Molecular Analysis of the Interaction between the Hematopoietic Master Transcription Factors GATA-1 and PU.1*†§

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GATA-1 and PU.1 are transcription factors that control erythroid and myeloid development, respectively. The two proteins have been shown to function in an antagonistic fashion, with GATA-1 repressing PU.1 activity during erythropoiesis and PU.1 repressing GATA-1 function during myelopoiesis. It has also become clear that this functional antagonism involves direct interactions between the two proteins. However, the molecular basis for these interactions is not known, and a number of inconsistencies exist in the literature. We have used a range of biophysical methods to define the molecular details of the GATA-1-PU.1 interaction. A combination of NMR titration data and extensive mutagenesis revealed that the PU.1-Ets domain and the GATA-1 C-terminal zinc finger (CF) form a low affinity interaction in which specific regions of each protein are implicated. Surprisingly, the interaction cannot be disrupted by single alanine substitution mutations, suggesting that binding is distributed over an extended interface. The C-terminal basic tail region of CF appears to be sufficient to mediate an interaction with PU.1-Ets, and neither acetylation nor phosphorylation of a peptide corresponding to this region disrupts binding, indicating that the interaction is not dominated by electrostatic interactions. The CF basic tail shares significant sequence homology with the PU.1 interacting motif from c-Jun, suggesting that GATA-1 and c-Jun might compete to bind PU.1. Taken together, our data provide a molecular perspective on the GATA-1-PU.1 interaction, resolving several issues in the existing data and providing insight into the mechanisms through which these two proteins combine to regulate blood development.

The development of the erythroid and myeloid blood cell lineages is controlled in part by the master transcriptional regulators GATA-1 and PU.1 (1, 2). GATA-1 plays an indispensable role in erythroid development, as indicated by the failure of embryonic erythropoiesis in GATA-1-null mice (3). Conversely, the inactivation of the PU.1 gene causes defects in the development of myeloid lineages and B and T lymphocytes (4, 5).

Considerable evidence exists to suggest that there is functional antagonism between GATA-1 and PU.1 and that this antagonism is important for determining lineage commitment (erythroid versus myeloid) during hematopoiesis (reviewed in Ref. 6). For example, forced GATA-1 expression leads to the reprogramming of myeloid precursors into erythroid cells or retrodifferentiation into multipotent progenitors (7, 8). Similarly, PU.1 expression can result in a block in erythroid differentiation in cellular assays (9, 10), and the block can be relieved by co-expression of GATA-1. Transient transfection assays in which either GATA-1 or PU.1 binding sites are placed in front of a reporter gene likewise reveal that each of the two proteins will repress activation by the other (11–13). In vivo observations also support this conclusion. Transgenic mice expressing PU.1 in erythroid cells develop erythroleukemias at a high rate (14), while GATA-1 expression has been detected in several myeloid and megakaryocytic tumors (tissues in which this protein is not normally expressed, Ref. 15). Thus, GATA-1 and PU.1 each appear to inhibit the transactivation activity of the other.

Recent chromatin immunoprecipitation (ChiP)4 data have established that GATA-1 and PU.1 can colocalize on promoters (16, 17), and that this colocalization is required for their antagonistic activity. These data have further demonstrated that the mechanism by which PU.1 inhibits the expression of GATA-1 target genes involves PU.1 recruiting chromatin-modifying proteins (including heterochromatin protein 1, C-terminal-binding protein and retinoblastoma protein [RB]) to genes to which GATA-1 is bound (16). The repressed chromatin struc-

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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4 The abbreviations used are: ChiP, chromatin immunoprecipitation; GST, glutathione S-transferase; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid; EMSA, electrophoretic mobility shift assay; ITC, isothermal titration calorimetry; CF, GATA-1 C-terminal zinc finger; PDB, Protein Data Bank, MBP, maltose-binding protein.
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A schematic of human GATA-1 and PU.1 are shown, indicating the conserved domains that have been identified in each protein. Amino acid numbering is shown. β, amino acid sequences of the human GATA-1 CF and the PU.1-Ets domains. Zinc-ligating residues are underlined, and differences between human and murine domains are indicated above each sequence. Residues in italics were not included in the constructs used previously to determine the NMR structure of PU.1 bound to DNA (PDB code: 1GAT, Ref. 31) or the x-ray structure of PU.1-Ets included in the constructs used previously to determine the NMR structure of GATA-1 CF that was synthesized chemically.

EXPERIMENTAL PROCEDURES

GST Pulldowns—Wild-type CF (murine GATA-1 residues 248–318), as well as a range of substitution and truncation mutants (CF300: residues 248–300 and CF308: residues 248–308), were overexpressed as GST fusion proteins and immobilized onto glutathione beads according to standard procedures (Amersham Biosciences). Similarly, GST fusions of wild-type and mutant PU.1-Ets domains (human PU.1 residues 158–270), MBP-PU.1-Ets and MBP-CF were also prepared. Amylose-agarose-bound MBP proteins were eluted with 10 mM maltose (in 50 mM Tris, 50 mM NaCl, pH 8). Glutathione beads with equal amounts of GST fusion proteins (as judged by SDS-PAGE) and the appropriate MBP fusion protein were mixed in pull-down buffer (50 mM Tris, 100 mM NaCl, 10% glycerol, 1% Igepal CA-630, 1.4 mM phenylmethylsulfonyl fluoride, 0.01% β-mercaptoethanol, 1 mg/ml bovine serum albumin, pH 8) and incubated at 4 °C for 1 h with shaking. The beads were washed repeatedly with pull-down buffer (omitting the bovine serum albumin). The bound proteins were released by boiling for 5 min in SDS-PAGE loading dye and analyzed by SDS-PAGE. The presence of the MBP-fusion proteins was detected by anti-MBP antiserum (New England Biolabs).

For pulldowns carried out in the presence of ethidium bromide, GST, GST-CF, and MBP-PU.1 Ets were overexpressed, and samples were prepared either in the absence or presence of EtBr. Soluble fractions treated with 100 μg/ml EtBr were incubated for 30 min at 4 °C. For EtBr-treated samples, the following steps were carried out in the presence of 30 μg/ml EtBr. GST-CF was immobilized onto glutathione beads for 1 h at 4 °C and washed with binding buffer, 50 mM Tris (pH 8), 50 mM NaCl, 1% Triton-X, 1.4 mM phenylmethylsulfonyl fluoride, and 1.4 mM β-mercaptoethanol. GST or GST-CF beads were mixed with MBP-PU.1-Ets lysate in binding buffer and incubated for 1 h at 4 °C. The beads were washed five times with wash buffer, 20 mM Tris, pH 8, 150 mM NaCl, 2 mM ZnCl2, 0.1% Nonidet P-40, and 4.5 mM β-mercaptoethanol. The bound proteins were eluted by heating at 85 °C for 5 min in SDS-PAGE loading dye.

Synthesis, Purification, and Acetylation of the CFtail Peptide—A peptide corresponding to residues Ala300–Gly318 of murine GATA-1 (CFtail) was synthesized manually according to the Merrifield technique using the standard Fmoc protocol (23). An additional tyrosine residue was incorporated at the N terminus of the peptide (giving the sequence YASGKGKKKRG) to permit concentration determination by A280. The crude peptide was purified by reverse phase HPLC using a C18 column and a water-acetonitrile (in 0.1% trifluoroacetic acid) gradient. To obtain the acetylated peptide, purified lyophilized peptide was resuspended in equal volumes of 3 M sodium acetate and acetic
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The pH of the mixture was adjusted to 8, and the reaction mixture shaken overnight at 4 °C. The acetylated mixture was then subjected to another round of rPHPLC, and the major species corresponded to CFtail peptide with five acetyl groups (Ac-CF$_{tail}$). The identity and purity of the purified peptides were confirmed by ESI-MS. A peptide corresponding to CF$_{tail}$ with the sole serine phosphorylated was purchased from AusPep (Melbourne) and used without further purification. Peptides were lyophilized and stored at -20 °C until use.

Protein Expression and Purification—The PU.1-Ets domain (residues 158–270 hPU.1) and CF (residues 237–317 of mGATA-1) were both cloned into the Ndel and BamHI sites of the pET3a vector (Novogen) and the plasmids transformed into Escherichia coli BL21 (DE3)-CodonPlus® (Stratagene) cells. Overexpression of the untagged proteins was induced by 0.4 mM isopropyl-1-thio-$\beta$-d-galactopyranoside at 25 °C. The proteins were initially purified using SP Sepharose$^\text{TM}$ Fast Flow beads (Amersham Biosciences) at pH 9 (in 50 mM HEPES, 50 mM NaCl, 1 mM DTT), using stepwise increases in salt concentration to elute the proteins. Fractions containing protein were then diluted in order to decrease the salt concentration and further purified on a MonoS$^\text{TM}$ HR 10/10 column (Amersham Biosciences). The purified proteins were generally concentrated and dialyzed into the buffer required for subsequent experiments. In some cases, the proteins were further purified by reversed phase HPLC using a C$_{18}$ column, then lyophilized and reloaded in the required buffer with the addition of stoichiometric Zn(II). The activation domain of human PU.1 (residues 1–71) was subcloned into the BamHI and EcoRI sites of pGEX-2T (Amersham Biosciences), overexpressed at 37 °C, and purified by glutathione affinity chromatography and reversed phase HPLC (following cleavage from the GST tag using thrombin). For the preparation of $^{15}$N- and $^{13}$C-labeled proteins, the procedure of Cai et al. (24) was used. Far-UV circular dichroism and one-dimensional $^1$H NMR spectroscopy were used to assess the folding of each domain.

NMR Spectroscopy—NMR data were acquired on a Bruker 600 MHz spectrometer and Varian Innova 750-MHz and 800-MHz spectrometers. Samples (CF and Ets) were dialyzed into one of the following buffers: (a) 5 mM Tris-HCl, pH 7.4, 150 mM NaCl, (b) 50 mM phosphate pH 7, 1 mM DTT, or (c) 50 mM MES, pH 6, 100 mM NaCl, 1 mM DTT. In all cases, buffers were made up in 95:5 H$_2$O:D$_2$O and 10 mM 2,2-dimethyl-2-silapentane-5-sulfonic acid was added as a chemical shift reference. Samples were concentrated to 400–800 $\mu$m using centrifugal concentrators and NMR data were recorded at 298 K. Backbone resonance assignments of both the Ets and CF domains were obtained using a combination of homonuclear and standard triple resonance experiments. Data were processed using either NMRPipe (25) or XINNMR and analyzed with XEASY (26), Sparky (27) and AUTOASSIGN (28). Automated assignments were verified by manual inspection and by comparison with previously reported assignments (29).

Chemical Shift Perturbation Experiments—For the titration of $^{15}$N[Ets with CF, samples of $^{14}$N-labeled PU.1-Ets alone (0.15 mM) and a mixture of $^{15}$N-labeled PU.1-Ets (0.15 mM) and unlabeled CF in a 1:13 molar ratio were prepared. The titration was performed at 25 °C by adding aliquots of the second sample to the first sample in eight intervals. In this way the concentration of $^{15}$N-labeled PU.1-Ets remained unchanged throughout the titration while the concentration of unlabeled CF was gradually increased, to a final concentration of 1.9 mM (13 molar equivalents). $^{1}$H HSQC spectra were recorded at each interval of the titration, and pH was carefully monitored. The reverse titration experiment was performed using an identical procedure. Manually placed cross-peaks were verified using the peak detection function of NMRRdraw (part of the NMRPipe software). Overall weighted average chemical shift changes ($\Delta$avg) were calculated for all residues using the equation: $\Delta$avg = $([\delta(H)\text{avg}^2 + (\delta(N) \times 0.154)^2]/2)^{1/2}$ (30) and fitted to a 1:1 binding model using nonlinear least squares regression.

The lyophilized CF$_{tail}$ peptides (wild-type, acetylated, and phosphorylated forms) were resuspended in 50 mM sodium phosphate, 50 mM NaCl, 0.5 mM DTT, pH 7. To a solution of 760 $\mu$m $^{15}$N-PU.1-Ets, wild-type CF$_{tail}$ (7.7 mM) was added to 0.5, 1, 2, 5, 10, and 12.4 molar equivalents, while acetylated CF$_{tail}$ (5.5 mM) was added to 0.5, 1, 2, 5, and 7.3 molar equivalents in a separate experiment. Phosphorylated CF$_{tail}$ was added to $^{15}$N[PU.1-Ets at levels of 1, 6, 17, 33, and 66 molar equivalents. Titrations were performed at 25 °C and $^{15}$N HSQC spectra were recorded after each addition.

Electrophoretic Mobility Shift Assays (EMSA)—Reactions were set up in a total volume of 30 $\mu$L, comprising 0.1 pg of $^{32}$P-labeled probe, 10 mM HEPES, pH 7.8, 50 mM KCl, 5 mM MgCl$_2$, 1 mM EDTA, 5% glycerol, 0.1 mg/ml dIdC, and 0.1 mg/ml bovine serum albumin. Quantitative EMSAs were carried out using either: (a) 50 pm $^{32}$P-labeled GATA dsDNA mixed with 0–250 nM of MBP-CF and 500 nM untagged PU.1-Ets or (b) 40 pm $^{32}$P-labeled Ets dsDNA mixed with 0–120 nM PU.1-Ets and 60 nM of MBP-CF. After incubation on ice for 10 min, samples were loaded onto a 6% native polyacrylamide gel made up in 0.5 × Tris borate/EDTA. The gel was then subjected to electrophoresis at 15 V/cm and 4 °C for 2 h, dried, analyzed, and quantified when necessary using a PhosphorImager (Molecular Dynamics). The probes used in the experiments were end-labeled according to standard procedures using poly nucleotide kinase. The sequence of GATA and Ets probes were 5′-GTT GCA GAT AAA CATT-3′ (the same sequence used in the determination of the structure of CF bound to DNA, Ref. 31) and 5′-TCG AAT AAA ATC AGG AAC TTG-3′, respectively. DNA binding affinities were estimated by nonlinear least squares analysis as described in Ref. 32.

Isothermal Titration Calorimetry (ITC)—Complementary 16-bp oligonucleotides containing the Ets consensus site (5′-AAAAAGGGGAAGTGGG-3′) were annealed by heating at 95 °C for 5 min and slowly cooling to room temperature in 10 mM Tris (pH 8) and 1 mM EDTA. DNA concentrations were measured using $A_{260}$. Titrations were carried out on a Microcal VPITC microcalorimeter at 25 °C. All samples were dialyzed extensively into a buffer composed of 20 mM MES (pH 6), 400 mM NaCl, and 1 mM DTT. CF (280 $\mu$m) was titrated into a solution of DNA (28 $\mu$m) or a solution of the 1:1 PU.1-Ets:DNA complex. Each titration consisted of 20 × 15-μl injections separated by 30-min or 8-min intervals. The reference power was set at 10 $\mu$cal s$^{-1}$ with continuous stirring at 310 rpm. Using the Microcal Origin software package (Microcal Software, North-
ampton, MA), data were analyzed and fitted using a single binding site model.

RESULTS

Identification of the PU.1 Binding Motif in GATA-1—In agreement with recent reports (11–13), preliminary GST pull-down assays indicated that the interaction between GATA-1 and PU.1 is mediated by the GATA-1 CF and the PU.1-Ets domain (data not shown). To pinpoint the specific elements within CF that are responsible for contacting PU.1, we constructed a library of CF substitution and deletion mutants that covered the entire surface of CF. These mutants were then tested for their ability to bind to the PU.1-Ets domain in GST pull-down assays. As shown in Fig. 2, none of the 35 point mutations (single and double mutations) significantly impaired the binding of CF to PU.1-Ets. However, truncation of the C-terminal basic tail region of CF (residues 309–318) essentially abrogated the interaction, indicating that this region is important for the interaction with PU.1. Interestingly, the panel of point mutants included a number of both single and double mutants in the basic tail region but these mutations did not eliminate the interaction in this assay.

To further probe the means through which GATA-1 recognizes PU.1, we carried out a chemical shift perturbation study, monitoring the 15N HSQC spectrum of 15N-labeled CF (residues 237–317 of mGATA-1) upon the addition of unlabeled PU.1-Ets (residues 158–270 of human PU.1). As shown in Fig. 3, small but significant changes are observed for several signals in the spectrum, while most signals remain unchanged. Chemical shift changes (averaged over 1H and 15N) are plotted versus residue number in Fig. 3B, revealing that many of the largest changes occur for residues toward the C-terminal end of the α-helix in CF (e.g. Gly283, Leu284, Leu288, His289, Gln290, Arg293). Smaller changes were also clustered in the N-terminal (Ile242, Met248, Ser251) and to a much lesser extent the C-terminal regions (Ala309, Ser310) of the domain.

It is notable that the magnitudes of the observed chemical shift changes are small overall and that the conversion between free and complexed forms of 15N-CF is in fast exchange on the chemical shift timescale. These observations indicate that the magnitude of the interaction between GATA-1 and PU.1 is probably less than 10^4 M^-1.

Using the chemical shift changes and the GST pull-down assays as a guide, we constructed three quadruple point mutants, in which two additional mutations were incorporated into the His289–Gln290 (→AA) double mutant: in turn, Ala309→Ser310, Gly311→Lys312 or Lys315→Lys316 were mutated (to VA, AA, or AA, respectively). Of these three quadruple mutants,
only the third exhibited any detectable decrease in binding to PU.1-Ets (data not shown).

Identification of the GATA-1 Binding Motif in PU.1—We next sought to determine which residues in the Ets domain of PU.1 are involved in recruiting GATA-1. We therefore carried out a 15N HSQC titration, adding unlabeled CF into 15N-labeled Ets (Fig. 4A). While the majority of the PU.1-Ets amide resonances remained unaffected, a few resonances underwent small (|Δ1H| < 0.1 ppm in the 1H dimension) chemical shift changes. An analysis of the changes in average amide chemical shifts of PU.1-Ets on titration with CF (Fig. 4B) indicated that the chemical environments of the backbone amides of residues Leu172, Tyr173, His205, Glu207, Lys227, Met228, and Lys245, together with the side chain indole NH of Trp213 and the side chain amide group of Asn219, underwent the largest changes in the presence of increasing amounts of CF (more than one standard deviation greater than the mean change). Again, the observed chemical shift changes were modest, consistent with a relatively weak interaction and only partial saturation of PU.1-Ets.

Additionally, 10 substitution mutants of PU.1-Ets were produced (Fig. 5A) as GST fusion proteins and tested for their ability to bind MBP-CF-(248–318). Unexpectedly, no discernible effect was observed in any case (Fig. 5B), even though the panel of mutants (i.e., Y173A, I215A, K237A, T238A, G239A, E240A, V241A, K243A, V244A, and K245A) included two implicated in our NMR titration experiment and others in the same vicinity. This result is similar to that observed above with the substitution mutants of CF.

Quantitation of the GATA-1-PU.1 Interaction—The chemical shift changes exhibited by several [15N]PU.1-Ets resonances following the addition of CF were analyzed by non-linear least squares regression to estimate the dissociation constant for the CF-Ets complex. The derived values for Kd all fall within a narrow range, giving an average of 3.3 ± 2.3 mM. Efforts to measure Kd using isothermal titration calorimetry were unsuccessful because of the negligible enthalpy changes observed upon mixing the two proteins, although a very weak binding event was evident from the raw data (not shown).

The C-terminal Basic Tail of the GATA-1 C-finger Is Required for PU.1 Binding—Because our GST pull-down data suggested that the basic tail region of CF was important in the GATA-1-PU.1 interaction, a peptide corresponding to this region (residues 309–318; CFtail) was synthesized chemically and tested for its ability to bind PU.1-Ets. NMR shift perturbation experiments showed that the titration of CFtail into [15N]PU.1-Ets resulted in the shift of several resonances (Fig. 6), namely Tyr173, Met185, Ile215, Gly218, Met223, Tyr225, Glu226, Lys227, Met228, Val244, and Leu248 (as well as the side chain groups of Trp213 and Asn219). This set of resonances overlaps signifi-
FIGURE 6. The basic tail of CF is able to bind PU.1-Ets. A, $^{15}$N HSQC spectra of $^{15}$NPU.1-Ets at the beginning (solid lines) and end (1:12 $^{15}$N[Ets:CFtail, dashed lines]) of the titration with the CF basic tail peptide CFtail. The same section of the spectrum expanded in Fig. 4A is shown here. Spectra are colored as indicated. B, plot of average chemical shift change versus residue number for $^{15}$N[Ets, following the addition of 12 molar equivalents of CFtail. The dotted line represents the mean chemical shift change ± one S.D. Note that only side chain NH pairs with average chemical shift changes larger than this cutoff have been shown. C and D show corresponding data for the titration of the acetylated CFtail (Ac-CFtail) into $^{15}$N[Ets. The spectra shown are the beginning (solid lines) and end (1:7.4 $^{15}$N[Ets:Ac-CFtail, dashed lines]) of the titration. E and F show corresponding data for the titration of the phosphorylated CFtail (P-CFtail) into $^{15}$N[Ets. The spectra shown are the beginning (solid lines) and end (1:66 $^{15}$N[Ets:P-CFtail, dashed lines]) of the titration. In A, C, and E, question marks represent unassigned peaks.
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The CF-Ets Interaction Is Not Acetylation- or Phosphorylation-dependent—The basic tail of the GATA-1 CF has been shown to undergo a number of post-translational modifications. The lysines Lys\(^{312}\), Lys\(^{314}\), and Lys\(^{315}\) can all be acetylated by the enzyme CBP (33, 34), while phosphorylation of Ser\(^{310}\) has been observed during MEL cell differentiation (35). Our observation that the presence of the CF\(_{\text{tail}}\) is required for the GATA-1-PU.1 interaction raised the possibility that such modifications might be relevant. To test this hypothesis, we first used acetic anhydride to peracetylate the CF\(_{\text{tail}}\) peptide, generating a derivative (Ac-CF\(_{\text{tail}}\)) that contained five acetyl groups, according to mass spectrometry data (most likely corresponding to acetylation of the four lysines and the N terminus). Remarkably however, this derivative exhibited extremely similar behavior in an HSQC titration experiment with \(^{15}\text{N}\)Ets (Fig. 6, C and D), suggesting that the GATA-1-PU.1 interaction is not dependent on the acetylation status of the former protein and is therefore not driven primarily by charge-charge interactions.

We also carried out an HSQC titration using \(^{15}\text{N}\)PU.1-Ets and a serine-phosphorylated version of the CF\(_{\text{tail}}\) peptide (P-CF\(_{\text{tail}}\)). While the interaction appeared to be slightly weakened, the chemical shift changes were similar overall to those observed in the other two titrations (Fig. 6), although some differences were noted. The residues that underwent significant changes were Tyr\(^{173}\), Ser\(^{188}\), His\(^{205}\), Glu\(^{207}\), His\(^{211}\), Arg\(^{212}\), Met\(^{223}\), Met\(^{228}\) and the side chain NH groups of Trp\(^{213}\) and Asn\(^{219}\). The additional perturbed residues (Ser\(^{188}\), His\(^{211}\), Arg\(^{212}\)) might represent the site where the phosphate group contacts PU.1-Ets.

The CF-Ets Binding Interface—Fig. 7A shows the structure of the chicken GATA-1 CF bound to DNA (1GAT, Ref. 31). High-
lighted as blue sticks are the residues corresponding to Gly309–Arg317 from human GATA-1, which we have demonstrated are essential for the interaction between GATA-1 and PU.1. These residues are disordered in the structure, displaying no nonsequential NOEs. The Cα atoms of the residues that were identified from HSQC titration data as being involved in PU.1 binding are shown as blue spheres. In Fig. 7C, the CF surface is shown, and it is clear that the two regions are somewhat separated in space, at least when the CF is bound to DNA. In Fig. 7B, the structure of PU.1-Ets is shown bound to DNA (1PUE, Ref. 36). The two C-terminal β-strands (β3/β4), which have been previously implicated in the interaction with GATA-1, are highlighted in magenta. The Cα atoms of the residues that were implicated in GATA-1 binding from our HSQC data are shown as yellow spheres. In Fig. 7D, the PU.1-Ets surface is shown, with the residues identified in our HSQC titrations colored yellow. Fig. 7, E and F shows the HSQC titration data for the titrations with CFtail and P-CFtail respectively. It can be seen that all of these experiments are consistent in that an overlapping region of PU.1-Ets is implicated in binding.

The Effect of the GATA-1-PU.1 Interaction on DNA Binding—Given that several of the residues in the CFtail are implicated in making direct contacts with DNA (31), we sought to determine quantitatively the effect of the CF and PU.1-Ets interaction on the ability of each domain to bind DNA. We carried out gel shift assays using MBP-CF and a 16-bp oligonucleotide containing a single GATA-1 site. Addition of PU.1-Ets reproducibly induced a modest increase in the affinity of CF for DNA (3-fold, from 5.3 ± 0.7 to 1.6 ± 0.2 nM, Fig. 8). In contrast, no change was observed in the strength of the PU.1-Ets-DNA interaction following the addition of CF (supplementary Fig. S1).

To assess whether the GATA-1-PU.1 interaction can promote the formation of a stabilized ternary complex on DNA sequences that contain both GATA and Ets binding sites, we constructed six double-stranded oligonucleotides that contain GATA and Ets sites with different spacings and orientations (supplementary Figs. S2 and S3). Gel shifts were carried out in which PU.1-Ets was titrated into the DNA at a constant concentration of CF. Cooperativity was not however observed in any case.

DISCUSSION
Regulation of GATA-1 Function—The data reported here reveal many of the molecular details of the interaction between the master transcriptional regulators GATA-1 and PU.1. A combination of GST pull-down and 15N HSQC data reveal that both the C-terminal end of the α-helix and a short motif to the C-terminal side of the GATA-1 CF are important for mediating the interaction between GATA-1 and PU.1.

Our findings explain puzzling results obtained in two previous studies. Both Zhang et al. (12) and Nerlov et al. (13) reported that the GATA-1 N-finger (NF) was not able to interact with PU.1. In contrast, Rekhtman et al. (11) concluded that the NF was able to contact PU.1, since deletion of the CF did not disrupt the GATA-1-PU.1 interaction. However, in the latter experiment only the structured part of the CF (Val250–His289) was deleted, leaving an important part of the interaction domain (Ala309–Gly318) intact and giving the impression that the NF could mediate an interaction. Similarly, Nerlov et al. (13) disrupted Zn2+ binding of the CF by mutating two Zn2+-coordinating cysteines. CD and NMR experiments show that the CF is completely unfolded in the absence of Zn2+ (data not shown), yet the two cysteine mutations failed to disrupt either the interaction with PU.1 or the ability of GATA-1 to inhibit transactivation by PU.1; this result was surprising at the time, but can be explained by the data obtained in our study, because the CFtail region remained intact and our results indicate that it is this region that makes a major contribution to the binding to PU.1-Ets.

Additionally, it has been shown that lysine residues in the CFtail region of GATA-1 (in the sequence Lys312–Ser313–Lys314–Lys315) are acetylated by the acetyltransferase CBP (33, 34), and

FIGURE 8. The DNA binding ability of GATA-1 CF is affected by PU.1-Ets. A, representative quantitative EMSAs of GATA-1 CF in the presence of a 16-bp GATA-containing oligonucleotide and varying amounts of PU.1-Ets domain. B, binding curves for the data in A, fitted to a simple 1:1 binding model.
that these residues are essential for normal erythroid development (33). Hong et al. (37) also demonstrated that PU.1 is capable of inhibiting the acetylation of GATA-1, although the mechanism through which this inhibition takes place was not elucidated. Our data reveal a possible molecular explanation for these observations, whereby the PU.1-Ets domain physically blocks access by CBP to the main acetylation sites on GATA-1.

Although several of the residues shown to be involved in PU.1 binding (Leu284, Leu288, His289, Ser310) have also been reported to make interactions with the phosphate backbone of DNA (31), these residues are relatively exposed to solvent (Fig. 7C). It is therefore quite possible that the GATA-1-PU.1 interaction can take place while the CF is bound to DNA. Consistent with this idea, our gel shift data indicated that the overall effect of the GATA-1-PU.1 interaction is to induce a small increase in the affinity of the GATA-1 CF for DNA. One point that should be noted regarding the gel shift data in Fig. 8 is that, whereas the CF-DNA interaction appeared to become more stable, no shift in the presence of Phox1, without the formation of a ternary complex (38). It was proposed that binding of Phox1 to SRF lowers the affinity of the CF to all oligonucleotides tested.

Previous mapping studies (40) localized the PU.1-binding motif of c-Jun to an 18-residue sequence within the DNA-binding basic domain of c-Jun (265–283 of the murine protein). A comparison of the sequence of this motif with the CFtail regions of GATA-1 and GATA-2 (Fig. 9C) reveals a high similarity among the three proteins. This similarity is consistent with the observation that GATA-1 and c-Jun compete for the same binding site on PU.1 (12) and also therefore provides strong support for the model in which the binding of GATA-1 to the PU.1-Ets domain disrupts the activity of the latter by preventing the formation of complexes with co-activators such as c-Jun (Fig. 9B).

Interestingly, the gene fusion product AML1-ETO, which results from the most common chromosomal abnormality in acute myeloid leukemia, has also recently been shown to bind to the β3/β4 region of PU.1, inhibiting PU.1 transcriptional activity by displacing c-Jun (42). In this way AML1-ETO can be seen to mimic the function of CF through interaction with a similar binding epitope on the PU.1-Ets domain. This might indicate that the binding site for CF on PU.1-Ets identified in this study represents a generally con-

**FIGURE 9. Model for GATA-1/PU.1 activity.** A and B, models for mechanisms through which GATA-1 and PU.1 each inhibit the activity of the other. GATA-1 is shown with shaded NF and CF domains; PU.1 is shown with white Ets and activation domains; c-Jun is shown as a dimer. C, sequences are taken from the murine proteins (P05627 for c-Jun, NP_032115 for GATA-1 and O09100 for GATA-2). The minimal PU.1-binding regions of c-Jun and GATA-1 are indicated with a dotted box.
served binding epitope for a broad range of proteins binding to Ets domains.

The CFtail Motif—A BLAST search using the CFtail sequence ASGKGGKRR reveals several other proteins that contain the motif SGKGGK, including small nuclear ribonucleoprotein D2 (Sm D2), a 13.5-kDa component of the hetero-heptameric Sm protein complex that forms an integral part of the pre-mRNA splicing apparatus. The Sm complex is found in each of the snRNP particles U1, U2, U4/6, and U5, and the structure of a dimeric subcomplex containing Sm D2 has been determined (43). Intriguingly, the Sm D2 structure is well ordered, except for a single, surface exposed loop region: the 10 residues containing the SGKGKKK motif. While there is no clear connection between GATA-1 and the spliceosome, it is possible that this sequence represents a point of regulation shared by the two proteins (e.g. through acetylation).

Weak Interactions in Transcriptional Regulation—According to our biophysical data, the interaction between GATA-1 and PU.1 is very weak. NMR titration experiments are extremely well suited to detecting such weak interactions (down to \(10^{-3} \text{ M}^{-1}\)), allowing both the determination of interaction affinities and the identification of the amino acids important for binding in a single titration experiment (see for example, Ref. 44). Note that the fact that no interaction could be measured by ITC indicates that the enthalpy change upon binding is too small to be readily detected, which is not surprising given the very low affinity constant.

Several other weak interactions have recently been reported, including those between GATA-1 and FOG \(1.0 \times 10^{-5} \text{ M}^{-1}\) (45) and between the PINCH LIM domain and Nck-2 \(1.0 \times 10^{2} \text{ M}^{-1}\) (46). These interactions might be effectively stronger in vivo, because of local concentration or avidity effects. In this context, GATA-1 and PU.1 sites have been found in close proximity in the transcription regulatory regions of various erythroid-specific genes (47, 48). Likewise Henkel and Brown (49) found that PU.1 and GATA factors collaborate to stimulate transcription of the interleukin-4 gene, which contains adjacent PU.1 and GATA binding sites in the promoter. The formation of such a ternary complex between DNA-bound GATA-1 and PU.1 might have a high affinity, as a consequence of the GATA-1–PU.1 interaction. Our gel shifts carried out with DNA containing both GATA and Ets sites argue against this latter possibility, although these experiments were not exhaustive, a different relative positioning of the two sites might be required. Alternatively, weak protein–protein interactions might represent mechanisms through which rapid changes in regulation are possible via fast off-rates for these complexes.

It was surprising that none of the single or double point mutations (and only one of the quadruple mutations) made to either CF or PU.1 abrogated the interaction between the two domains, despite clear evidence from NMR titration data that specific residues were involved in the interaction. It is perhaps relevant that all of the mutations made to residues identified as being important by NMR were mutations to alanine. While mutation to alanine will remove any specific side chain interactions, it is more conservative than charge reversal or size-increasing mutations, and in this case might not have been sufficiently disruptive to interfere with the CF–PU.1–Ets interaction.

In summary, we have defined the motifs involved in mediating the interaction between GATA-1 and PU.1. These data substantially improve our understanding of the molecular mechanisms through which these two transcription factors act to drive differentiation down the erythroid and myeloid lineages, respectively.

Acknowledgments—We thank Fiona Power, Ed Dram, and Jacqui Matthews for providing a number of GATA-1 CF mutants and Sigurður Kristjánsson for early work on GATA-1 CF constructs. We thank Bill Bubb for expert maintenance of the 600 MHz NMR spectrometer at the University of Sydney and Jens Kaalbye Thomsen and Flemming Larsen for expert maintenance of the 750 and 800 MHz NMR spectrometers at the University of Copenhagen. We also greatly appreciate the input and helpful discussions provided by Wolfgang Pieber, Kaare Teiulf, Nick Dixon, and Jason Kahn.

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