relate to the control of *Il-10* gene expression in cells that produce this cytokine (6–9). At the transcriptional level, Sp1 (10, 11), Sp3 (11), CCAAT/enhancer-binding protein β (12), IFN regulatory factor-1, and STAT3 (13) transactivate various constructs of the IL-10 promoter in reporter assays, both in mouse and human cell lines. Smad-4 (14) and Jun proteins (8) regulate IL-10 expression in primary Th1 and Th2 cells, respectively. Finally Ets-1 has been recently suggested to play a role in repressing the production of IL-10 in Th1 cells, as Ets-1-deficient mice show a marked increase in the production of this cytokine (15). However, a master regulator that initiates expression of the *Il-10* gene, by inducing remodeling of the *Il-10* locus to allow access and function of ubiquitous enhancing or silencing transcription factors, has not yet been identified.

Th2 cells, associated with eradication of helminths and other extracellular pathogens, express the cytokines IL-4, IL-5, and IL-13 (16). At the molecular level, the expression of these cytokines is controlled by the transcription factor GATA-3 (17–22), which also represses the Th1 cytokine IFN-γ (19, 21, 23). During in vitro Th2 development, GATA-3 induces changes in the chromatin structure at the *Il-4* locus (18, 22) by binding to sites localized upstream of the *Il-4* gene and in the *Il-4*/*Il-13* intergenic region (20, 24, 25). GATA-3 only weakly transactivates the IL-4 promoter (17, 20, 24) and an additional transcription factor, c-Maf (22, 26), is required for IL-4 production. Furthermore, GATA-3 binds and strongly transactivates the IL-5 (22, 27, 28) and IL-13 promoters (29). Inhibition by antisense transfection (17) or conditional deletion (30, 31) of GATA-3 in vitro and in vivo further demonstrates the instructive role of GATA-3 in initiating and maintaining the expression of the cytokines IL-4, IL-5, and IL-13.

Because Th2 cells also express high levels of IL-10 (1), a role for GATA-3 in the regulation of IL-10 in these cells has been suggested (32, 33). Antisense inhibition (17) or conditional deletion (30) of GATA-3 in established Th2 cells has been shown to reduce IL-10 levels. Conversely, overexpression of GATA-3 in a transgenic model caused an up-regulation of IL-10 production (17, 32). However, the mechanism whereby GATA-3 controls IL-10 expression is entirely unknown and could involve direct transactivation of the IL-10 promoter and/or remodeling of the *Il-10* locus at the chromatin level.

In this study, we showed that GATA-3 was present at different levels in all IL-10-producing CD4$^+$ T cell populations tested, and that the expression levels of GATA-3 correlated with levels of IL-10 expression. In contrast, expression of transcription factors implicated in *Il-10* gene regulation was also detected in non-IL-10-producing cells. Ectopic expression of GATA-3 induced IL-10 secretion by cells that do not normally express IL-10, via an IL-4-independent mechanism. Although GATA-3 did not transactivate

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[2] Abbreviations used in this paper: T_{reg}, T regulatory; WT, wild type; ChIP, chromatin immunoprecipitation; HSS, hypersensitivity site.

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the IL-10 promoter in in vitro reporter assays, it was recruited in vivo to multiple locations within the *Il-10* locus. Strikingly, the presence of GATA-3 induced changes in chromatin structure and switched the *Il-10* locus into transcriptionally active status, by inducing both remodeling of the *Il-10* locus and acetylation of histones H3 and H4. We show in our study for the first time that GATA-3 directly acts as a regulator of the *Il-10* locus, controlling the accessibility of the *Il-10* gene to transcription factors.

Materials and Methods

Animals

BALB/c mice (wild-type (WT) and IL-4$^{-/-}$) were bred and maintained under specific pathogen-free conditions at the National Institute for Medical Research (London, U.K.).

Reagents, cell lines, and plasmids

The reagents used for T cell preparation have been described before (34, 35). GATA-3 Ab was obtained from Santa Cruz Biotechnology, and anti-acetylation H3 and H4 histones was purchased from Upstate Biotechnology. PlatE cells were maintained in DMEM (10% FCS), 68-41 cells in RPMI 1640 medium (10% FCS, glutamine, HEPES, sodium pyruvate, and 2-ME), and D10 cells as described (22). pMXI-EGFP and pMXI-GATA-3 were used for retroviral preparation (21, 22). GATA-3 obtained from pMXI-GATA-3 was subcloned into pIRES-EGFP2 (Clontech Laboratories) for transient transfection studies. pRL-TK (Promega) and pGL3Basic IL-10-promoter (7) were used in luciferase reporter assays.

Isolation of T cell subsets and generation of polarized T cells

CD4$^+$ T cells were enriched from total spleen cell suspensions and purified as CD4$^+$CD62L$^+$CD45RB^{high} naive T cells (>98%), CD4$^+$CD62L$^+$ T cells (>99%), or CD4$^+$CD25$^+$ T$_{reg}$ cells (>96%) by MoFlo flow cytometer (DakoCytomation). Neutral, Th1, Th2 cells and IL-10-producing T$_{reg}$ cells (IL-10-T$_{reg}$) were derived in vitro in an APC-independent manner, as described (34, 35).

Cytokine measurement by intracellular staining and ELISA

T cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (10 μg/ml) and intracellularly stained and analyzed by FACS as described (34). Cytokine levels were measured with the Luminex 100 instrument and Beadlyte Mouse Cytokine assay (Upstate Biotechnology).

GeneChip preparation and expression analysis

Cells were Ficoll separated, and total RNA was prepared (Qiagen) from resting CD4$^+$ T cells and then quality controlled for quantitative and qualitative degradation on an Agilent Bioanalyzer 2100. Five micrograms of total RNA was further processed following one-cycle Affymetrix-recommended protocols (Roche Applied Science). Two independent biological replicates were prepared and used to generate each population profile. The GC-RMA (Robust Multiarray Average) algorithm was used to process probe level data (.cel file) and the expression probe set values were imported into GeneSpring version 7.0 software (Silicon Genetics) for further analysis. For data presentation, normalized median expression values were set to $\log_2 = 1.0$, and differential transcript values are shown as reported in comparison to other normalized conditions in the respective figure.

Real-time quantitative PCR

RNA from T cell populations was converted into cDNA and analyzed by real-time PCR as previously described (34, 35). Target cytokine gene mRNA expression was quantified using SYBR green (Applied Biosystems) and normalized to the ubiquitin mRNA levels (34, 35). The primer/probes sets (Applied Biosystems) were acquired for GATA-3 (Mm00484683_m1; assay no.), c-Jun (Mm00495062_s1), JunB (Mm00492781_s1), Ets-1 (Mm00468970_m1), and HPRT1 (hypoxanthine phosphoribosyltransferase; Mm00446968_m1). cDNA was amplified with TaqMan Universal PCR Master mix (Applied Biosystems) and primer/probes, and expression values were normalized to HPRT1.

Retroviral infection of T cells

Purified naive CD4$^+$ T cells from WT or IL-4$^{-/-}$ mice were infected as described before (21), differentiated for 7 days in neutral or Th1 conditions, and cytokine production was measured by intracellular staining upon restimulation. For RNA preparation and analysis and ELISA of cytokines in supernatants, the infected cells were purified by flow cytometry based on GFP expression.

Chromatin accessibility assay

IL-4$^{-/-}$ naive T cells were infected with a GATA-3 retrovirus or a mock retrovirus and 3 days postinfection nuclei were isolated and left untreated or digested with 1.0 μg/ml DNaseI (Sigma-Aldrich) as described before (36). The digested DNA was purified and 10 ng used as template for real-time PCR amplification with specific oligonucleotides for the indicated sites in the *Il-10* locus. The percentage of DNaseI digestion was calculated as previously described (37). The reported percentage of chromatin accessibility was calculated for each primer pair with the following equation: ((number of DNaseI$_{(gDNA)}$ − DNaseI-treated$_{(gDNA)}$)/(number of DNaseI$_{(gDNA)}$)) × 100.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed in unstimulated or stimulated primary T cells (1 h in the presence of PMA/ionomycin) as previously described (7). Chromatin extracts were precleared for 2 h with salmon sperm-blocked protein A beads (Amersham Biosciences) and then immunoprecipitated with anti-GATA-3, anti-acetylhistone H3, anti-acetylhistone H4, or control Abs (normal rabbit IgG) at 4°C overnight. Immunocomplexes, recovered by further incubation with protein A beads for 6 h at 4°C, were washed in low-salt buffer, and DNA was purified and used as a template for real-time PCR with specific oligonucleotides for the *Il-10* locus and the IL-5 promoter. Cycle threshold Ct values for GATA-3, anti-acetylhistone H3, anti-acetylhistone H4, or control Abs were normalized to the input Ct value (with same primer pair), and the relative expression is reported.

Luciferase reporter assays

D10 or 68-41 cells were transiently transfected with the pGL3Basic-IL-10-promoter linked to luciferase alone or in the presence of pIRES-EGFP2-GATA-3 recombinant plasmids in the presence of pRL-TK (Promega), using DEAE-dextran, rested for 4–6 h, and then left untreated or stimulated with PMA/ionomycin for 12 h. Firefly and Renilla luciferase activity in cell extracts were measured using the Dual Luciferase Reporter kit (Promega). The Firefly luciferase activity was normalized to the Renilla activity in all samples, and the ratio between the stimulated (12 h with PMA/ionomycin) and unstimulated samples was calculated and reported.

Results

GATA-3 is differentially expressed in IL-10-producing CD4$^+$ T cells

To investigate the regulation of the *Il-10* gene in T cells, we surveyed mRNA expression profiles of six populations of primary CD4$^+$ T cells known to produce IL-10 using the Affymetrix Mouse Genome 430 version 2.0 GeneChip. Mouse CD4$^+$CD62L$^+$ T cells were isolated and differentiated into homogeneous populations of Th1, Th2, and IL-10-T$_{reg}$ in the presence of IL-12, IL-4, or immunosuppressive drugs (vitamin D3 and dexamethasone) as previously described (34, 35). T cells cultured under the same conditions but in the absence of polarizing cytokines or immunosuppressive drugs and in the presence of blocking Abs to endogenous cytokines are referred to as "neutral." Additionally, two ex vivo populations were included in the panel: naive (CD4$^+$CD62L$^+$CD45RB^{high}) T cells and naturally occurring CD4$^+$CD25$^+$ T$_{reg}$ cells (CD25$^+$-T$_{reg}$). Th1 cells produced high levels of IFN-γ and no IL-4, whereas Th2 cells produced IL-4, IL-5, and IL-13, but no IFN-γ upon activation (Fig. 1*A* and data not shown). Th2 and IL-10-T$_{reg}$ upon activation produced IL-10 at very high levels, whereas Th1 cells expressed only low levels of this cytokine (Fig. 1*A*). As expected, activated naive T cells, neutral T cells, and CD25$^+$-T$_{reg}$ did not produce any Th1 or Th2 cytokines or IL-10 to a significant level (Fig. 1*A*).

A comprehensive analysis of transcription factors differentially expressed in IL-10- vs non-IL-10-producing T cells was also performed by GeneChip (Fig. 1*B*). Confirming previous observations

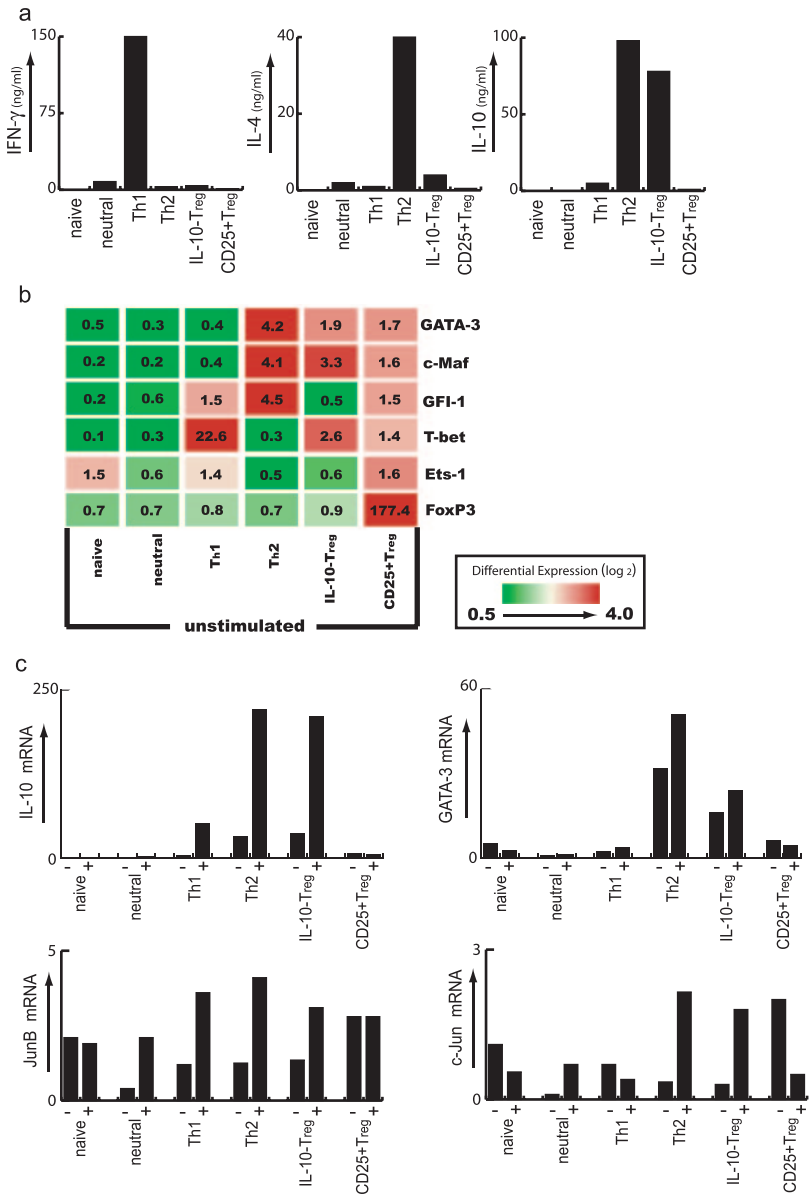

FIGURE 1. GATA-3 is differentially expressed in resting and activated IL-10-producing T cells. *A*, Luminex generated protein profiles for cytokines was obtained after stimulating each population for 48 h in cytokine or Ab-free medium. *B*, Whole genome expression analysis was performed with Affymetrix Mouse Genome 430 version 2.0 GeneChips. T cell populations were prepared as described and analyzed. Normalized values ($\log_2$) are shown. A one-way unsupervised hierarchical gene cluster was performed for labeled genes in resting (unstimulated) conditions as indicated. *C*, Real-time PCR was performed to validate the results from the GeneChips for IL-10, GATA-3, JunB, and c-Jun expression in unstimulated (−) or PMA/ionomycin-stimulated (+) T cell populations. Values represent relative mRNA expression normalized to ubiquitin B (IL-10) or to HPRT1 (GATA-3, JunB, and c-Jun) mRNA. Data shown are an accurate representation of three experimental replicates.

and validating the performance of the GeneChip, T-bet was up-regulated in the Th1 subset (38) with low level expression in IL-10-T_{reg} only (Fig. 1*B*), whereas GATA-3 was detected in Th2 cells (17), IL-10-T_{reg} cells, and to a lower extent in $CD25^+$-T_{reg} cells (Fig. 1*B*). FoxP3 expression was exclusively observed in $CD25^+$-T_{reg} (39–41) (Fig. 1*B*).

GATA-3 mRNA was significantly elevated within IL-10-producing populations (Fig. 1*B*), even in IL-10-T_{reg} when Th2-specific cytokines were not significantly expressed. We had previously shown that GATA-3 levels in IL-10-T_{reg} were low, but not absent (34). In this study we used both GeneChip data (differential expression profiles from six $CD4^+$ T cell populations) and primer/probes (as opposed to PCR primers alone as we used previously (34)), and therefore we now present a more sensitive and specific quantitative PCR GATA-3 expression validation. This model suggested a possible direct function for GATA-3 in the regulation of IL-10. Several other transcription factors with relevance to *Il-10* gene regulation were also examined in both Th2 and IL-10-T_{reg} cells (and are currently under investigation). The transcript for c-Maf was found up-regulated in both Th2 and IL-10-T_{reg} cells with low levels in $CD25^+$-T_{reg} (Fig. 1*B*). Ets-1 expression was down-regulated in both IL-10-producing T cell populations, consistent with previous reports that suggest a regulatory role of Ets-1 in suppressing IL-10 production (15) (Fig. 1*B*). Growth factor independent-1, a major early downstream transcriptional target for IL-4R/STAT6 signaling and a necessary proliferative factor for optimal Th2 differentiation (42), was only present in Th2 cells and not in IL-10-T_{reg} (Fig. 1*B*), suggesting that in these two populations discrete signaling pathways occur. We further confirmed and quantified, by real-time RT-PCR, the transcription of *IL-10* and GATA-3 in the T cell subsets, validating our GeneChip observations and reinforcing a correlation between the expression levels of GATA-3 and those of IL-10 expression (Fig. 1*C*). The expression of Sp1 and Sp3 was ubiquitous between $CD4^+$ T cell populations confirming previous reports (reviewed in Ref. 43) (data not shown). Smad-4 has been described to enhance IL-10 production in primary Th1-driven T cells (14), but we found Smad-4 was not differentially expressed in IL-10-producing T cells (data not shown).

Recently, the Jun family transcription factors c-Jun and JunB were shown to bind to a conserved noncoding sequence (CNS-3) at the 3′ end of the *Il-10* locus, positively regulating *Il-10* transcription in Th2 cells (8). In this study, we show that c-Jun and

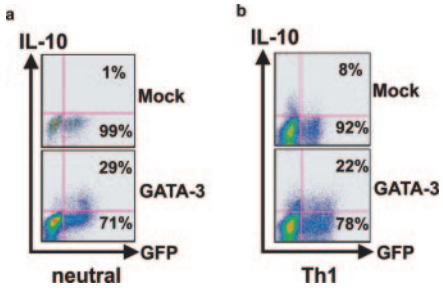

FIGURE 2. GATA-3 enhances IL-10 production in developing T cells. WT naive ($CD4^+CD62L^+CD45RB^{high}$) T cells were infected with a mock retrovirus or a GATA-3-expressing retrovirus and differentiated under neutral (*A*) and Th1 (*B*) conditions. Six days after infection, cells were stimulated with PMA/ionomycin for 4 h, intracellularly stained for IL-10 and analyzed by FACS. Cells were gated on GFP expression (*x*-axis) and the percentage of GFP^+ cells expressing IL-10 is shown. Data shown are an accurate representation of three experimental replicates.

JunB were not expressed exclusively in IL-10-producing T cells during unstimulated conditions but were also expressed in naive, Th1, Th2, and $CD25^+$-T_{reg}. However, c-Jun expression was up-regulated in both IL-10-producing conditions upon stimulation and down-regulated in $CD25^+$-T_{reg}, whereas JunB was up-regulated in neutral, Th1, Th2, and IL-10-T_{reg} upon activation (Fig. 1*C*).

Ectopic expression of GATA-3 induces IL-10 production in primary T cells

To further address the effect of GATA-3 on IL-10 production, purified naive T cells were transduced with a recombinant retrovirus expressing GATA-3 or with a mock retrovirus and cultured under neutral or Th1 conditions. The use of a retroviral vector containing an bicistronic IRES-GFP facilitated the monitoring of the expression of GATA-3 in the transduced cells at the single-cell vs intracellular cytokine production level by measuring GFP expression, which as we previously showed reflected GATA-3 expression in transduced cells (21). Cells cultured under neutral conditions and transduced with the mock retrovirus produced little to no IL-10 (Fig. 2*A*). However, ectopic expression of GATA-3 induced an increase in the number of IL-10-producing cells upon stimulation (29 vs 1%) (Fig. 2*A*) and increased levels of secreted IL-10 as measured by ELISA (data not shown). Similar to that observed under neutral conditions, overexpression of GATA-3 in naive T cells differentiated under Th1 conditions led to an increase of IL-10 production (Fig. 2*B*).

GATA-3 induction of IL-10 production is independent of IL-4

GATA-3 has been shown to induce changes in the chromatin structure at the *Il-4*/*Il-5*/*Il-13* locus (18, 22), with subsequent production of these Th2-type cytokines (17, 18, 21). Thus, the increased production of IL-10 observed in the presence of GATA-3 could result from indirect effects of Th2 cytokines (specifically IL-4 because IL-5 and IL-13 do not act directly on T cells) (44). To determine whether GATA-3 enhanced *Il-10* gene expression independently of IL-4, GATA-3 was transduced into naive T cells derived from $IL\text{-}4^{-/-}$ mice (45). Under neutral culture conditions, GATA-3 induced a similar number of IL-10-producing cells in the presence (WT) (Fig. 2*A*) or absence ($IL\text{-}4^{-/-}$) of IL-4 (Fig. 3*A*), showing that IL-4 is not necessary for IL-10 induction. However the number of IL-4- and IL-5-producing cells was much lower than IL-10-producing cells in the WT (data not shown) and the number of IL-5-producing cells much lower in the $IL\text{-}4^{-/-}$ cells (Fig. 3*B*). The effects on IL-4 and IL-5 were as previously reported (18, 19, 21).

Strikingly, transduction of GATA-3 in $IL\text{-}4^{-/-}$ naive T cells differentiated under Th1 conditions induced a greater number of IL-10-producing cells than that observed for WT cells (Figs. 2*B* and 3*C*). The IL-10 levels in the GFP-negative/GATA-3-transduced population were also increased, which was also observed at the mRNA level (data not shown). This probably reflects the loss of GFP expression in a proportion of GATA-3-transduced cells (21). Therefore, as in all the conditions, the most accurate comparison is between the GFP^+ mock retrovirus-transduced cells and GFP^+ GATA-3 retrovirus-transduced cells. Overexpression of GATA-3 induced similar changes in the percentage of IL-5-, IFN-γ-, and IL-2-producing cells in WT (data not shown) or $IL\text{-}4^{-/-}$ cells (Fig. 3*D*).

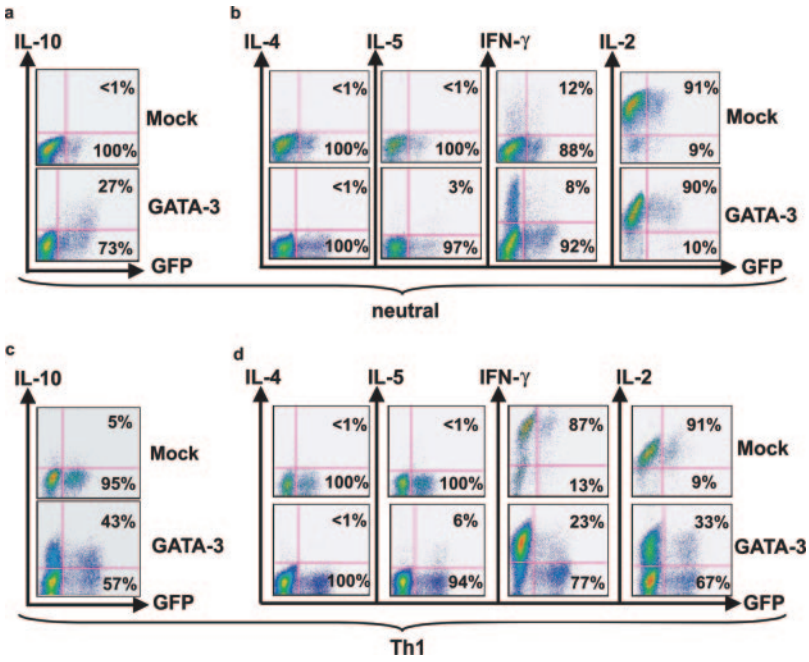

FIGURE 3. IL-10 production induced by GATA-3 is independent of IL-4. $IL\text{-}4^{-/-}$ naive ($CD4^+CD62L^+CD45RB^{high}$) T cells were infected with a mock retrovirus or a GATA-3-expressing retrovirus and differentiated under neutral (*A* and *B*) and Th1 (*C* and *D*) conditions. Six days after infection, cells were stimulated with PMA/ionomycin for 4 h, intracellularly stained for IL-10 (*A* and *C*) or for IL-4, IL-5, IFN-γ, and IL-2 (*B* and *D*), and analyzed by FACS. Cells were gated on GFP expression (*x*-axis), and the percentage of GFP^+ cells expressing the respective cytokines is shown. Data shown are an accurate representation of three experimental replicates.

To investigate the effect of GATA-3 on *Il-10* transcription in WT and IL-4$^{-/-}$ CD4^{+} T cells, we isolated GATA-3- or mock-transduced cells by flow cytometry on the basis of their GFP expression and quantified mRNA levels by real-time PCR for IL-10 and transcription factors implicated in *Il-10* gene regulation (Fig. 4). As expected, the levels of GATA-3 mRNA (Fig. 4) were higher in the GATA-3-transduced GFP^{+} T cells than in the mock-transduced GFP^{+} T cells. Independently of the polarizing conditions used and of the presence of IL-4, IL-10 transcription was significantly and consistently up-regulated by GATA-3 in keeping with the intracellular staining (Fig. 4). IL-5 mRNA was also consistently up-regulated in the presence of GATA-3, to a maximum amount in WT cells under neutral conditions (Fig. 4). Expression levels of IL-10 and of IL-4 (Fig. 4) confirmed the differential cytokine production shown by intracellular staining (Figs. 2 and 3 and data not shown). In IL-4$^{-/-}$ cells, overexpression of GATA-3 did not induce the transcription of c-Maf (Fig. 4), confirming that c-Maf expression requires IL-4 and strongly correlates with IL-4 signaling (18). Finally the expression of Ets-1 was down-regulated in the presence of GATA-3, independently of IL-4 production (Fig. 4).

GATA-3 binds to, but does not transactivate, the IL-10 promoter

Our findings that GATA-3 induced IL-10 mRNA and protein, in the absence of IL-4, suggested a direct, yet unknown effect of this transcription factor on the *Il-10* locus. Close comparison of the DNA sequence of the murine *Il-10* gene to that of other species (human, dog, rat, chicken) revealed two conserved sites for consensus GATA binding motifs (Fig. 5*A*). The existence of a highly conserved double GATA binding sites located in the proximal 5′ region of the *Il-10* gene (at position −0.865 bp relative to the *IL-10* starting site) suggested that GATA-3 may bind to this region of the *Il-10* gene. A second consensus GATA-3 binding site, located within intron 4 on the *Il-10* gene (at position +3.7 kbp relative to the *IL-10* starting site), was conserved between mouse, rat, dog, and chicken, but had no homology to the equivalent DNA region of the human locus. We assessed whether GATA-3 bound to these sites in vivo in primary IL-10-producing T cells, using ChIP. Cross-linked DNA from polarized Th2 cells was immunoprecipitated with a specific Ab for GATA-3 or with a control Ab. The immunoprecipitated DNA was then purified and amplified by real-time PCR using specific oligonucleotides for the sequence near or spanning the GATA-3 conserved putative binding sequences on the *Il-10* gene (previously described). Oligonucleotides specific for the *Il-5* gene 5′ region, where GATA-3 is known to bind (28), were included as a control. We show that the presence of GATA-3 at both consensus binding sites in the *Il-10* locus (Fig. 5, *B* and *C*), strongly supporting a direct role of GATA-3 in the expression of the *Il-10* gene. As controls, binding of GATA-3 as expected was

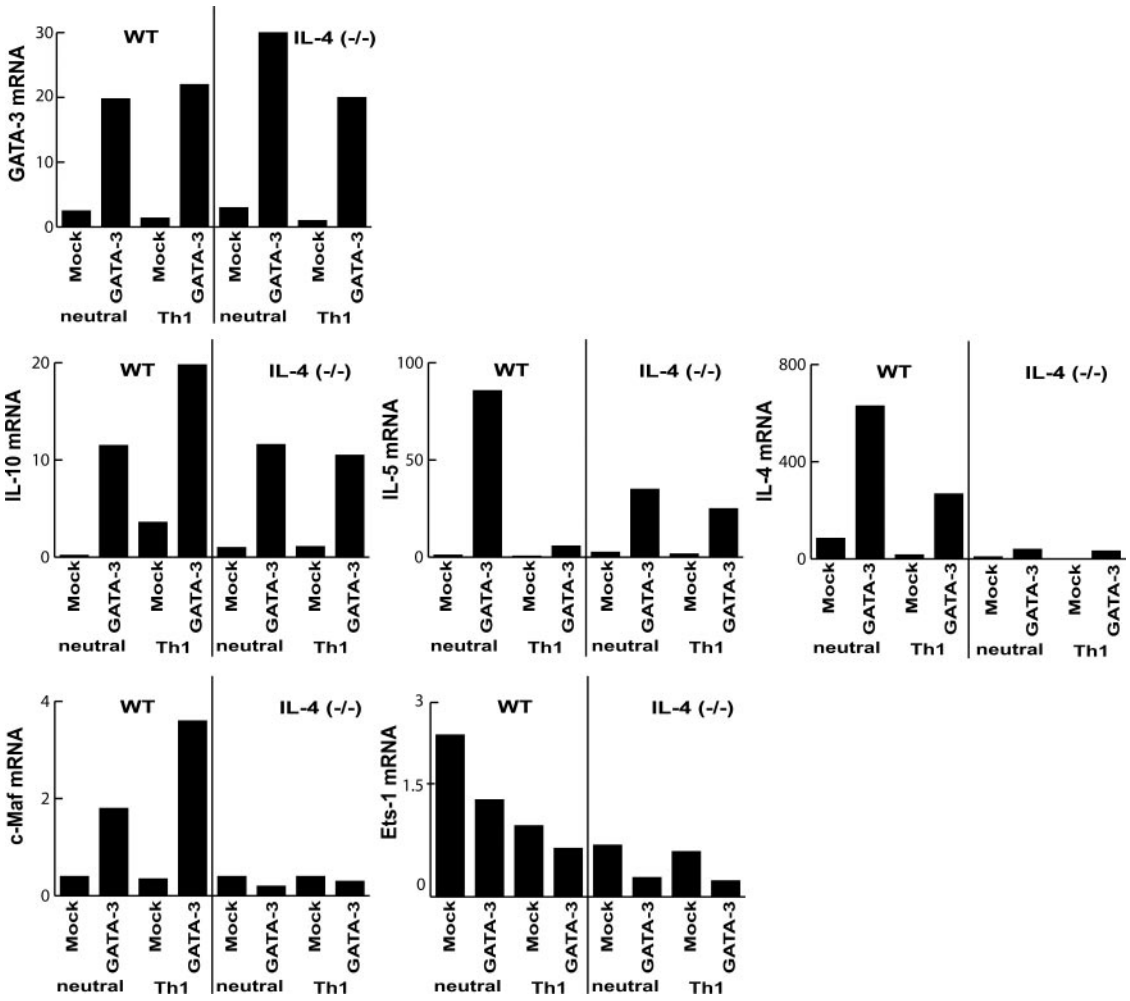

FIGURE 4. GATA-3 enhances IL-10 at the transcriptional level. CD4^{+} T cell populations described in Figs. 2 and 3 were purified by flow cytometry into GFP^{+} populations and activated for 3 h with PMA/ionomycin. Real-time PCR was performed to detect cytokine and transcription factor expression. Cytokine mRNA levels are expressed as relative normalized values to ubiquitin mRNA, and transcription factors transcripts are normalized to HPRT1 mRNA. Data shown are an accurate representation of three experimental replicates.

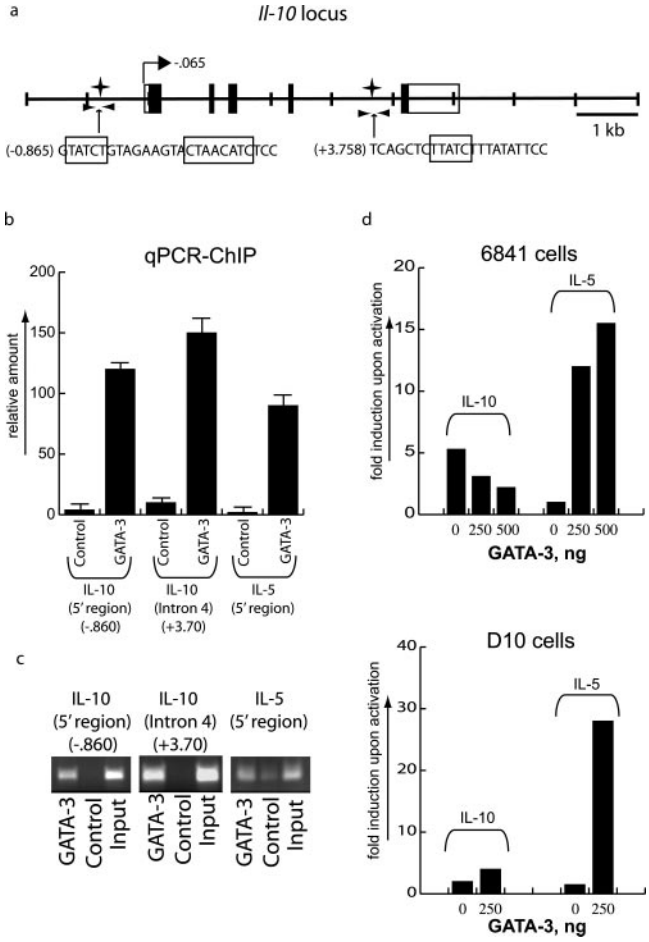

FIGURE 5. GATA-3 binds to the IL-10 promoter in vivo, but does not enhance its transcriptional activity. *A*, Conserved GATA-3 transcription factor binding sites in the mouse *Il-10* gene (+1 represents the start site) are depicted. Indicated are the IL-10 exons (■), the 5′ and 3′ untranslated regions (□), and the position of the oligonucleotides used in *B* and location of the conserved GATA-3 binding sites (✦). *B*, Cross-linked chromatin complexes from resting Th2 cells were immunoprecipitated with a GATA-3 or a control Ab. Specific oligonucleotides were used to amplify by real-time PCR the GATA-3 binding sites, in the *Il-10* gene or the IL-5 promoter, using the immunoprecipitated or untreated (Input) chromatin as template. Represented is the amount of PCR product obtained upon GATA-3 immunoprecipitation or control Ab immunoprecipitation normalized to the amount of PCR product obtained for the untreated chromatin. The error bars for each condition represent the SD from three biological replicates. *C*, The PCR products obtained as described in *B* were separated in a 2.5% agarose gel and stained with ethidium bromide. *D*, The 68-41 or D10 cells were transiently transfected with the IL-10- or IL-5 promoter luciferase constructs in the presence of increasing doses of pIRES2-GATA-3 construct and of pRL-TK. Cell lysates were prepared 12 h later from unstimulated or PMA/ionomycin-stimulated cells. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. Represented is the fold induction upon activation. Data shown are an accurate representation of three experimental replicates.

observed at the *Il-5* locus but was not observed in the region coding for the *Il-10* exon 5 (data not shown).

Given the high level of conservation of the GATA-3 site in the *Il-10* proximal 5′ region and its in vivo recruitment to this site, we then tested the ability of GATA-3 to transactivate the IL-10 promoter in reporter assays. For this, a 1.5-kb fragment upstream of the *Il-10* gene, containing the consensus double GATA-3 site (at position −0.865 relative to the IL-10 starting site), was cloned and linked to a luciferase reporter gene. This construct, that was similar to the one used in other *Il-10* transactivation studies (7), was transfected in the presence or absence of a GATA-3 expression vector into two different T cell lines: 68-41 and D10, a Th2 clone that expressed high levels of IL-10 (46). The induced luciferase activity was measured and normalized to the Renilla luciferase activity, in stimulated vs unstimulated cells. As shown in Fig. 5*D*, the presence of GATA-3 did not enhance the activity of the IL-10 promoter. This result was not due to the absence of other necessary IL-10 enhancers in the assay, as D10 cells endogenously expressed high levels of endogenous IL-10 upon stimulation (data not shown). In other experiments, we did transfect 500 ng of GATA-3-expressing vector into D10 cells, but still no transactivation was observed, and found in four experiments that the values of relative luciferase units obtained in unstimulated cells in the presence of absence of GATA-3 were similar, concluding that GATA-3 does not transactivate the IL-10 promoter (data not shown). As expected, and to control for the negative effect on the IL-10 promoter, GATA-3 was able to enhance the activity of the IL-5 promoter in keeping with previous reports (20, 22, 28). This finding indicates that the molecular mechanism for GATA-3-induced upregulation of the *Il-10* gene was not by direct activation of the IL-10 promoter.

GATA-3 induces changes in the chromatin structure at the Il-10 *locus*

Because transduction of GATA-3 into naive $CD4^+$ T cells and Th1 cells significantly induced IL-10 and yet GATA-3 did not transactivate the IL-10 promoter we investigated whether GATA-3 was able to induce changes in the chromatin structure at the *Il-10* locus. For this $CD4^+$ naive T cells from IL-$4^{-/-}$ mice were purified and transduced with a GATA-3-expressing or with a mock retroviral vector. This assay was performed at the earliest possible time (owing to transduction rates), which was with IL-$4^{-/-}$ cells day 3 posttransduction. Three separate biological replicates were assayed 3 days postinfection and nuclei isolated from resting GATA-3- or mock-transduced cells untreated or treated with DNaseI. Chromatin accessibility across the *Il-10* locus was then measured using real-time PCR (chromatin accessibility by real-time PCR; CHART-PCR) (37, 47). Recently we have described the existence of DNaseI hypersensitivity sites (HSS) at the *Il-10* locus in Th2 cells and other IL-10-producing cell populations (7). Based on that study, we designed specific oligonucleotide sets that span HSS of interest across the *Il-10* locus. These included HSS detected upstream of the *Il-10* start site (HSS −2.0, −0.860, −0.610), in intron 3 (HSS +1.65), and in intron 4 (HSS +2.98). In our analysis, we also included CNS-3, an HSS located downstream of the *Il-10* gene and recently described in another study (8, 9) and referred to here as HSS +6.40, in accordance to the nomenclature adopted for all other HSS (Fig. 6*A*). Of particular interest were the HSS detected in the 5′ proximal region of the *Il-10* gene and in intron 4 because we have shown in vivo recruitment of GATA-3 to these sites by ChIP. As a control for the quality and quantity of the template DNA used, the chromatin accessibility at exon 5 of the *Il-10* gene was also measured by PCR and expected to remain constant, as we have used low doses of DNaseI that will only reveal HSS.

First, to investigate changes in the chromatin structure at the *Il-10* locus induced by ectopic expression of GATA-3 in primary $CD4^+$ T cells, each primer set was used to amplify by real-time PCR 10 ng of genomic DNA from untreated or DNaseI-treated chromatin samples. As shown in Fig. 6*B*, DNaseI-treated samples from GATA-3-transduced samples show increased chromatin accessibility across the *Il-10* locus as shown by an increase in cycle threshold Ct values for HSS −0.860, −0.610, +1.65 and +6.40.

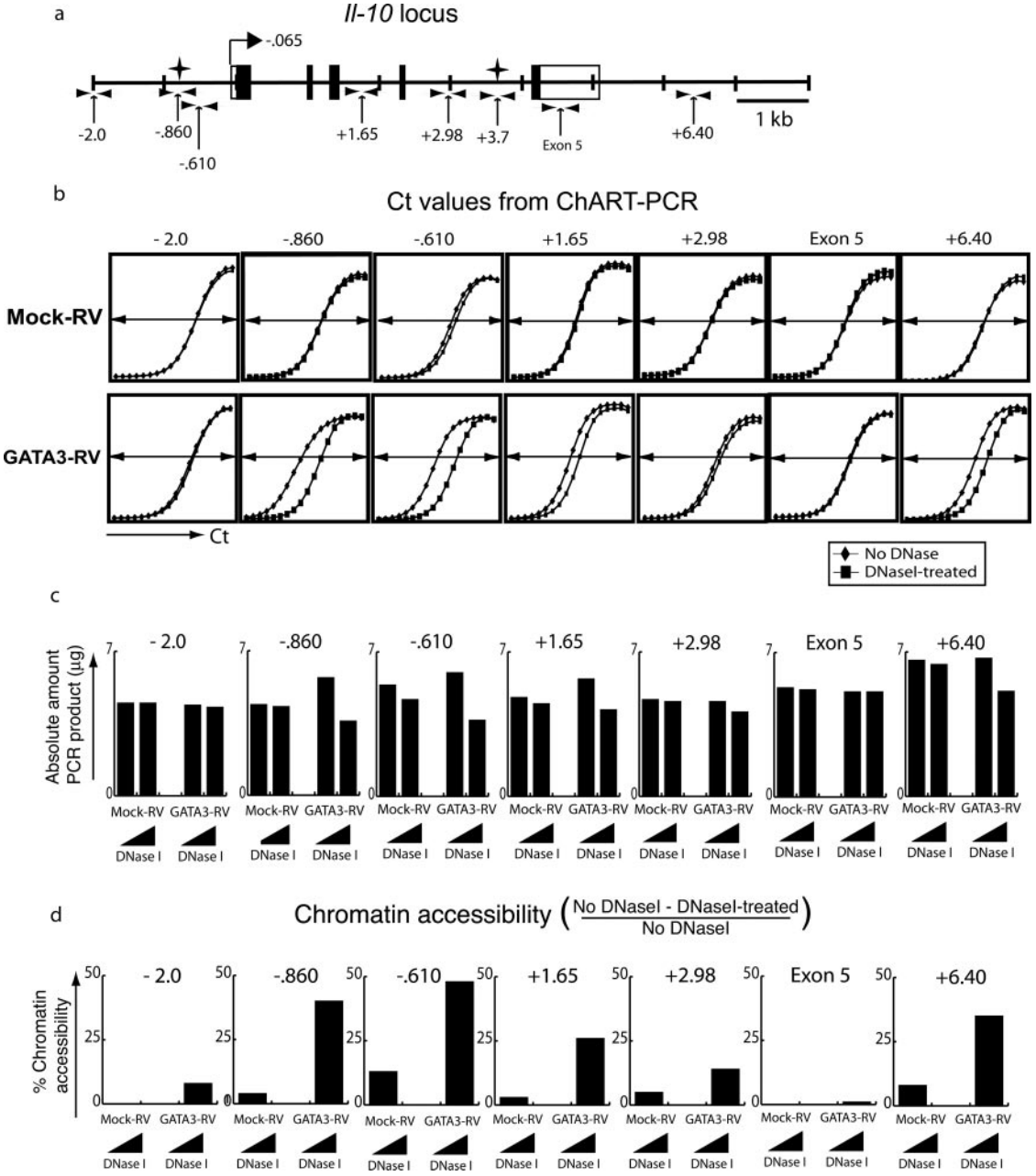

FIGURE 6. GATA-3 induces changes on the chromatin structure at the *Il-10* locus. *A*, Schematic of the fragment of the *Il-10* genomic locus analyzed in this study. *B*, IL-4$^{-/-}$ naive T cells were transduced with a mock retrovirus (Mock-RV) or a GATA-3-expressing retrovirus (GATA3-RV) and differentiated under neutral conditions. Three days after infection, cells were lysed, and the nuclei were isolated and left untreated (No DNaseI) or treated with DNaseI. The resulting DNA was purified and 10 ng used as template for SYBR Green real-time PCR amplification using specific oligonucleotides for the indicated sites. *C*, The cycle threshold (Ct) values obtained were converted to quantitative DNA amounts using a standard curve generated for each of the primer sets using genomic DNA as template (data not shown). *D*, The chromatin accessibility was calculated as described in *Materials and Methods* and expressed as a percentage of the untreated (No DNaseI) DNA. Our results were very consistent over the three distinct biological experiments.

We have generated a standard curve by amplifying known amounts of template genomic DNA with each set of primers and used it to convert our Ct values for untreated and DNaseI-treated samples (Fig. 6*B*) to absolute amount of PCR product obtained in each condition (Fig. 6*C*). In accordance with previous reports (37) the absolute amounts of PCR products were used to calculate the percentage of chromatin accessibility (Fig. 6*D*) across the *Il-10* locus. Following this calculation, when a particular site is exposed to DNaseI digestion, less absolute amount of PCR product is obtained with primers that span that site, and this result will be reflected in a higher percentage of chromatin accessibility.

In the presence of ectopic GATA-3 the chromatin accessibility at the *Il-10* locus was increased (Fig. 6*D*). This effect was most pronounced at sites located in the proximal 5′ region of the *Il-10* gene (HSS −0.860 and HSS −0.610) and described HSS within the *Il-10* introns, including sites that are near the putative GATA-3 binding site in intron 4 (Fig. 6*D*). Chromatin remodeling was also observed at HSS +6.40 (previously described as CNS-3 (8)), suggesting that GATA-3 may also induce long-range remodeling of known positive regulatory regions. No significant differences in PCR product amplified (Fig. 6, *B* and *C*) and on the percentage of DNaseI digestion (Fig. 6*D*) were observed at exon 5, suggesting

that the observed differences were specific and due to real changes in the chromatin accessibility at sites containing regulatory elements. Our data suggest that GATA-3 induced chromatin remodeling at specific sites of the *Il-10* locus, even in the absence of IL-4. This remodeling not only occurred at sites shown in this study to bind GATA-3 but also across the *Il-10* locus at sites known to positively regulate the transcription of the *Il-10* gene, as is the case for HSS +6.40.

GATA-3 induces acetylation of histones H3 and H4 to the Il-10 *locus*

We then assessed the acetylation status of histones H3 and H4 in primary $CD4^+$ T cells in the absence or presence of ectopic GATA-3. For this, naive $CD4^+$ T cells were transduced with GATA-3 or mock retrovirus, under neutral conditions and FACS-purified based on GFP^+ expression at day 6 posttransduction. Cross-linked chromatin was isolated and immunoprecipitated with Abs that specifically recognize acetylated residues of histones H3 and H4 vs control Abs. The immunocomplexes were further purified and DNA were then used as a template for real-time PCR with oligonucleotide sets that cover the predicted GATA-3 binding sites in the *Il-10* locus (HSS −0.860 and HSS +3.7) and the previously described AP-1 binding site (HSS +6.40) (8). Both histones were acetylated to a significantly higher level when cells had been transduced with GATA-3 (Fig. 7). The acetylation of histones H3 and H4 was readily observed in unstimulated GATA retrovirus cells, but was further increased upon activation at HSS −0.860 (Fig. 7).

Our finding that GATA-3 induced histone acetylation at the *Il-10* locus further supports a role for this transcription factor in instructing direct modifications of the *Il-10* locus that lead to IL-10 expression. This molecular mechanism is similar to, but independent of, the effects of GATA-3 in remodeling the chromatin at the *Il-4*/*Il-5*/*Il-13* locus and regulation of the Th2 cytokines (18, 22, 25). Taken together, our results suggest that GATA-3 acts as a direct regulator of the *Il-10* locus inducing changes in chromatin structure as shown by DNaseI digestion and histone acetylation.

Discussion

The regulation of IL-4, IL-5, and IL-13 is controlled by the transcription factor GATA-3 that has been shown to induce changes in the chromatin structure at the *Il-4* locus and subsequently transactivate the IL-5 and IL-13 promoters. In contrast to the regulation of the *Il-4* locus, little is known as to the factors that lead to chromatin remodeling and initiate transcription of the *Il-10* gene, a cytokine produced at high levels by Th2 cells. Because IL-10 is also produced by other $CD4^+$ T cell subsets, which produce little to no Th2-specific cytokines, we postulated that IL-10 may be regulated independently of the *Il-4*/*Il-5*/*Il-13* cluster. In this study, we showed that in primary T cells, GATA-3 plays a direct role in the first events leading to *Il-10* gene expression by inducing chromatin remodeling and acetylation of histones H3 and H4 at the *Il-10* locus, therefore switching the transcriptional status of the *Il-10* locus to a competent one.

To investigate the regulation of *Il-10* gene expression, we firstly compared the transcriptome of several $CD4^+$ T cell subsets, including two distinctly regulated T cell populations (Th2 and IL-10-T_{reg}) that expressed high levels of IL-10, but differed with respect to IL-4 expression. We found that the expression of GATA-3 was up-regulated in Th2 and IL-10-T_{reg} cells, but not in T cells that produced low levels of IL-10, which only expressed little, but detectable GATA-3. In keeping with this, recent studies showed that reduction of GATA-3 expression, by antisense or gene deletion in differentiated Th2 cells decreased the production of IL-10 in addition to a decrease in IL-4 (17, 30). Further, using a transgenic knock-in model in which GATA-3 is ectopically expressed in $CD4^+$ cells, induction of Th2 differentiation and subsequent IL-4, IL-5, and IL-10 production was observed (17, 32). In these studies however it was unclear whether GATA-3 was directly exerting its effect on the *Il-10* gene or whether the effects on IL-10 were mediated by variation of IL-4 levels, which has an instructive role in directing Th2 cell development that is accompanied by IL-10 production. We now show that ectopic expression of GATA-3 increased the transcription and secretion of IL-10 in primary naive $CD4^+$ T cells in the complete absence of IL-4. It is not clear at this

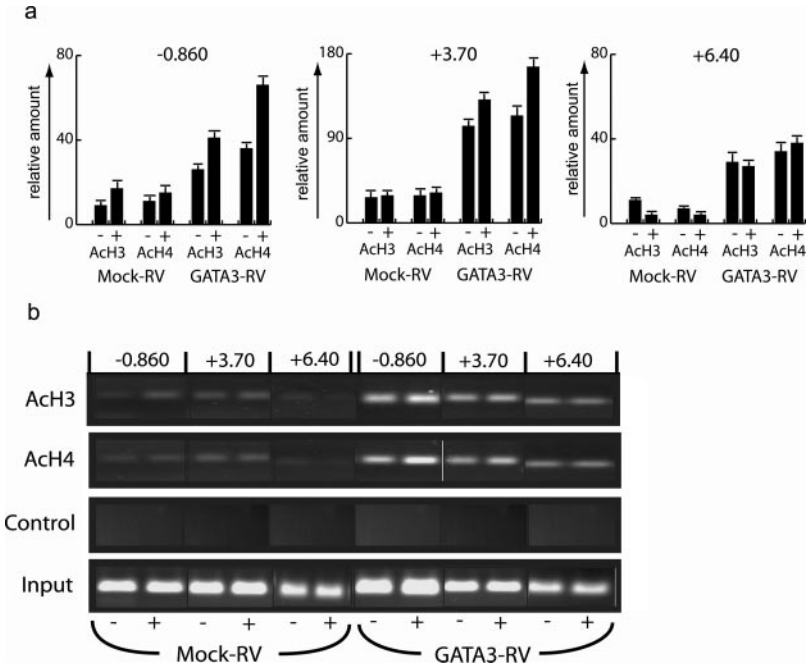

FIGURE 7. GATA-3 induces acetylation of histones H3 and H4 across the *Il-10* locus. *A*, Cross-linked chromatin complexes from resting (−) and activated (+) neutral cultured mock retrovirus (Mock-RV) and GATA-3 retrovirus (GATA3-RV) cells were immunoprecipitated with anti-acetylhistone H3 (AcH3), anti-acetylhistone H4 (AcH4), and control Abs. Specific oligonucleotides were used to amplify by real-time PCR the GATA-3 binding sites in the *Il-10* locus (−0.86 and +3.70 kbp) and HSS +6.70, using the immunoprecipitated or untreated (Input) chromatin as template. Represented is the amount of PCR product immunoprecipitated with specified Abs normalized to the amount of PCR product obtained for the untreated chromatin. The error bars for each condition represent the SD from three replicates. *B*, The PCR products obtained as described in *A* were separated in a 2.5% agarose gel and stained with ethidium bromide.

stage whether the remodeling of the *Il-10* locus induced by GATA-3, in the absence of IL-4, requires low level of activation of STAT6 or alternatively as with GATA-3 induced remodeling of the *Il-4* locus is STAT6-independent, as shown by Ouyang et al. (18). We consider this finding out of the scope of this study.

The molecular mechanism whereby GATA-3 affected the *Il-10* gene remained elusive. The questions are was GATA-3 recruited to the *Il-10* genomic locus and, if so could it transactivate the IL-10 promoter and/or directly induce remodeling of the chromatin? By comparing the DNA sequence of the *Il-10* locus between different species we identified putative conserved GATA-3 binding sites located at the 5′ proximal region (−0.865) and within intron 4 (+3.785). We then showed that GATA-3 is recruited to these sites in vivo in primary Th2 cells. However, unlike its effects on the IL-5 promoter, GATA-3 did not enhance the activity of the IL-10 promoter in in vitro reporter assays. Transactivation of the *Il-10* locus by GATA-3 via regions other than the IL-10 promoter is currently under study.

We used chromatin accessibility by real-time PCR (37) to measure and quantify chromatin accessibility at the *Il-10* genomic locus, and showed that GATA-3 induced changes in the chromatin structure at the *Il-10* locus, independently of IL-4. These changes were most pronounced in the vicinity of conserved GATA-3 binding sites in the 5′ region of the *Il-10* locus, positions −0.860 and −0.610 from the *Il-10* start codon. This result suggests that the binding of GATA-3 to this region enhanced its chromatin accessibility, possibly by stabilizing the *Il-10* locus in an open, euchromatic form. The chromatin at more distant sites of the *Il-10* locus, such as HSS +1.65 and HSS +2.98 that we have shown independently by Southern blot (7) to be remodeled in IL-10-producing cells, was also remodeled in the presence of GATA-3. The accessibility of a recently described positive regulatory region in the 3′ of the *Il-10* gene (HSS +6.40) (8) was significantly remodeled under the influence of GATA-3. These observations suggest that GATA-3 may also contribute to long-range changes of the chromatin structure at the *Il-10* locus and therefore to the stabilization of the chromatin conformation needed for the accessibility of transcription factors required for IL-10 expression. Positive regulatory transcription factors likely include the recently described Jun family members, c-Jun and JunB (8), which are shown to enhance IL-10 expression at these long-range sites (CNS-3) (8). However, as no GATA-3 binding was detected by ChIP at HSS +6.4 (data not shown), direct interaction between GATA-3 and this site is unlikely, although GATA-3 may induce changes in chromatin to allow its accessibility to Jun transcription factors.

In further support for an instructive role for GATA-3 in regulating the *Il-10* gene, we found that in the presence of ectopic GATA-3 the histones located in the vicinity of the GATA-3 conserved sites become acetylated, in cells that normally do not express IL-10. Our data suggest that in the presence of GATA-3, regulatory regions across the *Il-10* locus become accessible to transcription factors (and therefore DNaseI-sensitive) and that the histones around these regions become acetylated, reflecting a transcriptionally active environment.

Whereas changes in the chromatin structure at the *Il-10* locus were induced by GATA-3 before TCR stimulation (in resting cells), IL-10 transcription and secretion required secondary stimulation, even when ectopic GATA-3 was present. It is possible that GATA-3 instructs the changes at the *Il-10* locus necessary for the action of other transcription factors, such as Sp1, Sp3, Smad-4, c-Jun, JunB, and c-Maf, which have been described in different contexts to enhance IL-10 secretion. Similarly, the presence of GATA-3 may also expose the *Il-10* locus to the function of possible repressors, such as the transcription factor Ets-1 (15).

A recent study (48) showed that in primary macrophages and human monocytes stimulated with LPS, c-Maf enhanced IL-10 production; however, up-regulation of c-Maf depended on the addition of IL-4. In our study, T cells transduced with GATA-3 produced significant levels of IL-10 upon stimulation, but only low levels of c-Maf mRNA could be detected by real-time PCR in the complete absence of IL-4. This finding may suggest either that low levels of c-Maf are sufficient for *Il-10* expression in T cells or that in T cells and macrophages, IL-10 production is regulated via distinct mechanisms.

We also showed that GATA-3-transduced neutral or Th1-polarized T cells have a significant reduction in Ets-1 mRNA compared with the corresponding mock-transduced cells. This result is of interest because in Th1 cells derived from Ets-1-deficient mice, a marked increase of IL-10 production was observed (15), suggesting that the transcription factor Ets-1 may act as a repressor for *Il-10* gene expression. However, whether Ets-1 directly represses IL-10 is as yet unclear. It is possible that Ets-1 acts directly on a transcriptionally active *Il-10* locus to repress *Il-10* transcription, which may occur even in the presence of GATA-3. This possibility could partly explain why in GATA-3-transduced neutral or Th1 cells (15) the production of IL-10 is increased, but never to the extent observed in Th2 and IL-10-T_{reg} cells.

The possible interactions between several transcription factors may allude to multiple layers of combinatorial effects of transcriptional enhancers and repressors acting directly on the *Il-10* gene, GATA-3 being a key transcription factor that exposes the *Il-10* locus to all these putative regulators. This hypothesis is very attractive and could explain why we detected a basal level of GATA-3 expressed in all cells that produce IL-10 despite their variation on quantitative levels of IL-10 production. In this study, we systematically found that the expression levels of GATA-3 correlated with IL-10 secretion by different subsets of primary $CD4^+$ T cells. We showed that GATA-3 induced IL-10 expression in primary $CD4^+$ T cells under various differentiation conditions, even in the absence of IL-4. We also showed that GATA-3 was bound to and induced changes of the chromatin structure and acetylation status of histone H3 and H4 at the *Il-10* locus, although it did not transactivate the IL-10 promoter. Taken together our data suggests an instructive role for GATA-3 in the regulation of *Il-10* expression by directly remodeling the *Il-10* gene.

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Disclosures

The authors have no financial conflict of interest.

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