Interleukin-13 Gene Expression Is Regulated by GATA-3 in T Cells

ROLE OF A CRITICAL ASSOCIATION OF A GATA AND TWO GATG MOTIFS

Using a transgenic approach, we studied the role of GATA-3 in T cells. As previously shown, enforced GATA-3 expression in transgenic mice inhibits Th1 differentiation of CD4 T cells, but unexpectedly, both type 1 (interferon γ) and type 2 (interleukin (IL)-4 and IL-13) cytokine genes were activated in the transgenic CD8 T cells. Because IL-13 gene expression was highly enhanced in vivo by GATA-3 expression, we studied the human and the mouse IL-13 gene promoters and found an evolutionary-conserved association of a consensus GATA binding site and two GATG motifs. We showed that efficient GATA-3 binding to this regulatory sequence required these three motifs and that the affinity of the GATA zinc fingers for this association was five times higher than for the consensus GATA binding site alone. Transfections in a T cell line or transactivation by GATA-3 showed that the combination of the three sites was required for full transcriptional activity of the IL-13 gene promoter. Finally we showed that this association of binding sites causes a very high sensitivity of the IL-13 gene promoter to small variations in the level of GATA-3 protein. Altogether, these results indicate an important role of GATA-3 in CD8 cytokine gene expression and demonstrate that a critical network of GATA binding sites highly modulates GATA-3 activity.

Upon antigenic activation, naive CD4 T cells differentiate into at least two functionally distinct subsets of effector cells that differ in their cytokine expression pattern and in their effect in the regulation of the immune response (1). T helper 1 (Th1)CD4 cells produce interleukin-2 (IL-2), interferon γ (IFNγ), and tumor necrosis factor β and are implicated in cell-mediated immune response, whereas Th2 CD4 cells produce IL-4, IL-5, IL-10, and IL-13 and are mostly involved in humoral immunity. In several pathological conditions like allergy or autoimmune diseases, the balance between Th1 and Th2 responses seems to be impaired (2, 3). In asthma, Th2 CD4 T cells are increased in the Airways of patients after antigen challenge, and Th2 cytokines seem to be required for the development of airway eosinophilia and elevation of IgE serum level (4).

How the Th1/Th2 differentiation of CD4 T cells is regulated at the transcriptional level has been the subject of intensive research these last years. Among transcription factors, an essential role has been assigned to the signal transducer and activator of transcription 4 (STAT4) and 6 (STAT6) in the IL-12- and IL-4-mediated differentiation of CD4 T cells toward Th1 or Th2. In addition, Th1 commitment requires the expression of T-bet, a T box transcription factor that induced IFNγ and IL-12 receptor β2 gene expression, whereas nuclear factor IL-6 and c-Maf are expressed in Th2 cells and regulate IL-4 gene expression (5, 6). Finally, numerous reports demonstrate that GATA-3 is a major regulator of Th1/Th2 polarization (7–11). GATA-3 is a member of the GATA family of transcription factors whose common structure is a conserved double zinc finger motif that binds to the consensus DNA sequence 5′-A(G/T)GATA(A/G)-3′. GATA-3 is widely expressed during mouse embryonic day 12 (12–14). In adults, its expression is essentially restricted to T and natural killer cells. GATA-3 is absolutely required for the development of the T cell lineage and for the Th1/Th2 differentiation of naive CD4 T cells (15, 16). The increase of GATA-3 mRNA level during Th2 development is induced by the IL-4/STAT6 pathway, whereas its decrease during Th1 development is under the control of the IL-12/STAT4 pathway (8). In CD4 T cells, GATA-3 induces the expression of type 2 cytokines like IL-4 or IL-5 and represses type 1-specific genes like the IFNγ and the IL-12 β2 receptor subunit genes (7–9).

In contrast to CD4 T cells, CD8 T cells have been described as a homogeneous class of cells producing IFNγ and tumor necrosis factor β and are implicated in cytotoxicity (17). However, some studies indicate that CD8 T cells can also differentiate toward two different phenotypes, leading to the concept of Tc1 and Tc2 CD8 T cells (18, 19). Tc2 cells have been detected in AIDS patients and in lepromatous leprosy, and the relative in vivo roles of the Tc1 and Tc2 subsets in immune regulation are currently being investigated in pathological conditions such as autoimmunity, viral infections, or bone marrow graft (20–22).

To study the consequences of GATA-3 overexpression in CD4 and CD8 T cells, we generated transgenic mice where GATA-3 expression is under the transcriptional control of the human CD2 promoter and locus control region, a cassette that allows an expression restricted to T cells in adult (23). The expression pattern of the cytokines genes in transgenic mice CD8 T cells indicated that the role of GATA-3 might be different in CD8
and in CD4 T cells and showed a function of GATA-3 in the transcriptional regulation of the IL-13 gene through a complex network of motifs characterized in this study.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs and Transgenic Mice**—The CD2-hGATA-3 construct was obtained by inserting the human GATA-3 (hGATA-3) cDNA at the EcoRI cloning site of the CD2 minigene cassette (22). After a SalI and XhoI digestion of the recombinant plasmid, the 12-kbp DNA fragment containing the transgene was purified by agarose gel electrophoresis and microinjected into the pronuclei of the protected oocytes from a cross of B6D2F1 animals (C57BL/6 × DBA/2). Southern blot analysis of tail DNA was used to identify transgenic animals, to determine the copy number, and to assess integration patterns.

**Northern Analysis**—Total RNA of thymus was prepared using TRIZOL (Invitrogen). 20 µg of each sample were electrophoresed in a 1% agarose gel containing 2% formaldehyde in 10 mM phosphate buffer, pH 7, and then transferred to a Nylon+ membrane (Hybond-N+) by capillary blotting. The blot was probed with a BglII-BamHI DNA fragment complementary to the 3’ end of the transgenic RNA.

**Western Analysis**—Cellular extracts of thymus were obtained and Western blotting was performed as previously described (24) using the mouse anti-hGATA-3 antibody (HGS-35, Santa Cruz Biotechnology) and a rabbit anti-mouse horseradish peroxidase-linked antibody (Promega). Immunoblots were then developed using electrochemiluminescence (ECL, Amersham Biosciences).

**Purification and Activation of Splenic T Lymphocytes**—Spleens were removed from mice and homogenized in RPMI 1640 medium containing 1% fetal calf serum, 50 µM β-mercaptoethanol, and 1% penicillin and streptomycin. Red blood cells were lysed by osmotic shock in lysis buffer (NH4Cl 155 mM, KHCO3 10 mM, EDTA 0.1 mM). Non-dissociated cells and tissue debris were filtered on a cell strainer 70-µm nylon (Falcon, 35-2350). Spleenic B cells were depleted using sheep anti-mouse IgG microbeads (Dynal 11201), and CD4 and CD8 T cells were further purified by positive selection after staining, respectively, with fluorescein isothiocyanate-conjugated anti-mouse CD3 (clone 145–2C11) and Rhodamine-conjugated anti-mouse CD4 (clone GK1.5) or fluorescein isothiocyanate-conjugated anti-mouse CD8 (clone 53-6.7) by sorting on a flow cytometer (Epics Coulter). Both T cell subsets were more than 95% pure. These T cells were cultured in RPMI 1640 medium supplemented with Glutamax, penicillin, and streptomycin and containing 10% fetal calf serum, 50 µM β-mercaptoethanol, 10 ng/ml phorbol 12-myristate 13-ace tone (PMA), 500 µM anti-CD3, and 50 units/ml human IL-2 (human lymphocyte IL-2, R&D System). Cells were cultured in 1% agarose gel containing 2M formaldehyde in 10 mM phosphate buffer, pH 7 and then treated with DNase I (Roche Molecular Biochemicals). cDNA was prepared from 10^5 cells/200 µl of total RNA using a oligo-dT primer and the local of the slope of the best linear fit as described previously. The ratio cytokine mRNA levels was calculated from the concentration of bound DNA in mol/liter. The dissociation constants (K) were determined by Scatchard analysis from the reciprocal of the slope of the best linear fit as described previously. The sequences of the binding sites (sense strands) were as follows.

**RT-PCR Reactions**—Total RNA from thymus, spleen, or purified and activated CD4 or CD8 T cells was purified with TRIzol (Invitrogen) and concentrated by ethanol precipitation. RNAs were then treated with DNase I (Roche Molecular Biochemicals). cDNA was prepared from 1 µg of total RNA using a oligo-dT primer and the Superscript II reverse transcriptase (Invitrogen) in 50 µl. All RT-PCR reactions were standardized by level of murine hypoxanthine phosphoribosyltransferase (mhprt) expression. Conditions of PCR were chosen according to the primers used. Amplification of cDNA was performed using the following primers: mhprt 5’, GTAATGATCAGTCAACGGGG-GAC, and 3’, GTAATGATCAGTCAACGGGG-AC; mhprt 5’, GTAATGATCAGTCAACGGGG-GAC, and 3’, GTAATGATCAGTCAACGGGG-AC; and mhprt 5’, GTAATGATCAGTCAACGGGG-GAC, and 3’, GTAATGATCAGTCAACGGGG-AC.

**Determination of Dissociation Constants**—Titrations with the GATA-2 DNA binding domain and various oligonucleotides were performed in 10 µl of 50 mM Tris, pH 7, 0.0125% Triton, 3.2% Ficoll, 2 mM EDTA, and 4 µg/ml polyclonal antibodies. Samples were electrophoresed on 8% acrylamide gels in 10 mM HEPES, 10 mM Tris, and 1 mM EDTA. The amount of bound (B) versus free (F) DNA was determined with a Molecular Dynamics PhosphorImager, and the B/F ratio was plotted against the concentration of bound DNA in mol/liter. The dissociation constants (K) were determined by Scatchard analysis from the reciprocal of the slope of the best linear fit as described previously. The sequences of the binding sites (sense strands) were as follows.

**RT-PCR Reactions**—Total RNA from thymus, spleen, or purified and activated CD4 or CD8 T cells was purified with TRIzol (Invitrogen) and concentrated by ethanol precipitation. RNAs were then treated with DNase I (Roche Molecular Biochemicals). cDNA was prepared from 1 µg of total RNA using a oligo-dT primer and the Superscript II reverse transcriptase (Invitrogen) in 50 µl. All RT-PCR reactions were standardized by level of murine hypoxanthine phosphoribosyltransferase (mhprt) expression. Conditions of PCR were chosen according to the primers used. Amplification of cDNA was performed using the following primers: mhprt 5’, GTAATGATCAGTCAACGGGG-GAC, and 3’, GTAATGATCAGTCAACGGGG-AC; mhprt 5’, GTAATGATCAGTCAACGGGG-GAC, and 3’, GTAATGATCAGTCAACGGGG-AC; and mhprt 5’, GTAATGATCAGTCAACGGGG-GAC, and 3’, GTAATGATCAGTCAACGGGG-AC.

**Determination of Dissociation Constants**—Titrations with the GATA-2 DNA binding domain and various oligonucleotides were performed in 10 µl of 50 mM Tris, pH 7, 0.0125% Triton, 3.2% Ficoll, 2 mM EDTA, and 4 µg/ml polyclonal antibodies. Samples were electrophoresed on 8% acrylamide gels in 10 mM HEPES, 10 mM Tris, and 1 mM EDTA. The amount of bound (B) versus free (F) DNA was determined with a Molecular Dynamics PhosphorImager, and the B/F ratio was plotted against the concentration of bound DNA in mol/liter. The dissociation constants (K) were determined by Scatchard analysis from the reciprocal of the slope of the best linear fit as described previously. The sequences of the binding sites (sense strands) were as follows.

**RT-PCR Reactions**—Total RNA from thymus, spleen, or purified and activated CD4 or CD8 T cells was purified with TRIzol (Invitrogen) and concentrated by ethanol precipitation. RNAs were then treated with DNase I (Roche Molecular Biochemicals). cDNA was prepared from 1 µg of total RNA using a oligo-dT primer and the Superscript II reverse transcriptase (Invitrogen) in 50 µl. All RT-PCR reactions were standardized by level of murine hypoxanthine phosphoribosyltransferase (mhprt) expression. Conditions of PCR were chosen according to the primers used. Amplification of cDNA was performed using the following primers: mhprt 5’, GTAATGATCAGTCAACGGGG-GAC, and 3’, GTAATGATCAGTCAACGGGG-AC; mhprt 5’, GTAATGATCAGTCAACGGGG-GAC, and 3’, GTAATGATCAGTCAACGGGG-AC; and mhprt 5’, GTAATGATCAGTCAACGGGG-GAC, and 3’, GTAATGATCAGTCAACGGGG-AC.

**Determination of Dissociation Constants**—Titrations with the GATA-2 DNA binding domain and various oligonucleotides were performed in 10 µl of 50 mM Tris, pH 7, 0.0125% Triton, 3.2% Ficoll, 2 mM EDTA, and 4 µg/ml polyclonal antibodies. Samples were electrophoresed on 8% acrylamide gels in 10 mM HEPES, 10 mM Tris, and 1 mM EDTA. The amount of bound (B) versus free (F) DNA was determined with a Molecular Dynamics PhosphorImager, and the B/F ratio was plotted against the concentration of bound DNA in mol/liter. The dissociation constants (K) were determined by Scatchard analysis from the reciprocal of the slope of the best linear fit as described previously. The sequences of the binding sites (sense strands) were as follows.
disrupted (the mutations are as described under “Nuclear Extracts and DNA Binding Assays”) were generated by the use of different 5′ primers during PCR amplification. All constructs were checked by DNA sequencing.

**Transient Transfections and Luciferase Assays**—10^7 Jurkat or HeLa cells were electroporated as described (25). Ten μg of reporter gene plasmid, 2 μg of pRL-TK plasmid (Promega) for normalization of transfection efficiency, and for HeLa cells, various amounts of pECE-hGATA-3 expression plasmid or empty pECE vector were used. Two hours after electroporation, Jurkat cells were activated by 50 ng/ml PMA and 1 μg ionomycin. Cells were harvested 24 h after transfection, and luciferase activities were determined using the dual-luciferase reporter assay system as indicated by the manufacturer (Promega).

**RESULTS**

**CD2-GATA-3 Transgenic Mice**—Transgenic constructs were made by inserting the human full-length GATA-3 cDNA into the EcoRI site of an hCD2 minigene (23) containing 4.5 kb of 5′-flanking sequence, the first exon and intron as well as the last exon of the human CD2 gene, and 5.5 kb of 3′ CD2 locus control region (Fig. 1A). The CD2-GATA-3 DNA fragment was purified and microinjected into the pronuclei of fertilized (C57BL/6 × DBA/2) F1 oocytes. Two transgenic lines were generated, line 1 (L1) and line 3 (L3). Southern analysis of tail DNA showed that L1 contained 8 copies of the transgene, whereas L3 contained 3 copies (data not shown).

Northern blot analysis showed the presence of transgenic hGATA-3 mRNA in the thymus of L1 heterozygous and homozygous mice (Fig. 1B), whereas a very faint signal was obtained with mRNA from L3 thymus (data not shown). To precisely define the difference in the expression of the transgene in the two transgenic lines, we used RT-PCR analysis and found that the hGATA-3 mRNA level was about eight times lower in the thymus of line 3 (Fig. 1C). Transgenic hGATA-3 protein expression was studied by Western blot analysis using protein extracts from the thymus of L1 mice. As shown in Fig. 1D, a higher level of GATA-3 was found in both heterozygous and homozygous L1 mice, and similar results were obtained using EMSA (data not shown). The GATA-3 protein found in the thymus of wild type (WT) mouse is accounted for by the cross-reactivity of the antibody against human GATA-3 with mouse GATA-3. In purified CD4 or CD8 T cells isolated from the spleen of homozygous L1 mice, hGATA-3 expression was studied by RT-PCR, and a higher level of expression was found in CD8 T cells (Fig. 1E).

Transgenic mice were healthy and of normal fertility in the two lines. Thymus and spleen of transgenic mice were of similar size compared with wild type mice. The percentage of immature double negative CD4−CD8−, double positive CD4+CD8+, or single positive CD4+ or CD8+ was similar in the thymus of transgenic and WT mice as was the percentage of CD4+ or CD8+ T cells in the spleen (data not shown).

**Cytokines Expression after Activation of CD4 T Lymphocytes from Transgenic Mice**—Numerous studies have shown that overexpression of GATA-3 in CD4 T cells leads to an elevation of type 2 cytokines and a reduction of type 1 cytokines. It has also been demonstrated that GATA-3 overexpression mediated by retroviral transduction induced an increased expression of the endogenous GATA-3 gene in murine T cells (11, 26). To validate the transgenic CD2-GATA-3 model we have generated, we compared the expression level of mGATA-3 mRNA in the thymus of transgenic mice and of two known GATA-3 targets, IL-4 and IFN-γ, in CD4 lymphocytes isolated from the spleen of homozygous L1 mice or of non-transgenic age- and sex-matched control mice. Cell-sorted purified CD4 T cells were activated for 6 days with PMA and ionomycin and further stimulated for 24 h with immobilized anti-CD3 in the presence of IL-2. The mRNA expression level of mGATA-3, IFN-γ and IL-4 was studied by RT-PCR. As shown in Fig. 2, the mGATA-3 level was not modified by the presence of the transgenic hGATA-3 protein, but the IFN-γ mRNA level was lower in CD4 lymphocytes of homozygous mice compared with WT CD4 T cells, whereas IL-4 mRNA level
was higher in the CD4 lymphocytes of these transgenic mice. These results agreed with previous reports and indicate that the CD2-GATA-3 mice produced are suitable to study the consequences of GATA-3 overexpression in T cells.

**Cytokine Expression after Activation of CD8 Lymphocytes from Transgenic Mice**—We therefore used these mice to determine the consequence of GATA-3 overexpression in CD8 lymphocytes. Cell-sorted purified CD8 T cells from the spleen of L1 or L3 mice or from non-transgenic age- and sex-matched control mice were activated for 6 days by PMA and ionomycin and further stimulated for 24 h with immobilized anti-CD3 in the presence of IL-2. The mRNA levels of mGATA-3, IL-4, IL-13, and IFNγ were first studied by RT-PCR. As shown in Fig. 3A, although the mRNA level of mGATA-3 was identical in WT and transgenic CD8 T cells, the expression level of type 2 cytokines (IL-4 and IL-13) mRNAs was higher in the transgenic CD8 lymphocytes. Surprisingly, the level of IFNγ mRNA was also higher, albeit to a lesser extent. The same results were obtained using CD8 lymphocytes from an L1 heterozygote mouse (Fig. 3B), and an elevation of IL-13 mRNA was also demonstrated in CD8 lymphocytes from a L3 homozygous mouse (data not shown).

To quantify the respective expression of the different cytokine mRNAs after CD8 lymphocyte activation, RNase protection assay was performed. IFNγ mRNA was highly expressed and could be easily detected, whereas among Th2 cytokines, IL-13 mRNA was the only mRNA whose level was high enough to be detected by RNase protection assay (Fig. 4A). After quantification of the different signals, the IFNγ mRNA level in the CD8 T cells from the homozygous L1 mice was found to be twice the level of control mice, whereas the IL-13 mRNA was 13-fold higher. IFNγ was also evaluated at the protein level by intracellular staining with a monoclonal antibody to IFNγ and flow cytometry. The percentage of CD8 T cells expressing IFNγ was 22% for control mouse and 34% for transgenic mouse (Fig. 4B), indicating that hGATA-3 expression did not impair IFNγ expression. Altogether, these results indicated that the consequences of GATA-3 overexpression are different in CD4 and in CD8 T cells and that the IL-13 gene is regulated by GATA-3 in vivo.

Because it has been demonstrated that in CD4 T cells GATA-3 expression induced type 2 cytokines expression (7) and reduced IFNγ expression under Th1-skewing conditions (9), we studied, under Th1- or Th2-polarizing conditions, the mRNA levels of IFNγ and IL-13, which is the only highly expressed type 2 cytokine in CD8 T cells (Fig. 4A). The mRNA levels of the endogenous mGATA-3 and of the transgenic hGATA-3 were also studied (Fig. 5). Under Th1 conditions mGATA-3 mRNA level was nearly undetectable, whereas the same level of mGATA-3 mRNA was observed in WT or transgenic CD8 T cells under Th2 conditions. Under Th1 conditions, no IL-13 mRNA was seen in WT, whereas a high expression of this mRNA was detected in transgenic CD8 T cells. Under Th2 conditions, we found no obvious difference of the IL-13 mRNA level between transgenic and non-transgenic CD8 T cells. Finally, no clear difference of the IL-13 mRNA expression under Th1- or Th2-polarizing conditions was observed. Thus, ectopic expression of hGATA-3 in CD8 T cells induced expression of IL-13 under Th1-polarizing or neutral activation conditions. Under Th2-polarizing conditions, the mGATA-3 level is high, and ectopic expression of hGATA-3 has no obvious effect.

**The Mouse or Human IL-13 Gene Promoter Contains Three Clustered GATA Binding Sites**—Because the IL-13 mRNA level was markedly increased by the overexpression of hGATA-3 in CD8 T cells, we wondered if IL-13 gene transcription was directly regulated by GATA-3. We first looked at the sequences of the mouse and human IL-13 gene promoters (27) and found a region of homology between human and mouse, respectively, located between −125 to −77 (human) and −107 to −59 (mouse) upstream of the site of transcription initiation. This region contains a consensus GATA binding site (site 1) and two GATG motifs (sites 2 and 3) highly conserved between human and mouse (Fig. 6A).

We first studied the binding of hGATA-3 to the consensus GATA binding site using a T cell nuclear extract and a short oligonucleotide that does not contain either of the two GATG motifs and found very weak hGATA-3 binding (data not shown). This result prompted us to study hGATA-3 binding to the mouse −110 to −70 DNA fragment that contained the three putative GATA binding sites. This fragment specifically bound hGATA-3, as shown by competition (Fig. 6B, lane 1) and supershift (Fig. 6B, lane 3) experiments, and displayed a much higher affinity for hGATA-3 than the consensus GATA binding site alone (Fig. 6B, compare lanes 4 and 5). Disruption of the consensus GATA binding site led to weak hGATA-3 binding (lane 6) and, although disruption of site 3 (lane 8) had a modest

![Fig. 2. Effect of hGATA-3 on mGATA-3, IFNγ, and IL-4 genes expression in CD4 T cells.](http://www.jbc.org/)

![Fig. 3. Effects of hGATA-3 expression on the mGATA-3 and cytokines genes expression in CD8 T cells activated under neutral conditions.](http://www.jbc.org/)
from the EMSA experiment, the association of the three sites or quantification, IFNγ, and IL-13 and IFNγ genes expression in CD8 T cells activated under Tc1 or Tc2 conditions. Sorted splenic CD8 T cells were fixed, permeabilized, stained with anti-CD8 and anti-IFNγ monoclonal antibodies, and analyzed by flow cytometry. The percentage of cells expressing IFNγ was determined in gated CD8 cells. Both experiments were conducted three times, and the result of a representative experiment is shown.

A

B

IFNγ

WT

HO

Cell number

Effect of hGATA-3 expression on mGATA-3, IL-13, and IFNγ genes expression in CD8 T cells activated under Tc1 or Tc2 conditions. Sorted splenic CD8 T cells were fixed, permeabilized, stained with anti-CD8 and anti-IFNγ monoclonal antibodies, and analyzed by flow cytometry. The percentage of cells expressing IFNγ was determined in gated CD8 cells. Both experiments were conducted three times, and the result of a representative experiment is shown.

Affinities of the Different IL-13 Promoter GATA Binding Sites for the GATA Zinc Fingers—To precisely evaluate the relative binding affinities of the three GATA sites found in the IL-13 gene promoter, we determined by Scatchard analysis their affinity for a GATA-2 recombinant peptide that contained the DNA binding domain of GATA-2. Among the GATA factors, the DNA binding domains of GATA-2 and -3 are very similar to one another. Unlike most GATA factors, the N-fingers of both proteins can bind to DNA independently (28), and the specificity of GATA-2 and -3 was shown to be virtually identical in binding site selection experiments (29). Each of the three sites displayed a low affinity, the dissociation constant being, respectively, 10, 67, and 73 nM for sites 1, 2, or 3 (Fig. 7A). As expected from the EMSA experiment, the association of the three sites or of the consensus GATA binding site with the site 2 enhanced affinity between 5- and 10-fold (Fig. 7B). These experiments demonstrated that the GATA binding motif present in the IL-13 promoter is a high affinity motif resulting from the association of low affinity binding sites.

Using higher peptide concentrations, a second more slowly migrating complex (2:1 complex) can be seen with the wild type IL-13 probe or with sites 1 and 3 (Fig. 7B). This complex is due to the association of two molecules of peptide with the DNA and occurs with the probes containing the first and the last GATA sites. No 3:1 complexes were observed.

It can be seen in Fig. 7A that the migration of complexes with probes containing only site 1 or 2 is slower than that of the wild type or the site 3 probe. However, combining site 1 and 2 on a single probe results predominantly in a complex that migrates in the position of the wild type probe and faster than either single site alone (Fig. 7B). This suggests that the presence of both binding sites on a single DNA allows the GATA-2 peptide to bind in a different conformation than when only one site is available. In contrast, the combination of site 1 and site 3 appears additive since both the slow migrating (site 1) and fast migrating (site 3) complexes are visible with the 1,3 probe in the 1:1 complex. However, adding site 2 to the probe (wild type) causes a reduction of the slow migrating form (Fig. 7B), again suggesting a change in complex conformation. As previously shown, the N-finger of GATA proteins may participate in binding to double sites, causing conformational changes in the protein-DNA complexes. In conclusion, sites 1 and 3 can each independently bind a molecule of protein, whereas site 2 increases the affinity, probably through binding of the N-finger.

Expression Driven by the IL-13 Promoter in a T Cell Line—To study the function of the GATA binding sites of the IL-13 gene promoter, a 176-bp DNA fragment of the murine IL-13 promoter spanning nucleotides −110 to +66 relative to the transcription initiation start site was amplified and cloned upstream from the firefly luciferase coding sequence in the pGL3 basic vector. We also constructed different mutated promoters in which one or several of the GATA binding sites were disrupted. The resulting plasmids were transiently transfected in Jurkat T cell line stimulated by PMA and ionomycin, and
firefly luciferase activity was determined after 24 h. The transfection efficiency of each assay was evaluated by cotransfection of a plasmid expressing Renilla luciferase under the control of the herpes simplex virus thymidine kinase promoter (pRL-TK). When the WT promoter was transfected, transcriptional activity was 4.2-fold higher than when the three GATA sites were disrupted (Fig. 8). Disruption of site 2 did not affect the transcriptional activity of the IL-13 gene promoter, whereas disruption of site 3 or sites 2 and 3 resulted in a 40% decreased transcriptional activity. These results demonstrated that full activity of the IL-13 gene promoter in T cells is dependent on the presence of at least two GATA binding sites. However, the role of sites 2 and 3 in their association with site 1 does not seem to correlate with their affinity for GATA proteins since site 2, which increases GATA-3 binding affinity, seems dispensable for the promoter activity in Jurkat cells.

Transactivation by hGATA-3 of the IL-13 Gene Promoter in a Heterologous Cell Line—To determine directly the ability of hGATA-3 to activate the IL-13 gene promoter and to define the relative role of the different GATA-3 binding sites in this activation, we studied the function of the previously described promoters (WT or mutated) in a heterologous cell line (HeLa) that does not express hGATA-3 (Fig. 9). In the absence of hGATA-3, all the constructs displayed a similar transcriptional activity, which was less than twice the transcriptional activity of the empty vector (data not shown). When hGATA-3 was co-transfected with the WT promoter, its transcriptional activ-

---

**FIG. 6.** The human and mouse IL-13 genes promoters contain three clustered GATA binding sites that can bind hGATA-3. A, comparison of the 5' regions of the human and mouse IL-13 genes. The −125/90 (human) and −107/72 (mouse) regions of the IL-13 gene shared a consensus GATA binding site (site 1) and two GATG motifs (site 2 and 3). B, binding of hGATA-3 to the mIL-13 −110/−70 5' region. EMSAs with Jurkat T cell nuclear extracts were performed using oligonucleotide probes that contain the three GATA binding sites (lanes 1–4), site 1 only (lane 5), sites 2 and 3 (lane 6), sites 1 and 3 (lane 7), or sites 1 and 2 (lane 8). This binding was performed in the presence of a 100-fold molar excess of the unlabeled “three GATA sites probe” (lane 1) or in the presence of anti-hGATA-3 antibody (lane 3). The black arrow indicates the hGATA-3 complex, the hatched arrow indicates the hGATA-3 supershifted complex, and the empty arrow indicates the free probe.

**FIG. 7.** Affinity of IL-13 promoter GATA binding sites for GATA zinc finger. Approximately 6 pmol of GATA-2 double finger peptide was titrated with 32P-labeled oligonucleotides (0.25, 0.5, 1, 1.5, and 2 pmol) containing the three IL-13 gene GATA binding sites in various combinations as shown. The numbers above the gels indicate which GATA sites are present on each individual probe. The dissociation constants (Kd), as determined by Scatchard analysis, are shown below the gel: N is the number of experiments, SD is the standard deviation, and ND is not determined. A, titrations are with probes containing a single GATA site. B, titrations are with probes containing the wild type site or two of the three sites. For this gel, 20 pmol of peptide was used, but the Kd determinations were from experiments with less peptide.

**FIG. 8.** Role of the three GATA binding sites in the IL-13 gene promoter activity. The various pGL3 promoter constructs together with pRL-TK were transiently cotransfected into Jurkat T cell line activated by PMA and ionomycin as described under “Experimental Procedures.” The firefly luciferase (LUC) activities were determined, and normalization for transfection efficiency was obtained by determination of Renilla luciferase activity. Data represent the fold increase in luciferase activity over that obtained for cells transfected with the IL-13 gene promoter construct containing none of the GATA binding sites (mean results of three transfections). The hatched box represents the consensus GATA binding site, whereas the dotted box and gray box represent the two GATG sites.
Fig. 9. Transactivation of the human IL-13 gene promoter by hGATA-3. HeLa cells were cotransfected with the different IL-13 promoter constructs, pRL-TK and 1 µg of pECE-hGATA-3 or empty pECE vector. Luciferase (LUC) activities were determined as described under “Experimental Procedures” and Fig. 8. The fold increase by GATA-3 transactivation was calculated as the ratio of luciferase activity in the presence of pECE hGATA-3 or in the presence of the empty pECE vector (mean results of three transfections). The hatched box represents the consensus GATA binding site, whereas the dotted box and gray box represent the two GATG sites.

Fig. 10. Effect of hGATA-3 protein level on the transcriptional activity of the human IL-13 gene promoter. Two constructs containing the three GATA binding sites (WT) or only the consensus GATA binding site (Site 1 alone) were co-transfected into HeLa cells with pRL-TK and increasing amounts of the pECE-hGATA-3 expression vector. The transcriptional activities of the two promoters were evaluated as described under “Experimental Procedures” and Fig. 8. The fold increase of the transcriptional activity of the two promoters was calculated as the ratio of luciferase activity in the presence of the different amounts of pECE hGATA-3 or in the presence of the same amount of the empty pECE vector. The two constructs reached 50% of their full activity with 200 ng for the WT construct and 400 ng for the construct containing the consensus GATA binding site only.

Quantitative Requirement of hGATA-3 in the IL-13 Gene Promoter Activity—During T lymphocyte activation, the level of the hGATA-3 protein is regulated as a consequence of the different stimuli leading to differentiation toward the Th1 or the Th2 pathway. Thus, we thought that it was of interest to evaluate the quantitative requirement of the hGATA-3 protein for the function of the different elements of the IL-13 gene promoter. Precisely, we wanted to test whether site 1 alone or sites 1, 2, and 3 behaved similarly in response to the amount of hGATA-3 protein expressed. These two constructs were cotransfected with increasing amounts of hGATA-3 expression vector, and after normalization, the reporter gene activity was determined. As shown in Fig. 10, the two promoter constructs displayed a very different dependence on hGATA-3 protein level. The WT promoter reached 50% of its full activity when less than 200 ng of the hGATA-3 expression vector was cotransfected and was sensitive to variations of hGATA-3 concentration under 50 ng, whereas the mutated IL-13 gene promoter, which only contained site 1, required 400 ng of hGATA-3 expression vector for 50% transcriptional activity and started to be sensitive to hGATA-3 expression only when more than 200 ng of the expression plasmid was used. Altogether, these results demonstrated that the association of several sites of low affinity for hGATA-3 could lead to a fine tuning of the transcriptional activity of the IL-13 gene promoter, which becomes highly sensitive to minute variations of GATA-3 protein level.

DISCUSSION

In this paper we used transgenic mice that expressed hGATA-3 under the CD2 locus regulatory region to study the effects of enforced hGATA-3 expression on the transcription of the cytokine genes in CD4 and CD8 T cells. We found no difference between transgenic and wild type control mice in the T lymphoid populations of thymus or spleen, and none of the transgenic mice obtained developed a thymic lymphoma. These results are different from the ones recently published using the same CD2 locus region but containing a mouse GATA-3 cDNA tagged with three hemagglutinin epitopes (30). Using this construct, Nawjin et al. have shown a significant reduction of CD8 T cells in the spleen and lymph nodes and the induction of thymic lymphoma in 50% of the transgenic mice (30). The differences between these results and the ones shown in this paper could be accounted for 1) by the presence of the hemagglutinin tag that seems to affect post-transcriptional regulation of the mouse GATA-3 protein and 2) by the genetic background, which is C57BL/6 for the transgenic mice we studied and FVB for the mice previously described. Indeed genetic backgrounds can lead to completely different phenotypes, as shown by the studies done on different strains of mice carrying the same gene inactivation (31).

We first validated the mouse model generated by studying the CD4 T cells. We showed that after activation by PMA-ionomycin and reactivation by anti-CD3, i.e., under nonpolarizing conditions allowing commitment to both Th1 or Th2 phenotype, the CD4 T cells of transgenic mice displayed a shift in the Th1/Th2 balance, as demonstrated by an increased expression of IL-4 mRNA associated with a decreased expression of IFNγ mRNA in the transgenic mice. These results are in a good agreement with the ones obtained by retroviral tagging of splenic lymphocytes with GATA-3 or by enforced GATA-3 expression in CD4 T cells using the CD4 gene promoter or the CD2 locus control region and further demonstrated that GATA-3 is a major regulator of the Th1/Th2 differentiation of CD4 T cells (7, 8, 32). No activation of the endogenous mGATA-3 gene could be detected, and this result is different from previous studies (11, 26). One explanation for this discrepancy might be the origin of ectopic GATA-3 expression, i.e., retroviral-mediated expression or transgenic mice. Because the CD2 cassette used also drove expression of the transgene in CD8 T cells, we studied the expression of the cytokine genes in these cells. After activation, the CD8 T cells of wild type mice displayed a type1 phenotype (expressing essentially IFNγ mRNA), as classically described, whereas the CD8 T cells of the transgenic mice displayed over an intermediate phenotype with simultaneous expression of both IL-13 and IFNγ mRNAs, whose levels were, respectively, more than 10- and 2-fold higher than in control-activated CD8 T cells. This pattern of expression was maintained under Tc1 conditions. The IFNγ
gene regulation is different in CD4 and CD8 T cells since CD4 T cells required STAT4 activation to express IFNγ, whereas CD8 T cells displayed a STAT4-independent activation of this gene (33). At the molecular level, two regulatory elements located in the IFNγ gene promoter have been implicated in the induction of IFNγ gene transcriptional activity during T cell activation, and the function of these two elements is different in CD4 and CD8 T cells (34). Both elements are active in primed CD4 T cells, whereas only the distal element, which contained a consensus GATA binding site, is active in primed CD8 T cells (35). The results presented in this paper corroborated the differential regulation of the IFNγ gene in CD4 and CD8 T cells and showed that the GATA-3 expression level also participates in this differential regulation. However, these results are different from the results recently published (32) where CD8 T cells from GATA-3-expressing transgenic mice displayed a lower level of positive cells for intracellular expression of IFNγ than control mice. The differences between the transgenic mice used (genetic background, hemagglutinin tag) and the differences in the protocols of activation might again account for the discrepancy. WT and transgenic CD8 T cells have a similar cytokine expression pattern under Tc2 conditions. This result could be explained by the high level of expression of mGATA-3 in these conditions and is in accordance with recent results demonstrating the inhibitory effect of IFNγ transduction pathway on GATA-3 expression and IL-4 production (36).

A major finding of this study is that GATA-3 could directly enhance IL-13 gene expression in CD8 T cells under neutral or Tc1 conditions. This result is different from the transgenic data of Zheng and Flavell (7) in CD4 T cells showing a major effect of GATA-3 on IL-4 gene expression but minimal effects on IL-5 and IL-13 genes. However, our result is in perfect accordance with the up-regulation of IL-13 in T cells that overexpressed GATA-3 and the inhibition of IL-13 genes seen in transgenic mice expressing a dominant-negative mutant of GATA-3 (8, 37). Many reports have described the regulation of the IL-4 and IL-5 genes. As expected, proximal and distal cis-acting elements regulate their expressions and GATA-3, c-Maf, and nuclear factor AT are among the major trans-acting factors that control these two genes (5). The IL-5 promoter is directly trans-activated by GATA-3, and two distal regulatory elements are necessary for the transactivating effect of GATA-3 on IL-4 gene (7, 37–39). Conversely, and despite the importance of IL-13 in diseases such as asthma and allergy, there is very limited information on the regulation of the IL-13 gene. During the immune responses, the co-expression of IL-4, IL-5, and IL-13 in conditions associated with the appearance of Th2 cells indicated a co-regulation of these cytokines genes consistent with their clustered position over 150 kb of genomic DNA in both human and mouse (40). Recently, an IL-4/IL-13 intergenic regulatory region that might be involved in the co-regulation of the IL-4 and IL-13 genes has been characterized, and GATA-3 together with STAT6 seems to induce chromatin remodeling of this regulatory region (41). However, no direct role of this regulatory region on IL-13 gene expression has been shown. Because the IL-13 mRNA level was increased in the hGATA-3 transgenic mice we studied, we first analyzed the IL-13 gene promoter and found an evolutionary-conserved consensus GATA binding site possibly involved in the GATA-3-mediated activation of the IL-13 gene. Using EMSA, we showed that this GATA binding site alone bound GATA-3 with poor affinity, whereas the combination of this motif and two GATG sequences located immediately upstream constituted a high affinity sequence for GATA-3. A similar result was obtained in the study of the IL-5 promoter, where a consensus GATA binding site is of weak affinity, whereas an overlapping and inverted TGATT motif dramatically increased the GATA-3 affinity and trans-activation mediated by the consensus GATA binding site (37). Using peptides that represent the two GATA-2 zinc fingers, we were able to determine that the presence of multiple GATA sites increases the binding affinity and alters the conformation of the complexes formed on the IL-13 gene promoter. Two molecules of protein can bind to the IL-13 gene GATA sequences through sites 1 and 3 but not to other pairs of sites. The addition of site 2 increases the affinity but does not result in the binding of a third protein molecule, suggesting the participation of the N-finger. The role of site 2 could be to ensure that sites 1 and 3 are both occupied by increasing the local concentration of GATA-3 or by stabilizing the binding of two molecules of GATA-3 through formation of a more favorable protein/DNA conformation. At high GATA-3 concentrations site 2 may be unimportant, but it could be critical at stages of development when GATA-3 is limiting. Although the sites 1 and 2 association lead to a higher affinity for GATA proteins than the sites 1 and 3 association, we showed that sites 1 and 3 association is transcriptionally more efficient. This result together with our Scatchard analysis indicated that the number of GATA-3 molecules bound on this region is more important than their DNA binding affinities in the transcriptional activity of the IL-13 promoter and is in line with a recent study that showed that high affinity binding sites for GATA-1 did not always lead to high transactivation (42).

The association of consensus and non-consensus GATA binding sites found in the IL-13 or the IL-5 genes promoters might be important for the fine regulation of the expression of cytokine genes during the immune response. This hypothesis was tested by transfections using increasing amounts of the hGATA-3 expression vector and IL-13 promoters containing the consensus GATA binding site alone or the association of motifs found in the IL-13 gene promoter. Indeed, we found that the wild type promoter was highly sensitive to minute amounts of hGATA-3, whereas the promoter containing only the consensus GATA binding site required a higher amount of hGATA-3 for trans-activation. Such results have already been found for other trans-acting factors but not for members of the GATA family (43). Because most of the studies performed on the functions of the GATA family members have been done on constitutively expressed target genes, the results presented in this study might reveal an arrangement of cis-acting motifs that mediate inducible expression by GATA-3.

Several studies indicate that GATA-3 might be a potential therapeutic target for the treatment of pathologies such as asthma and allergy (44, 45). These studies are based on inhibition of GATA-3 activity in T cells by a dominant negative mutant of GATA-3 or by a blockade of GATA-3 expression using antisense RNA. In this paper we show that the production of cytokines by CD8 T cells is also modulated by the expression level of IL-13 but, at least for IFNγ, in a different way than in CD4 T cells. Thus, depending on the target T cell, the blockade of GATA-3 might have different consequences on specific cytokine expression and immune response. As for the IL-13 gene, our results demonstrate that it is a true GATA-3 target gene that is highly sensitive to GATA-3 expression level. Recently, it has been shown that inhibition of IL-13 production results in a severe inhibition of mucus production and airway hyperresponsiveness in a murine model of asthma (46, 47). Our results indicate that modulation of GATA-3 expression might be a promising therapy in the treatment of this disease.

Acknowledgments—We thank Dr. Helene Jouault, Isabelle Bouchaert, and Nicolas Lebrun for flow cytometry, Frank Letourneur and Nicolas Lebrun for sequencing, Christophe Dez for maintenance of
mice colonies, and Drs. Sophie Ezine, Flora Zavala, and Yves Levy for assistance and helpful discussions.

REFERENCES

1. Abbas, A. K., Murphy, K. M., and Sher, A. (1996) *Nature* **383**, 787–793
2. Romagnani, S. (1994) *Annu. Rev. Immunol.* **12**, 227–257
3. Liblau, R. S., Singer, S. M., and McDevitt, H. O. (1995) *Immunol. Today* **16**, 34–38
4. Robinson, D. S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A. M., Corrigan, C., Durham, S. R., and Kay, A. B. (1992) *N. Engl. J. Med.* **326**, 298–304
5. Rincon, M., and Flavell, R. A. (1997) *Curr. Biol.* **7**, 729–732
6. Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., and Glomcher, L. H. (2000) *Cell* **100**, 655–669
7. Zheng, W., and Flavell, R. A. (1997) *Cell* **89**, 587–596
8. Ouyang, W., Ranganath, S. H., Weindel, K., Bhattacharya, D., Murphy, T. L., Sha, W. C., and Murphy, K. M. (1998) *Immunity* **9**, 745–755
9. Ferber, I. A., Lee, H. J., Zain, F., Heath, V., Mui, A., Arai, N., and O’Garra, A. (1999) *Clin. Immunol.* **81**, 134–144
10. Lee, H. J., Takemoto, N., Kurata, H., Kamogawa, Y., Miyatake, S., O’Garra, A., and Arai, N. (2000) *J. Exp. Med.* **192**, 105–115
11. Ouyang, W., Lohning, M., Gao, Z., Assenzacher, M., Ranganath, S., Radbruch, A., and Murphy, K. M. (2000) *Immunology* **12**, 27–37
12. George, K. M., Leonard, M. W., Roth, M. E., Lieue, K. H., Kiousis, D., Grosveld, F., and Engel, J. D. (1994) *Development* **120**, 2673–2686
13. Pandolfi, P. P., Roth, M. E., Karis, A., Leonard, M. W., Dzierszak, E., Grosveld, F. G., Engel, J. D., and Lindenbaum, M. H. (1995) *Nat. Genet.* **11**, 40–44
14. Lim, K. C., Lakshmanan, G., Crawford, S. E., Gu, Y., Grosveld, F., and Engel, J. D. (2000) *Nat. Genet.* **25**, 209–212
15. Hattori, N., Kawamoto, H., Fujimoto, S., Kune, K., and Katsura, Y. (1996) *J. Exp. Med.* **184**, 1137–1147
16. Ting, C. N., Olson, M. C., Barton, K. P., and Leiden, J. M. (1996) *Nature* **384**, 474–478
17. Feng, T. A., and Mosmann, T. R. (1990) *J. Immunol.* **144**, 1744–1752
18. Erenf, F. Wild, M. T., Garcia-Sanz, J. A., and Le Gros, G. (1983) *Science* **260**, 1820–1825
19. Sad, S., Marcotte, R., and Mosmann, T. R. (1995) *Immunology* **2**, 271–279
20. Paganelli, R., Scala, E., Anstoguri, I. J., Astito, C. M., Halapi, E., Fanes- Belgas, E., D’Ozirri, G., Mezzaroma, I., Pandolfi, F., Fornili, M., et al. (1995) *J. Exp. Med.* **181**, 423–428
21. Fowler, D. H., Breglio, J., Nagel, G., Eckhaus, M. A., and Gress, R. E. (1996) *J. Immunol.* **157**, 1411–1420
22. Vizier, C., Berovici, N., Heurtier, A., Pardon, N., Goude, K., Bailly, K., Convadiere, C., and Liblau, R. S. (2000) *J. Immunol.* **165**, 6314–6321
23. Greaves, D. R., Wilson, F. D., Lang, G., and Kiousis, D. (1989) *Cell* **58**, 886–896
24. Leroy-Viard, K., Vinit, M. A., Lecointe, N., Jouault, H., Hibber, U., Romeo, P. H., and Mathieu-Mahul, D. (1995) *EMBO J.* **14**, 2341–2349
25. Max-Audit, I., Eleouet, J. F., and Romeo, P. H. (1995) *J. Biol. Chem.* **268**, 5431–5437
26. Ranganathan, S., and Murphy, K. M. (2001) *Mol. Cell. Biol.* **21**, 2716–2725
27. McKenzie, A. N., Li, X.,Largepada, D. A., Sato, A., Kaneda, A., Zurawski, S. M., Doyle, R. L., Milavich, A., Franchise, U., Copeland, N. G., et al. (1995) *J. Immunol.* **156**, 5436–5444
28. Pedone, P. V., Omichinski, J. G., Neny, P., Trainor, C., Gronenborn, A. M., Clere, G. M., and Felsenfeld, G. (1997) *EMBO J.* **16**, 2874–2882
29. Ko, L. J., and Engel, J. D. (1993) *Mol. Cell. Biol.* **13**, 4011–4022
30. Nawijn, M. C., Ferreira, R., Dingjan, G. M., Kahre, O., Drabek, D., Karis, A., Grosveld, F., and Hendriks, R. W. (2001) *J. Immunol.* **167**, 715–723
31. Suzuki, A., da La Pompa, J. L., Stambolic, V., Elia, A. J., Sasaki, T., del Barco Barrantes, I., Hu, A., Wakeham, A., Rie, A., Khoo, W., Fukumoto, M., and Mak, T. W. (1998) *Curr. Biol.* **8**, 1169–1178
32. Aune, T. M., Linex, L. A., Rincon, M. R., and Flavell, R. A. (1997) *Mol. Cell. Biol.* **17**, 199–208
33. Zhang, Y., Apilado, R., Coleman, J., Ben-Sasson, S., Tsung, S., Hu-Li, J., Paul, W. E., and Huang, H. (2001) *J. Exp. Med.* **194**, 165–172
34. Zhang, D. H., Yang, L., and Ray, A. (1998) *J. Immunol.* **161**, 3817–3821
35. Lee, H. J., O’Garra, A., Arai, K., and Arai, N. (1998) *J. Immunol.* **160**, 2343–2352
36. Lee, G. R., Fields, P. E., and Flavell, R. A. (2001) *Immunology* **14**, 447–459
37. Dolganov, G., Bort, S., Lovett, M., Burr, J., Schubert, L., Short, D., McGurn, M., Gibson, C., and Lewis, D. B. (1996) *Blood* **87**, 3316–3326
38. Takemoto, N., Kamogawa, Y., Jun Lee, H., Kurata, H., Arai, K. I., O’Garra, A., Arai, N., and Miyatake, S. (2000) *J. Immunol.* **165**, 6687–6691
39. Trairiris, C. D., Gurtlerando, R., and Simpson, M. A. (2000) *J. Biol. Chem.* **275**, 28157–28166
40. Yang, D., Lu, H., Hong, Y., Jinks, T. M., Estes, P. A., and Erickson, J. W. (2001) *Mol. Cell. Biol.* **21**, 1581–1592
41. Zhang, D. H., Yang, L., Cohn, L., Parkyn, L., Homer, R., Ray, P., and Ray, A. (1999) *Immunology* **111**, 473–482
42. Foresto, S., De Santis, G. T., Lehr, H. A., Herz, U., Buerke, M., Schipp, M., Bartsch, B., Areya, R., Schmitt, E., Galle, P. R., Renz, H., and Neurath, M. F. (2001) *J. Exp. Med.* **193**, 1247–1260
43. Grunig, G., Warnock, M., Waki, A. E., Venkayya, R., Brombacher, F., Rennick, D. M., Sheppard, D., Mohrs, M., Donaldson, D. D., Locksley, R. M., and Corry, D. B. (1998) *Science* **283**, 2261–2263
44. Wills-Karp, M., Layimbazi, J., Xu, X., Schofield, B., Neben, T. Y., Karp, C. L., and Donaldson, D. D. (1998) *Science* **282**, 2258–2261
