Transcriptional Regulation of Gata3 in T Helper Cells by the Integrated Activities of Transcription Factors Downstream of the Interleukin-4 Receptor and T Cell Receptor*

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GATA3 is a critical transcription factor for many developmental processes. During T helper (Th) cell differentiation, GATA3 induces the Th2 and suppresses the Th1 pathway. Stimulation of the T cell receptor (TCR) of naive Th cells in the presence of interleukin 4 (IL-4) induces robust expression of GATA3; however, it is unclear where these signals integrate. Gata3 encodes two transcripts that differ in their alternative, untranslated first exons. We show here the involvement of the TCR-inducible transcription factor NFAT1 in the transcriptional regulation of both Gata3 transcripts following TCR stimulation of naive and differentiated Th2 cells. We also show that IL-4 is important for the initiation and establishment of Gata3 transcription in developing Th2 cells, especially from the distal promoter. The early function of IL-4 can be STAT6 dependent or independent. However, the establishment of the activity of the distal promoter is totally dependent on STAT6, whereas it is likely that the proximal promoter has additional activation mechanisms that are STAT6 independent. Our findings suggest that different combinations of transcription factors downstream of the IL-4 receptor (IL-4R) and TCR finely modulate Gata3 gene expression from its two promoters for optimal Th2 differentiation.

When naive Th2 cells encounter an antigen in the periphery for the first time, they can differentiate into several lineages. Th1 and Th2 cells are characterized by their respective production of interferon γ and IL-4 following stimulation (1, 2). GATA3, a member in a family of six transcription factors, is a powerful inducer of Th2 and suppressor of Th1 differentiation (3–6). GATA3 also regulates the lineage determination of T cells in the thymus (7–14) and of other cells (15) in the nervous system (16, 17), ear (18, 19), hair follicle (20, 21), kidney (22), and mammary gland (23, 24). The disruption of Gata3 in the mouse germ line is lethal (16), and human Gata3 haploinsufficiency causes hypoparathyroidism, sensorineural deafness, and renal anomaly syndrome (25). Subtle regulation of GATA3 expression is crucial during development: whereas GATA3 is necessary for T-cell differentiation (26), its overexpression impairs this pathway (27–29). Transgenic mice expressing GATA3 in the thymocytes and T cells develop thymic lymphomas (14), and show the spontaneous formation of committed Th2 cells (30). Dysregulated or mutated GATA3 also appears to be associated with various human cancers, including those of the breast (31–33), prostate (34), and cervix (35).

GATA3 epigenetically modifies the Il4 gene and induces its expression. When it is ectopically expressed in developing Th1 cells, GATA3 reconstitutes the DNase I hypersensitivity pattern (4, 36, 37), interferes with the methyl-CpG binding domain protein-2 (38), and promotes histone hyperacetylation on the Il4 locus (39, 40). The function of GATA3 is regulated by its interaction with other factors, such as ROG (41), FOG-1 (42, 43), and T-bet (44). GATA3 can also be modulated by post-translational modifications, phosphorylation (45), and ubiquitination (46), but the functional significance of these modifications is still poorly understood.

Naive Th cells express a low level of GATA3, which is elevated during Th2 and down-regulated during Th1 differentiation (3, 6, 47, 48). Robust GATA3 expression can be achieved through stimulation of both the TCR and IL-4R (49). GATA3 is degraded by the proteasomal machinery following ubiquitination, but is stabilized by the Ras-extracellular signal-regulated kinase (ERK)mitogen-activated protein kinase (MAPK) pathway activated by the TCR stimulation (50). However, it is unclear whether the TCR signaling pathway also regulates GATA3 expression at other levels, such as transcription. The transcription factor STAT6, which is activated by the IL-4R, induces Gata3 mRNA expression (51), but whether this is a direct effect is also unknown. Th2 differentiation can still be elicited in the absence of IL-4 (52–54), and a small percentage of Stat6-deficient GATA3-expressing Th2 cells occur (4). Alternative pathways for the induction of Gata3 expression probably involve the activation of receptors such as Notch, whose ligands are expressed on antigen-presenting cells (55–57).

Gata3 encodes two transcripts with alternative untranslated first exons (49, 1a and 1b. The proximal promoter, which drives expression of the exon 1b-containing transcript, is selectively active in the thymus but not in the brain, whereas the distal promoter, which is located 10-kb upstream and drives the 1a-con-
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taining transcript, is active in the brain but not in the thymus, and gradually increases its activity during Th2 development (49). The distal promoter was recently identified as a direct target of Notch during Th2 differentiation (55, 57). To date, very little is known about the transcriptional regulation of Gata3 and the differential activity of the proximal and distal promoters. Here we quantified the dynamic expression of both Gata3 transcripts during the differentiation and after the restimulation of Th cells, and tested their transcriptional dependence on inputs downstream of the TCR and IL-4R.

EXPERIMENTAL PROCEDURES

Mice—Four-week-old female Balb/c mice were purchased from Harlan Biotech, Israel. Stat6−/− mice were purchased from The Jackson Laboratory and maintained under pathogen-free conditions in the animal facility of the Faculty of Medicine, Technion-Israel Institute of Technology. These studies were reviewed and approved by the Inspection Committee on the Constitution of Animal Experimentation at Technion.

In Vitro Th Cell Differentiation—Th cell differentiation was carried out as previously described (58). CD4+ T cells were purified from the spleen and lymph nodes of 4-week-old mice, with magnetic beads (Dynal). For Th cell differentiation, the cells were stimulated with 1 μg/ml purified anti-CD3ε antibodies (145.2C11) and 1 μg/ml anti-CD28 antibodies (37.51, BD Pharmingen) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, L-glutamine, penicillin-streptomycin, nonessential amino acids, sodium pyruvate, vitamins, HEPES, and 2-mercaptoethanol, in a flask coated with 0.3 mg/ml goat anti-hamster antibodies (MP). For Th1 differentiation, the cells were stimulated in the presence of 10 ng/ml recombinant mouse IL-12 (R&D Systems) and 10 μg/ml purified anti-IL-4 antibodies (11B11). For Th2 differentiation, the cells were stimulated in the presence of 1000 units/ml mouse recombinant IL-12 (R&D Systems) and 10 μg/ml purified anti-IL-12 antibodies (C178). After 2 days, the medium was expanded (4-fold) in the absence of anti-CD3ε and anti-CD28 antibodies, but in the continued presence of the cytokines and antibodies, which included 12 units/ml IL-2. The medium was then expanded every other day. After 8 days, the differentiated Th cells were left unstimulated or stimulated with PMA (15 nM) and ionomycin (0.75 μM). For short stimulation of naive cells, 4 × 107 cells were incubated in 1 μg/ml anti-CD3ε and 1 μg/ml anti-CD28 antibodies in the presence of 160 μl of protein G beads coated with goat anti-hamster IgG for 45 min. Unstimulated cells were cultured without antibodies in the presence of the beads.

RNA Extraction and Real-time PCR—RNA was extracted using TriReagent (Sigma), according to the manufacturer’s instructions, and was reverse-transcribed with Moloney murine leukemia virus Reverse Transcriptase (Promega) using random primers (Amersham Biosciences). Quantitative PCR was performed with Absolute Blue SYBR-Green ROX Mix (Thermo Scientific, ABgene) according to the manufacturer’s instructions, with the Rotor-Gene™ 6000 system (Corbett Research, Sydney, Australia) and its software, version 1.7. The amounts of transcript were normalized to that of β2-μ-globin. Melting curves were determined to ensure the amplification of a single product. The primers used for the exon-specific quantitative PCR analysis were: 1a, 5′-GAGCCTTCAGCACAACGTGAA-3′ and 5′-CCACACTGCACACTGATTC-3′; 1b, 5′-CAATCTGACCGGCAAGGT-3′ and 5′-CAGAGACGGTGTCCCTCCG-3′; 2b-μ-globin, 5′-TTCTGGTGCTTTGCTCCTGA-3′ and 5′-CATGTTGTGGCGTTCCTCCATT-3′.

Chromatin Immunoprecipitation (ChIP)—ChIP analysis was carried out as previously described (58). Cells (10−24 × 10^6) were cross-linked on ice for 20 min, by adding a one-tenth volume of 1% formaldehyde solution (0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES, pH 8.0) directly to the medium. Glycine was then added to a final concentration of 0.125 M. Following an incubation in 100 mM Tris·HCl (pH 9.4), 10 mM dithiothreitol, the cells were washed and resuspended in 0.5 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris·HCl, pH 8.1, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 25 μg/ml leupeptin, and 10 μl iodoacetamide) and sonicated three times with 1-min intervals at 4 °C. The samples were centrifuged at 14,000 × g at 12 °C, and the cleared supernatants were diluted with an equal volume of dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris·HCl, pH 8.1, and protease inhibitors). Aliquots containing ~3 × 10^7 cells were stored at −80 °C. The samples were thawed in 1 ml of dilution buffer and pre-cleared with 40 μl of a slurry of salmon sperm DNA-coated Protein A- or G-Sepharose beads for 2 h at 4 °C (the beads were incubated first with 100 μg/ml salmon sperm DNA). After 10 min of high-speed centrifugation, the cleared samples were incubated overnight at 4 °C with 10 μg of specific antibody followed by a 3-h incubation with the salmon sperm DNA beads. The specific antibodies used were mouse anti-GATA3 (HG 3.31, Santa Cruz), goat anti-GATA3 (n-16, Santa Cruz), rabbit anti-STAT6 (M-20, Santa Cruz), rabbit anti-NOTCH1 (C-20R, Santa Cruz), rabbit anti-NFAT1 (T2B1 and 67.1 (59)), and rabbit anti-acetyl-histone H3 K-9 and K-14 (C-20, Upstate). After the immunoprecipitation, washes, and reverse cross-linking, the samples were extracted twice with phenol/chloroform and once with chloroform, and then ethanol precipitated in the presence of 30 μg of glycogen.

20 μl (out of the resuspended 130 μl) was used for PCR: 2.5 min at 95 °C, 28–30 cycles (30 s at 95 °C, 30 s at 48 °C, and 30 s at 72 °C), completed by 10 min at 72 °C. As a control, PCR was performed directly on input DNA purified from the chromatin before immunoprecipitation. Selected input samples were also amplified with each pair of specific primers. The PCR products were resolved on 3% NuSieve agarose gels and visualized with ethidium bromide. The following primers were used: Il4 enhancer Vα, 5′-AGGGCATTACATATTCC-3′ and 5′-ACGCTCTAGGACTTCC-3′ (239-bp product); Il4 site V, 5′-TGTGTGCCTGGAACGACG-3′ and 5′-AGCTTCCACAATGTTCC-3′ (239-bp product); Gata3 distal promoter, (b) 5′-ACACTGGATTTATCGG-3′ and 5′-ACTTTACCC-ACGTTCGC-3′ (295-bp product), (c) 5′-GTGTTTTACTCCAGGACGG-3′ and 5′-ATTGCTGTGCTTCAATCC-3′ (229-bp product), (d) 5′-TGACCTGTCCCCATAGAC-3′ and 5′-GCC-
TGAAATGTGACCTGC-3’ (186-bp product). Gata3 proximal promoter, (a) 5’-TTATACGACATAGCCC-3’ and 5’-GCAAGCCGAGATTCATTCG-3’ (186-bp product), (b) 5’-ATGCCTGTCTCAGAAGG-3’ and 5’-AGCCTAGGCGCTATGGTGC-3’ (263-bp product), (c) 5’-ATCCGTGTTGCTACCATGAAGG-3’ and 5’-AAGATTTGCTCCGAACC-3’ (205-bp product), (d) 5’-ATCCACTGTGCTCAGAAGG-3’ and 5’-CCGAAAAGACTTCGGGT-3’ (220-bp product), (e) 5’-CTCTGTCCTGCTCACATATTGCCAAAGCCGATTCATTCG-3’ (263-bp product); Gata3 distal promoter, (a) 5’-GATA3GAGAAGTGGCAGG-3’ and 5’-ATGGGGAGAACC-3’ (253-bp product); CNS I, (a) 5’-CATTGGGAGCTGAAAGCC-3’ and 5’-CACCAGTGTGTATTTGCGC-3’ (220-bp product), (b) 5’-CTACCACTTGGTCTACCATGAAGG-3’ and 5’-CAAATGAATCTGATGGTGG-3’ (241-bp product); CNS II, 5’-ATGTTCATCTGGGCATGG-3’ and 5’-CGCGTTGTCACTAAGG-3’ (224-bp product); CNS III, (a) 5’-AAGGAGATGGGAGAACC-3’ and 5’-AGTGAAGAATCCGGAGAACG-3’ (228-bp product), (b) 5’-GAGTGATTCACGCCAACATCC-3’ and 5’-CCAATGTCTCGTTACCG-3’ (211-bp product); CNS IV, 5’-TCCAGGGCCATGTTACC-3’ and 5’-CTCTGATGTTACCAGG-3’ (239-bp product); CNS I, (a) 5’-TGCGAACCCAAAACTACC-3’ and 5’-CGCCAAGAATGCAACTG-3’ (254-bp product), (b) 5’-AGCCTCTCGGAGACTG-3’ and 5’-CTGGAATCCGCTTGAGG-3’ (293-bp product).

4 μl (out of the resuspended 120 μl) was used for the real-time PCR. Each sample was normalized to the value obtained from the input material for that sample without immunoprecipitation. The values of the controls without antibody were subtracted from the samples. Melting curves were determined to ensure the amplification of a single product. The following primers were used: Il4 enhancer Vα5’-GACTGAGAACCCACAGAGATGC-3’ and 5’-GCCCTGGTCTTCTATCATATCATCATGACT-3’ (79-bp product); Gata3 distal promoter, 5’-TGCCCTATGATAATGGCCCATTC-3’ and 5’-CTGCTCTGCTGGTGCTCAAAG-3’ (101-bp product); Gata3 proximal promoter, (a) 5’-ATGCAATGCGTTGTCTACATAA-3’ and 5’-CCAAACCTTCTCCGGAAGG-3’ (104-bp product), (b) 5’-AAACGTCTTGCCTGGAATCCT-3’ and 5’-AGATTATCCGGTACGAGTG-3’ (122-bp product); Exon 1b 5’-CTGGGGCCGCTGCTGAGATTT-3’ and 5’-GGTCAAGATGCGTATGCTCTTATGT-3’ (123-bp product); CNS I, (a) 5’-AAACCTTCTCCGGAAGG-3’ and 5’-TCTGTCTCCTGCTCACATATCC-3’ (119-bp product).

Nuclear/Cytoplasmic Extraction and Western Blotting—Cells were centrifuged for 7 min at 2000 × g, 4 °C, and the pellet was collected with 1 ml of ice-cold phosphate-buffered saline and centrifuged for 5 min at 6000 × g. The supernatant was discarded, followed by another short centrifugation. The pellet was resuspended in 9 volumes of HL buffer (10 mM HEPES-KOH, pH 7.5, 10 mM KCl, 3 mM MgCl2, 0.05% Nonidet P-40, 1 mM EDTA, pH 8.0) and incubated for 30 min on ice, followed by centrifugation for 5 min at 6000 × g, 4 °C. 8 volumes of supernatant was collected into a new tube containing an equal volume of 2× GSLB buffer (90 mM HEPES-KOH, pH 7.9, 500 mM KCl, 0.15% Nonidet P-40, 0.2 mM EGTA, pH 8.0, 20% glycerol), and defined as the “cytoplasmic fraction.” The rest of the supernatant was removed, and the pellet was washed with 9 volumes of HL buffer followed by spinning for 5 min at 6000 × g, 4 °C. The pellet was resuspended in 1× GSLB buffer (50 mM HEPES-KOH, pH 7.9, 250 mM KCl, 0.1% Nonidet P-40, 0.1 mM EDTA, pH 8.0, 0.1 mM EGTA, pH 8.0, 10% glycerol), followed by incubation for 30 min on ice. The tubes were then centrifuged for 10 min, maximum speed, at 4 °C, and the supernatant was collected into a new tube, and defined as the “nuclear fraction.” The samples were fractionated by electrophoresis on 12% SDS gels, followed by transfer onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were stained with anti-GATA3, anti-Lamin-B (Santa Cruz), and anti-γ-tubulin (Sigma) antibodies.

RESULTS

Dynamic Changes in the Expression Levels of Gata3 Transcripts—The first alternative exons of the Gata3 transcripts (1a and 1b) are spliced to a common second exon, which contains the translation start site (49) (Fig. 1A). To quantify the ratio between the expression levels of the Gata3 transcripts during Th cell development and in the differentiated Th cells, we performed an exon-specific quantitative PCR analysis. First, the mRNA amounts were monitored in developing Th2 cells during the first 2 days following stimulation with anti-CD3 and anti-CD28 antibodies (Fig. 1B). The level of both transcripts was increased more than 10 times after 48 h; the expression of the 1b-containing transcript was augmented gradually, whereas the 1a-containing transcript was up-regulated significantly during the second day. Then, after 8 days of differentiation, Th1 and Th2 cells were left unstimulated or stimulated with PMA and ionomycin for the indicated time periods between 1 and 4 h, and the mRNA levels were evaluated (Fig. 1C). As expected, the Th1 cells expressed negligible amounts of the Gata3 transcripts. At 4 h after stimulation, the expression level of both transcripts had increased ~1.5 times in the Th2 cells. In both developing and differentiated Th2 cells, the 1b-containing transcript was more abundant than the 1a-containing one (Fig. 1D); however, the ratio was dynamic: ~600 in naive cells, peaking between 4 and 24 h to an average value of ~2000 and then decreasing to ~20 in resting differentiated cells. Fig. 6 below shows that the dramatic decline in the ratio is the result of a sharp induction in the expression of the 1a-containing transcript after the second day, whereas the 1b-containing transcript reaches its expression peak earlier.

To test whether the stability of the transcripts plays a role in their differential expression rates, we terminated transcription with Act D. Act D was added to (i) differentiating Th2 cells 48 h after stimulation with anti-CD3 and anti-CD28 antibodies (Fig. 1E, left panel), (ii) 8-day resting differentiated Th2 cells (middle panel), and (iii) differentiated Th2 cells 30 min after restimulation (right panel). At the indicated time points, between 0 and 4 h after Act D was added, the cells were harvested and the amount of both transcripts was determined. No major changes were found in the degradation rates of either the 1a- or 1b-containing transcript in developing, resting, or restimulated cells. Therefore, the differential amounts of the transcripts is most likely a result of selective transcriptional rates, although we cannot rule out the possibility that Act D terminates the transcription of an inducible regulator of the Gata3 mRNA stability.

A ChIP assay showed that the proximal promoter is preferentially acetylated on histone H3 (AcH3) in naive cells (Fig. 1F,
**FIGURE 1.** The expression of Gata3 transcripts is dynamically regulated during Th2 differentiation. A, schematic illustration of the Gata3 distal and proximal promoters and the primer sites for amplification of the 1b-containing transcript (1b + AS2 primers) and 1a-containing transcript (1a + AS2 primers). B, naive CD4+ cells from 4-week-old mice were stimulated with anti-CD3 and anti-CD28 antibodies for the indicated time points under Th2 conditions. The Gata3 mRNA levels transcribed from the proximal (left panel) and distal (right panel) promoters were measured by exon-specific quantitative PCR analysis. The expression in naive cells was set as 1. C, 8-day-differentiated Th1 and Th2 cells were stimulated with PMA and ionomycin for the indicated time periods, and the levels of the Gata3 transcripts were measured as in B. The expression in resting Th2 cells was set as 1. Data in B and C are the mean ± S.D. from three independent experiments. D, the ratio between the transcripts, as evaluated in B and C, was calculated as 2^n - (Ct_{1b} - Ct_{1a}) for each time point ± S.D. The amplification efficiencies for both the 1a and 1b as was calculated from the slopes were close to 2.2. The scale for the differentiated cells is magnified in the right graph. E, Act D was added to (i) differentiating Th2 cells 48 h after stimulation with anti-CD3 and anti-CD28 antibodies (left panel), (ii) 8-day resting Th2 cells (middle panel), and (iii) 8-day Th2 cells 30 min after stimulation with PMA and ionomycin (right panel). At the indicated time points after Act D was added, the cells were harvested and the expression levels were measured as in B. The expression level just before Act D was added was set as 1. Data are the mean of two independent experiments. F, ChIP assay assessing the H3 acetylation of the Gata3 promoters in naive and 24-h differentiating Th1 and Th2 cells (left panel), and in 8-day differentiated Th1 and Th2 cells using anti-acetylated histone H3 K-9, K-14 antibodies (αAcH3). Real-time PCR was performed to quantify the acetylation status. The expression in naive (left panel) and resting Th1 cells (right panel) was set as 1. Data are the mean of two independent experiments.
This level of acetylation was maintained for 24 h in differentiating Th1 cells and increased in both the proximal and distal promoters during Th2 differentiation. The proximal promoter was more acetylated than the distal one also in the 24-h differentiating Th2 cells (left panel) and in the 1-week differentiated Th2 cells (right panel). Thus, differences in the acetylation status might underlie the dominance of the proximal promoter. Taken together, these results show that during in vitro Th2 differentiation, the proximal promoter is more active, especially during the early developmental stages, but also in the differentiated cells, although less strikingly.

Identification of Conserved Non-coding Sequences (CNSs) at the Gata3 Locus—Next, we wanted to test whether transcription factors downstream of the IL-4R and TCR are directly bound to Gata3, and whether they have differential effects on the activity of the Gata3 promoters. To extend our search for potential binding sites for transcription factors beyond the Gata3 promoters, we performed a comparative sequence analysis (60). Several CNSs were found ~4–8 kb upstream of the distal promoter, CNSs I-IV, and another one, CNS 1, ~2.5 kb upstream of the proximal promoter, (Fig. 2A). The latter region is reported to be a DNase I hypersensitivity site in Jurkat human T cells (61), and is bound by the trithorax protein MLL in memory Th2 cells (62), and weakly by Notch during Th2 differentiation (57). Several other CNSs appeared downstream of the last exon and inside introns. A ChIP assay confirmed that CNSs I-IV and CNS 1 were hyperacetylated on histone H3 in Th2 but not in Th1 cells, strengthening the assumption that these elements are Th2-specific (Fig. 2B). Sequence inspection revealed that the CNSs contained closely spaced putative binding sites for the transcription factor NFAT, many for GATA3, and several for Notch/RBPJ and STAT (see below). The regions of both promoters possessed binding sites for these factors as well. Fig. 2C delineates the potential binding sites for these factors in CNS I.

NFAT1 Binds to the Gata3 Locus, and the Expression of both Gata3 Transcripts Is Calcineurin-dependent—NFAT1 is a TCR-inducible transcription factor that, following stimulation, binds to cytokine genes in a differential manner: the Il4 gene in Th2 and the Ifng gene in Th1 cells (63). We assessed the binding potential of selected NFAT sites, with the core sequence of GGAA, on the Gata3 promoters and CNSs by ChIP experiments. Fig. 3A shows that NFAT1 bound to the Gata3 promoters and CNSs in differentiated Th2 cells with the expected inducible and selective pattern; as to the control the Il4 enhancer V_A. CsA interferes with the dephosphorylation of NFAT by calcineurin, and as a consequence inhibits its nuclear translocation (64). CsA reduced the expression of both Gata3 transcripts in stimulated Th2 cells to the level of resting cells (Fig. 3B). The induction of Gata3 expression was also calcineurin-independent early in Th2-cell development, as the expression of both Gata3 transcripts was strongly inhibited by CsA during the first 48 h (Fig. 3C). Moreover, the binding of NFAT to both Gata3 promoters was induced shortly (45 min) following stimulation of naive cells (Fig. 3D). Because the conditioned medium contains exogenous IL-4, the necessity for NFAT probably does not reflect the function of NFAT in the transcriptional regulation of Il4 (64). These results strongly suggest the involvement of NFAT1 in the transcriptional regulation of both...
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Gata3 transcripts following TCR stimulation of naive and differentiated Th2 cells.

Notch receptors are stimulated by Notch ligands. The binding of the ligand triggers the release of the intracellular domain of the Notch receptor, which translocates into the nucleus, where it modulates transcription via RPBj (65). Notch ligands are expressed on antigen-presenting cells; however, the TCR stimulation of naive cells also induces the activation of Notch under neutral conditions might use endogenous IL-4 in an autocrine manner. To avoid that, we used cells derived from Stat6-deficient mice (Fig. 4C, lower panels). In the absence of STAT6, we observed a further reduction in the level of the 1b-containing transcript under neutral conditions, indicating that early Gata3 expression from the proximal promoter was induced by the IL-4 in an autocrine manner. Interestingly, 18 h after stimulation with anti-CD3 and anti-CD28 antibodies, the level of both transcripts was higher under Th2 conditions than CD28 antibodies increased the expression of both Gata3 transcripts, and their effect was clearly synergistic 18 h after stimulation. The expression of the 1b-containing transcript increased slightly, even under neutral conditions, in the context of anti-CD28, whereas the expression of the 1a-containing transcript was totally dependent on exogenous IL-4. Cells under neutral conditions might use endogenous IL-4 in an autocrine manner. To avoid that, we used cells derived from Stat6-deficient mice (Fig. 4C, lower panels). In the absence of STAT6, we observed a further reduction in the level of the 1b-containing transcript under neutral conditions, indicating that early Gata3 expression from the proximal promoter was induced by the IL-4 in an autocrine manner. Interestingly, 18 h after stimulation with anti-CD3 and anti-CD28 antibodies, the level of both transcripts was higher under Th2 conditions than

**FIGURE 3.** The expression of the 1a- and 1b-containing transcripts is calcineurin-dependent. A, ChIP assay assessing the binding activity of NFAT1 to the Gata3 promoters, indicated CNSs, and I4 enhancer V4 in resting and 2-h stimulated differentiated Th1 and Th2 cells. Data are representative of two independent experiments. B, the mRNA level of the Gata3 transcripts in 1.5-h stimulated 8-day differentiated Th2 cells with or without 1 μM CsA was measured by exon-specific quantitative PCR analysis. The level in resting cells was set as 1. Data are the mean of two independent experiments. C, naive CD4+ cells were stimulated with anti-CD3 and anti-CD28 antibodies for the indicated time periods under Th2 conditions in the presence or absence of CsA. The Gata3 mRNA levels transcribed from the proximal (left panel) and distal (right panel) promoters were measured. Data are representative of two independent experiments. D, ChIP assay assessing the binding activity of NFAT1 to the Gata3 promoters in resting and 45-min stimulated naive CD4+ cells. The cells were stimulated with anti-CD3 and anti-CD28 antibodies. Real-time PCR was performed to quantify the binding activity. The expression in resting naive cells was set as 1. E, ChIP assay assessing the binding activity of Notch1 at the Gata3 locus in resting and 2-h stimulated 8-day differentiated Th1 and Th2 cells. Data are representative of two independent experiments.

Notch bound to these sites in differentiated Th2 but not Th1 cells (Fig. 3E). Although Notch was bound to these regulatory elements under resting conditions, its binding activity was strongly induced upon restimulation. The selective and inducible binding pattern of Notch was reminiscent of the binding pattern of NFAT1, and we are currently examining the potential cross-talk between these two signaling pathways.

**IL-4 Synergizes with CD28 in Promoting the Early Transcription of Gata3 with Differential Effects on Its Promoters**—We next assessed whether the IL-4R-inducible transcription factor STAT6 also binds directly to Gata3. CNS I and the regions of both promoters contain non-conventional binding sites for STAT6 with spaces of 5,6 nucleotides instead 3,4 (TTGNNNGAA) (71). A ChIP assay showed that STAT6 bound to these regions in resting Th2 cells cultured in the presence of IL-4, and following restimulation (Fig. 4A). The presence of IL-4 during restimulation did not change the expression levels of the 1a- or 1b-containing transcripts (Fig. 4B). To test the importance of IL-4 for the expression of Gata3 transcripts early in differentiation, naive Th cells were stimulated for 12 and 18 h in the presence (Th2) or absence (neutral; N) of exogenous IL-4 (Fig. 4C, upper panels). The cells were stimulated with anti-CD3 antibodies alone or with a combination of anti-CD3 and anti-CD28 antibodies. IL-4 and the anti-CD28 antibodies increased the expression of both Gata3 transcripts, and their effect was clearly synergistic 18 h after stimulation. The expression of the 1b-containing transcript increased slightly, even under neutral conditions, in the context of anti-CD28, whereas the expression of the 1a-containing transcript was totally dependent on exogenous IL-4. Cells under neutral conditions might use endogenous IL-4 in an autocrine manner. To avoid that, we used cells derived from Stat6-deficient mice (Fig. 4C, lower panels). In the absence of STAT6, we observed a further reduction in the level of the 1b-containing transcript under neutral conditions, indicating that early Gata3 expression from the proximal promoter was induced by the IL-4 in an autocrine manner. Interestingly, 18 h after stimulation with anti-CD3 and anti-CD28 antibodies, the level of both transcripts was higher under Th2 conditions than...
under neutral conditions, even in the absence of STAT6. These results indicate the involvement of another IL-4R-inducible factor(s) in the initiation of Gata3 expression. However, in the absence of STAT6, the expression of the 1a-containing transcript was entirely ablated in 1-week differentiated cells in the presence or absence of exogenous IL-4 (Fig. 4D). The expression of the 1b-containing transcript was less dependent on STAT6, although its expression was significantly reduced in the absence of STAT6. Its expression was further moderately reduced under neutral conditions.

FIGURE 4. Differential effect of IL-4 on Gata3 promoters. A, ChIP assay assessing the binding activity of STAT6 to the Gata3 promoters and CNS I in resting and stimulated Th1 and Th2 cells at the indicated time points. The sequences of the binding sites are: CNS 1, TTCAGCTGAA; promoter 1a, TTCCTAGAA; promoter 1b, TTCGAGCTGAA; Il4 site Vα, TTCAGAGAA. Data are representative of two independent experiments. B, the mRNA level of the Gata3 transcripts in 8-day resting or 2-h stimulated Th2 cells in the presence or absence of IL-4. The level in resting cells was set as 1. Data are the mean of two independent experiments. C, naive CD4+ Balb/c (upper panels) or Stat6-deficient (lower panels) cells were stimulated with either anti-CD3 alone or with a combination of anti-CD3 and anti-CD28 antibodies for the indicted time periods under Th2 or neutral (N) conditions (without the addition of external IL-4). The Gata3 mRNA level of the 1b-containing transcript (left panels) or the 1a-containing transcript (right panels) was measured. The expression in naive cells was set as 1. The data are the mean ± S.D. from three independent experiments. D, 1-week differentiated Th1 and Th2 cells derived from Balb/c or Stat6-deficient mice were left unstimulated (0) or stimulated for 1 h (1), and the mRNA level of the Gata3 transcripts was measured. Data are the mean of two independent experiments.
IL-4 synergistically increased the expression of the Gata3 mRNAs from both promoters. Although STAT6 bound to both promoters, the expression of the 1a-containing transcript was more dependent on STAT6 and IL-4 during Th2 development than that of the 1b-containing transcript. IL-4 has STAT6-dependent and -independent effects on Gata3 expression, especially early in Th2 differentiation.

**STAT6 Is Necessary for the Binding of GATA3 at the Distal Promoter**—Recently, GATA3 was found to be associated with the distal promoter and CNS1 during Th2 development (57). There are also putative binding sites in the regions of the proximal promoter and CNSs I-IV. We found by ChIP analysis that GATA3 was bound to both promoters, CNS I, and exon 1b, in resting differentiated Th2 cells (Fig. 5A), that activity can underlie the ability of GATA3 to autoregulate its own expression (4, 36), and to maintain it even in unstimulated cells (Fig. 1C). GATA3 bound the proximal promoter stronger than the distal promoter (data not shown). The binding activity of GATA3 on exon 1b (and slightly on other sites) was increased upon restimulation, possibly, at least partially, as a consequence of the elevation in GATA3 mRNA (Fig. 1C) and protein expression levels (Fig. 5B). The GATA3-binding activity was more greatly induced at the IL-4 enhancer VA, which might reflect the differences in the degree of inducibility between these two genes (resting Th2 cells express negligible amounts of IL-4). As expected, the binding activity of GATA3 was reduced in the Stat6−/−-derived Th2 cells (Fig. 5A), because under these conditions the 1b-containing transcript is selectively expressed and at a lower level (Fig. 4D). However, GATA3 bound the distal promoter in Stat6−/−-deficient Th2 cells more weakly than in Th1 cells (as a background), whereas at the proximal promoter the binding activity was higher than in Th1 cells. These results suggest that STAT6 is necessary for the binding activity of GATA3 at the distal promoter. This might be a result of either STAT6-dependent modulation of the accessibility of the promoter or the necessity for a higher concentration of GATA3 for it to bind to the distal promoter, which is not achieved in the absence of STAT6. ChIP analysis showed that GATA3 was bound to the CNSs II, III, IV, and 1 in differentiated Th2 cells, as well (Fig. 5C).

**The Activity of the Distal Promoter Is More Dependent on IL-4 Than That of the Proximal Promoter, Even at Later Stages of Th2 Cell Development**—To evaluate the role of IL-4 in the expression of the alternative transcripts after the first 48 h, when the cells have already differentiated down the Th2 pathway, we compared the expression levels of the 1a and 1b transcripts in cells under Th2 conditions (Th2) with their expression levels in cells that were cultured for the first 48 h.
under Th2 conditions to allow differentiation, and then cultured without IL-4 and with antibodies against IL-4 (Th2 224 N) (Fig. 6). Th1 cells as a negative control did not express Gata3. The expression level of the 1b-containing transcript was higher on the second day than on the fifth or the eighth days (left panel). Its expression decreased in the presence or absence of exogenous IL-4, although the reduction was moderately greater in the absence of IL-4. In contrast, the expression of the 1a-containing transcript was increased dramatically after the second day, and was much higher under the Th2 conditions (right panel, note the different scales for 1a and 1b). These results, in addition to the data in Fig. 4D, show that the 1a-containing transcript is dependent on IL-4 both early in Th2 development and at later stages of Th2 differentiation, when the 1b-containing transcript has already reached its peak of expression.

DISCUSSION

Tight regulation of GATA3 expression is critical for many developmental processes, and its aberrant expression is associated with diseases in humans and mice (14, 16, 25, 27, 28, 30). Therefore, it is not surprising that GATA3 expression is regulated at more than one step to ensure that its levels are accurate, with the appropriate cellular and developmental specificity. Here we showed that, in addition to stabilizing the GATA3 protein (50), signaling pathways downstream of TCR/CD28 regulate Gata3 transcription. Our results strongly suggest the involvement of NFAT1 in the transcriptional regulation of both Gata3 promoters during early Th cell differentiation and after the stimulation of the differentiated cells. NFAT1 bound to the Gata3 promoters and CNSs in Th2 cells in a differential and inducible manner, similar to its regulation of the Il4 gene (63). The inducible expression of both Gata3 transcripts was inhibited by CsA in differentiated cells upon restimulation. CsA did not interfere with the constitutive expression of Gata3, and therefore other factors must maintain the Gata3 transcription in resting differentiated cells. CsA strikingly inhibited the initiation of the expression of both transcripts following TCR stimulation during early differentiation, and NFAT1 bound both Gata3 promoters in stimulated naive cells. In contrast to NFAT, the TCR/CD28-inducible transcription factor NF-κB is important for the expression of GATA3 in developing cells but not in differentiated cells (72). Several sequences in the Gata3 promoters and CNSs contain closely spaced binding sites for GATA3 and NFAT that may facilitate the cooperation of these factors, such as at the Il4 enhancer V_\lambda (59).

We showed that in differentiated Th2 cells, Notch1 bound to both Gata3 promoters as well as to selectively tested CNSs. Under physiological conditions, Notch binding is probably induced upon the interaction of Th cells with antigen-presenting cells (56); however, the TCR stimulation of naive cells (56, 66, 67) or restimulation of differentiated cells with PMA and ionomycin (here) also induces Notch-binding activity. Because stimulated Th cells express Notch and Notch ligands (56, 67, 73), mutual cellular interactions are feasible (74). The requirement for stimulation to induce Notch-binding activity may reflect the up-regulation of Notch ligands and receptors, a post-translational modification such as glycosylation (75), or a potential, as yet unknown, conformational change. However, it is also possible that the activation of Notch by the TCR is cell autonomous, and the co-localization of Notch1 with CD4 following stimulation transduces the signals into the cells (76). There are several examples of cross-talk between the Notch and calcineurin/NFAT signaling pathways, and the outcomes of these interactions are context-dependent (77, 78). The binding sites of both NFAT and RPBjκ partially overlap, and both contain the core element GGAA (see the example in CNS I in Fig. 2C), raising the option of competition/cooperation in the binding activity of these factors, similar to that of RPBjκ and NF-κB (79–83). Further research is necessary to determine whether these pathways interact in Th cells, and if so, whether the effect is antagonistic or synergistic.

CD28 synergistically cooperates with IL-4 and is critical for the initiation of the expression of both the 1a- and 1b-containing transcripts, especially in Stat6-deficient cells. The costimulatory molecule CD28 enhances Th2 differentiation and responses (84–90), and can augment IL-4 production (88, 91);
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however, because exogenous IL-4 was provided under the Th2 conditions, the up-regulation in the level of the Gata3 transcripts, in the context of anti-CD28 antibodies, was IL-4-independent. It is possible that the activity of CD28 is necessary to regulate the duration and amplitude of the NF-κB response (92).

The early endogenous expression of IL-4 during Th cell differentiation was sufficient for low expression of only the 1b-containing transcript, because in the absence of exogenous IL-4, under neutral conditions, up-regulation of the 1a-containing transcript was totally absent. It is possible that the reduced level of GATA3, which is expressed from the proximal promoter in the absence of exogenous IL-4, is below the required threshold to initiate the expression from the distal promoter. Indeed, in the absence of STAT6, the binding activity of GATA3 to the distal promoter was severely impaired. The activity of the distal promoter was dependent on IL-4 even after the first 48 h of differentiation, when the expression of the 1b-containing transcript was already established; the depletion of IL-4 strongly inhibited the dramatic up-regulation of the 1a-containing transcript during the first week of differentiation. IL-4 increased the expression of Gata3 transcripts in early differentiating Th2 cells even in STAT6-deficient cells, probably as a result of additional activity downstream of the IL-4R that is STAT6-independent (93, 94). However, this initial activity was not established in the absence of STAT6, especially from the distal promoter, which was totally silent in 1-week differentiated Stat6−/−-derived cells. Taken together, these findings indicate that IL-4 is important for the initiation and establishment of Gata3 expression in developing Th2 cells, especially from the distal promoter. The early activity downstream of the IL-4R can be STAT6 independent, but the establishment of the function of the distal promoter is totally dependent on STAT6. The proximal promoter has probably additional activation mechanisms that are STAT6 independent.

The ratio between the levels of the alternative Gata3 transcripts changed dynamically during Th2 differentiation. The expression of the 1a-containing transcript was elevated dramatically and continuously throughout the first week. However, under our experimental conditions, although the ratio of 1b to 1a transcripts decreased significantly from its peak on the second day, when the 1b-containing transcript reached its peak, the 1b-containing transcript was more abundant than the 1a-containing transcript, even on the eighth day and after restimulation. Perhaps, under different circumstances, such as under stimulation in the context of Notch ligands, the 1a-containing transcript prevails. We could not detect any differences in the degradation rate of the transcripts; therefore, the differences in the levels of the transcripts most likely reflect differential activity of the promoters. Recent evidence suggests that the alternative promoters use in mammalian cells is very general, and almost one-half of the protein-coding genes contain more than one promoter (95). Both of the Gata3 transcripts are translated from the same initiation site at the second exon. There is a stop codon at the second exon, before the translation initiation site, that prevents the translation of the alternative first exons. Alternative promoters that encode identical proteins can regulate cell type-specific transcription; such as the Gata3 proximal promoter is active in the thymus, whereas the distal promoter is active in the brain (49). Alternative promoters can also be regulated differentially even within the same cell by differential responses to environmental cues, as the distal Gata3 promoter is induced after the activation of IL-4R and/or Notch, whereas the expression of the 1b-containing transcript is less dependent on these receptors. Heterogeneity in the 5′ non-coding end of the transcripts can also confer the potential for rapid and fine-tuned post-transcriptional regulation. As mentioned above, we could not detect differences in the stability of Gata3 transcripts; however, these transcripts may have differential translation rates, cellular locations, or other differences directing their selective fates. Although both transcripts contain exon 2, we cannot exclude the possibility that the different first exons promote alternative downstream splicing patterns. Using commercially available anti-GATA3 antibodies, we recognized only one band by Western blot analysis.

Because the 1b-containing transcript is the prominent transcript in the thymus and naive cells (49), it might be already poised for transcription in naive cells and therefore be induced faster during Th2 development and less dependent on IL-4 and Notch. The activation of the distal promoter may provide a mechanism for the irreversible commitment to Gata3 transcription in terminally differentiated Th2 cells, in contrast to the potential to reverse the transcriptional status in thymocytes and naive T cells. This is consistent with the activity pattern of the distal promoter in more differentiated cells, such as in the brain and terminally differentiated T cells (49).

Several regulatory elements of the murine and human Gata3 genes (between −10 and +7 kb) have been found in different cell types by transient transfection assay, transgenic mice, and DNase I hypersensitivity analysis (15, 61, 96–99). We found CNSs upstream of the distal and proximal promoters that were bound by the TCR- and IL-4-inducible transcription factors. However, even a yeast artificial chromosome containing 625 kb of the murine Gata3 gene was not sufficient to recapitulate its full transcriptional pattern in the thymus of transgenic mice, suggesting that the boundaries of the locus extend even beyond this large region (100), and that probably the transcriptional regulation of Gata3 is more complicated than it appears so far.

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