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Key Residues Characteristic of GATA N-fingers Are Recognized By FOG*

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Protein-protein interactions play significant roles in the control of gene expression. These interactions often occur between small, discrete domains within different transcription factors. In particular, zinc fingers, usually regarded as DNA-binding domains, are now also known to be involved in mediating contacts between proteins. We have investigated the interaction between the erythroid transcription factor GATA-1 and its partner, the 9 zinc finger protein, FOG (Friend Of GATA). We demonstrate that this interaction represents a genuine finger-finger contact, which is dependent on zinc-coordinating residues within each protein. We map the contact domains to the core of the N-terminal zinc finger of GATA-1 and the 6th zinc finger of FOG. Using a scanning substitution strategy we identify key residues within the GATA-1 N-finger which are required for FOG binding. These residues are conserved in the N-fingers of all GATA proteins known to bind FOG, but are not found in the respective C-fingers. This observation may, therefore, account for the particular specificity of FOG for N-fingers. Interestingly, the key N-finger residues are seen to form a contiguous surface, when mapped onto the structure of the N-finger of GATA-1.

Individual eukaryotic transcription factors rarely work alone to activate gene expression. Diverse collections of proteins are typically found assembled at promoters and enhancers, where they are thought to act by recruiting the basal transcriptional machinery and/or by influencing chromatin structure (for review, see Refs. 1–3). Many different types of protein-protein interactions are required for the coordinated formation of an active transcription complex. These interactions can be grouped into 4 main classes: homotypic interactions, such as the dimerization of leucine zippers; short-range interactions, involved in the cooperative binding of factors at adjacent DNA elements; local interactions between DNA-bound proteins and their ancillary factors, such as coactivators or corepressors; and long range interactions between factors bound at widely spaced control elements, which may mediate DNA looping. Although much is now known about transcription factor dimerization and the way in which proteins contact DNA, much less is known about the molecular interactions made between different transcription factors.

It is now apparent that some of the well defined structures known to be involved in protein-DNA interactions are also involved in mediating contacts between different proteins. In particular, there are now several examples of zinc finger domains which are involved in protein-protein interactions (4). In some cases, such as GATA fingers (see below), these domains are thought to make both protein-protein (5–9) and protein-DNA contacts (10), whereas in other cases, such as the C-terminal fingers of the Ikaros family proteins, the zinc fingers appear to be exclusively dedicated to protein-protein contacts (11). We have investigated the molecular basis of the interaction between the zinc finger proteins GATA-1 (12, 13) and FOG (Friend Of GATA) (7), as a prototype for understanding the molecular details of zinc finger-finger interactions.

GATA family transcription factors typically bind to (A/T)GATA(A/G) motifs in DNA and contain one or two Cys-X_2-Cys-X_{17}-Cys-X_2-Cys zinc fingers. The founding member of the family, the erythroid transcription factor GATA-1, is thought to be involved in the expression of most, if not all, genes transcribed specifically in red blood cells (for review, see Refs. 14 and 15) and knockout experiments in mice have confirmed that GATA-1 is essential for red blood cell development (16–19). GATA-2 and -3 also play key roles in hematopoiesis (20–22), while other members of the family, GATA-4, -5, and -6, regulate gene expression in the heart and gut (23–25). Additional GATA family proteins play fundamental roles in other organisms: AreA is involved in nitrogen fixation in *Aspergillus* (26), Ash1 in cell division control in yeast (27, 28), StkA in stalk formation in *Dyctyostelium* (29) and the *Drosophila* GATA factors, Pannier (30), Serpens (31), and dGATAc (32) are involved in the control of cell fate during development.

Recently, it has been suggested that GATA factors act in conjunction with partner proteins of the FOG family. FOG is a zinc finger protein, which was originally isolated in a screen for proteins that could interact with the zinc finger domain of GATA-1 (7). A second member of the FOG family, U-shaped (33, 34), was subsequently identified as a partner of the *Drosophila* GATA factor, Pannier. Both FOG and U-shaped contain 9 putative zinc fingers, 4 of which appear to be classical (TFIIIA-like) CCHH fingers and 5 of which are variant CCHC fingers. FOG is believed to work in concert with DNA-bound GATA-1 to activate gene expression (7), whereas U-shaped appears to counter the action of Pannier. Knockout experiments in mice have recently confirmed the essential role of FOG in hematopoiesis (35) and studies of mutations in *Drosophila* attest to the importance of the interaction between U-shaped and Pannier in proneural gene expression and bristle cell formation (34).

We have studied the molecular contacts made between murine GATA-1 and FOG in order to gain insights into the general mechanisms by which the two families of transcription factors interact to regulate gene expression. We confirm that one key region of FOG, finger 6, is a true zinc-binding domain and have investigated its interaction with the N-finger of GATA-1. We show that association is reliant on intact zinc-coordinating

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residues in both GATA-1 and FOG and that the interacting regions map precisely to zinc-binding subdomains within the two proteins. Noting that FOG recognizes the N-finger but not the highly related C-finger of GATA-1, we have explored the basis for this specificity by utilizing a panel of N/C microchimera mutants. This mutagenic analysis identifies key residues within the GATA-1 N-finger that are required for contact with FOG. These residues are conserved within the N-fingers, but not the C-fingers, of all GATA family proteins known to bind FOG. These results confirm the GATA-FOG interaction as an unequivocal finger-finger interaction and have implications for the mechanisms by which these two proteins cooperate to activate gene expression.

EXPERIMENTAL PROCEDURES

Metal Binding Studies on FOG-Finger 6—FOG-finger 6 (residues 694–723) was obtained as a crude product from a solid-phase synthesis carried out by Chiron Mimotopes (Clayton, Victoria, Australia), and was purified using reversed phase HPLC (rpHPLC)[1] on a Vydac analytical C_{18} column (5 μm), employing linear water/acetonitrile gradients. The identity of the purified peptide was established using positive-ion electrospray mass spectrometry (M_{exp} = 3687.8 Da, M_{obs} = 3687 Da), and taken together, the electrospray mass spectrometry and rpHPLC data indicate that the peptide was >95% pure.

Circular Dichroism Spectropolarimetry—Circular dichroism (CD) spectra were recorded on a Jasco J-720 spectropolarimeter using a 1-mm quartz cuvette. FOG-finger 6 (25 μM) was dissolved in a buffer containing Tris (10 mM) and tris(2-carboxyethyl)phosphine (TCEP, 250 μM) at pH 8.0. $ZnCl_2$ was made up in water at a concentration of 20 mM. CD spectra (190–260 nm) were recorded following the addition of successive 5-μM aliquots of Zn^{2+}. The final spectrum in each case is the sum of five separate spectra with a step size of 0.5 nm, a 1-s response time, a 20-nm min^{-1} scan rate, and a 1-nm bandwidth. Data were acquired at 20 °C and were baseline corrected by subtraction of the Tris/TCEP buffer.

UV/Visible (UV/VIS) Spectrophotometry—FOG-finger 6 was prepared as for CD spectropolarimetry, and Co^{2+} was made up as a 20 mM solution of $CoCl_2$ in water. UV and VIS absorption spectra (250–500 and 500–800 nm, respectively) were recorded on a Cary 3 double-beam spectrophotometer using 1-cm matched quartz cuvettes. Co^{2+} was titrated into both the sample cell and the reference cell (the latter containing the Tris/TCEP buffer only) in 5 μM aliquots. Data were recorded at a speed of 100 nm min^{-1} with a signal averaging time of 0.1 s.

Site-directed Mutagenesis and Plasmid Construction—Site-directed mutagenesis was performed using either overlap polymerase chain reaction or single primer mismatch polymerase chain reaction with *Pfu* polymerase (Stratagene) and mutant oligonucleotide primers (Life Technologies, Inc.). Details of oligonucleotides and plasmids are available on request.

Yeast Two-hybrid Assays—*In vivo* interactions between GATA-1 and FOG were analyzed using a yeast two-hybrid system (CLONTECH). GATA-1 truncations and mutants were cloned in-frame into the Gal4 DNA-binding domain encoding plasmid, pGBT9. The Gal4 activation domain encoding plasmid, pGAD10, was used as the cloning vector for in-frame FOG constructs, both truncations and mutants. Competent HF7c yeast cells were transformed simultaneously with both the appropriate pGBT9 and pGAD10 constructs (CLONTECH Two-hybrid Matchmaker system protocol) and the transformants selected on Leu^- Trp^- minimal media plates by growth at 29 °C for 3 days. Transformants were then patched onto His^- Leu^- Trp^- plates and monitored for growth for up to 3 days.

Western Blotting—Western blotting was employed to confirm the expression of GATA and FOG Gal4 fusion proteins that gave negative results in the two-hybrid assay. Yeast protein extractions (CLONTECH Yeast protocol manual) were performed on overnight cultures of HF7c containing the appropriate construct and run on an SDS-polyacrylamide gel. After overnight blotting onto nitrocellulose, Western analysis was performed with antibodies to either the Gal4 activation domain (FOG-GAD10 constructs) or the Gal4 DNA-binding domain (GATA-GBT9 constructs), according to the manufacturer's instructions (CLONTECH). Secondary antibody was detected using an ECL kit (Amersham).

GST Fusion Protein Binding Assays—The N-finger (residues 200–254, 200–248, 200–243, 200–239, 200–235, 200–231, and 200–227), C-finger (residues 249–318), and mutants of the N-finger of GATA-1 (residues 200–248) were generated by polymerase chain reaction and subcloned in-frame into the expression vector pGEX2T. The expression of both GST fusion proteins and GST alone was performed using *Escherichia coli* strain DH5α and purification was carried out as described previously (36). ^{35}S-Labeled FOG (residues 279–760) (7) was prepared by *in vitro* transcription/translation using the TNT system according to the manufacturer's instructions (Promega). *In vitro* binding assays were performed in 0.3 ml of buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.1% Nonidet P-40, 10 μM $ZnSO_4$, 0.25% bovine serum albumin, 1 mM β-mercaptoethanol, and 1.5 mM phenylmethylsulfonyl fluoride) with 1 μg of fusion protein attached to glutathione beads and 3 μl of radiolabeled FOG. In all cases, levels of the various GST fusion proteins were confirmed by Coomassie staining. Reaction mixtures were incubated for 1 h at 4 °C and the beads were then washed repeatedly with binding buffer. Samples were boiled in loading buffer and subjected to SDS-polyacrylamide gel electrophoresis. The gel was then dried and any FOG retained by the beads was detected using a PhosphorImager.

Gel Shift Assays—10-cm diameter plates of COS cells were transfected with 3 μg of pXMGATA-1, pXMGATA-1 ΔN-finger (37), or 3 μg of pXMGATAE203V, V205T, GA208/209QT, and HY222/223DP by the DEAE-dextran method. (38). 48 h later, nuclear extracts were prepared as described previously (39). Gel shift reactions were performed using 1 ng of ^{32}P-labeled oligonucleotide probe containing the mouse α-globin GATA site (GATCTCCGGCAACTGATAAGGATTCCCTG) as described previously (40). 1 μl of N6 monoclonal anti-GATA-1 antibody (Santa Cruz) was added where indicated.

Transactivations—To study GATA-1 activity alone, NIH3T3 cells were transfected with 2 μg of the GATA-dependent reporter M1αGH (37) and 2 μg of transactivator plasmid, pXMGATA-1, pXMGATA-1 ΔN-finger, or pXMGATA-1 mutants using the calcium phosphate method (38). To examine the synergistic activation of FOG and GATA-1 on the p45 (NF-E2) promoter, 5 μg of p45GH reporter (7) was transfected, along with 2 μg of PMT2FOG (7) and 0.5 μg of pXMGATA-1, pXMGATA-1 ΔN-finger, or pXMGATA-1 mutants. Growth hormone assays were carried out using Nichols Institute Allegro GH assay kits according to the manufacturer's instructions. All cell culture data is the result of three separate experiments and has been normalized to LacZ levels derived from a co-transfected β-galactosidase-encoding plasmid, EF1α-LacZ.

RESULTS

Minimal Interacting Domains of FOG and GATA-1 Map Precisely to Putative Zinc Finger Regions—The smallest FOG cDNA clone isolated in the initial two-hybrid screen against the GATA-1 N-finger (residues 200–254), contained residues 563–859, encompassing FOG-finger 6, which is known to bind GATA-1, as well as fingers 5 and 7 (7). We sought to determine whether fingers 5 and 7 could also bind GATA-1 and to delineate the minimal region of finger 6 required for the interaction. A series of deletion mutants were prepared and tested for their ability to interact with the GATA-1 N-finger in the yeast two-hybrid assay. Clones containing finger 6 were capable of binding GATA-1, whereas clones containing either finger 5 alone or finger 7 alone, could not (Fig. 1). Further deletions defined the minimal region required for the interaction to a 40-residue region (690–729), centered around the zinc-binding domain (Cys^{698} to Cys^{719}). Note that the residues (690–697) immediately before the first cysteine of the finger appear to be necessary for the interaction. These results localize the minimal region of FOG required to bind GATA-1 to finger 6.

GATA-type fingers are composed of a zinc-binding fold, followed by a basic tail region. In the case of GATA-1, the tail region of the C-finger has been shown to be important for DNA binding (10) and has also been implicated in self-associations between GATA proteins (5). To determine whether the tail of the N-finger is required for contact with FOG, we prepared a series of N-finger truncation mutants and tested their ability to

[1] The abbreviations used are: rpHPLC, reversed phase high performance liquid chromatography; TCEP, tris(2-carboxyethyl)phosphine; GST, glutathione *S*-transferase.

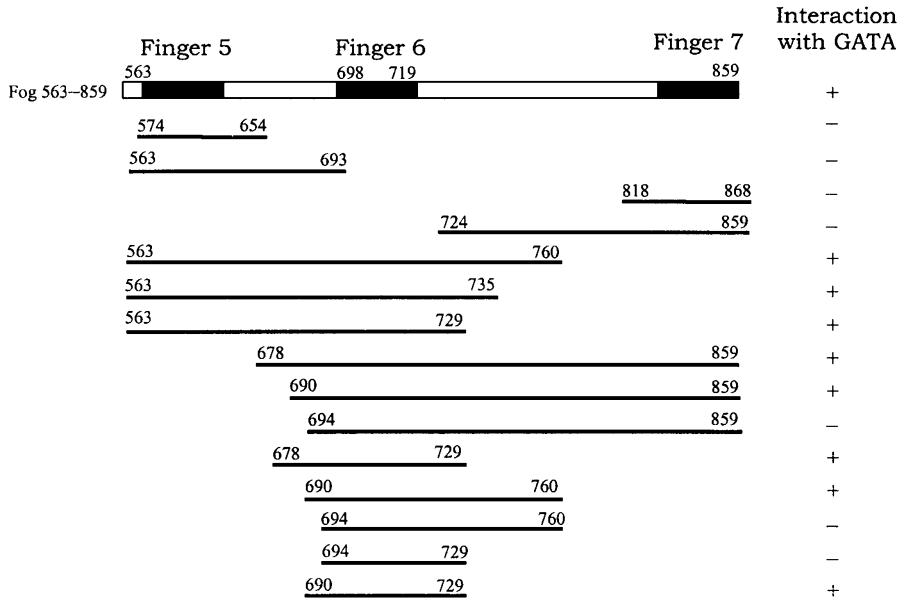

FIG. 1. **Delineation of the minimal region of FOG that can interact with the N-finger of GATA-1.** Regions of the original FOG clone, FOG M10 (7), which contains fingers 5 to 7 (residues 563–859), were tested for their ability to interact with the N-finger of GATA-1 (residues 200–254) using the yeast two-hybrid assay. Yeast strain HF7c was co-transformed with GATA-1 derivatives harbored in pGBT9, and FOG derivatives in pGAD10. Transformants were selected on Leu^- Trp^- plates and patched onto Leu^- Trp^- His^- plates. + indicates clear growth on Leu^- Trp^- His^- plates when incubated at 29 °C for 48 h, – indicates no growth, (see Fig. 7*B* for an example of actual yeast growth).

bind FOG in the yeast two-hybrid system. As shown in Fig. 2, the entire tail region was dispensable for FOG binding. Binding was abolished only when the deletion extended from the tail into the zinc-binding region (*i.e.* when one of the zinc coordinating cysteines, Cys^{228}, was deleted). GST pull-down experiments, using the same series of constructs, confirmed this result *in vitro* (data not shown). These findings indicate that a 32-amino acid peptide (residues 200–231) encompassing the zinc-binding region of the N-finger is sufficient for recognition by FOG.

FOG Finger 6 Is a True Zinc Finger—As can be seen from Table I, FOG-finger 6 appears related to classical (TFIIIA-like) zinc fingers, in that it contains the characteristic conserved hydrophobic residues and three of the four zinc chelating residues. FOG-finger 6 is unusual, however, in that it has a CCHC rather than a CCHH configuration of putative zinc-ligating residues. In order to determine whether FOG-finger 6 was a genuine metal-binding domain we carried out CD spectropolarimetry and UV/VIS spectrophotometry experiments in the presence of Zn^{2+} and Co^{2+}, respectively.

Fig. 3*A* shows far-UV CD spectra of HPLC-purified FOG-finger 6, recorded in the presence of increasing molar ratios of Zn^{2+}. In the absence of metal, FOG-finger 6 displays a spectrum characteristic of an unfolded polypeptide, with a pronounced minimum below 200 nm. The red shift of the minimum to 208 nm and the appearance of a second minimum at 220 nm, following the addition of 1 molar equivalent of Zn^{2+}, are indicative of the formation of secondary structure. The lack of change after the addition of more than 1 equivalent of Zn^{2+} establishes the stoichiometry of the Zn^{2+}·FOG-finger 6 complex as 1:1 (data not shown) and the general form of the spectrum is comparable to those previously observed in studies of CCHH classical zinc fingers (41, 42).

In order to probe the geometry of metal ion ligation and the identity of the metal-coordinating residues, we carried out UV/VIS spectrophotometry experiments in the presence of Co^{2+} (a metal which is commonly used as a spectroscopic probe for zinc-binding proteins, due to its favorable electronic properties (42–45). The appearance of bands at 310 and 350 nm is indicative of cysteinate ligation (42), while the signals at 600, 650, and 720 nm show both that the metal coordination sphere is tetrahedral and that at least one nitrogen ligand is involved in the metal ligation (43, 45) (Fig. 3*B*). Taken together, the CD and UV/VIS data suggest that the FOG finger is capable of folding around zinc, with one histidine and three cysteine residues ligating the metal.

The Integrity of FOG Finger 6 Is Required for Binding to GATA-1—We next sought to investigate whether the integrity of the FOG zinc finger was required for its interaction with GATA-1. We prepared two FOG-finger 6 (residues 678–760) mutants in which zinc-coordinating cysteine residues were replaced by alanine, $Cys^{698} \rightarrow$ Ala and $Cys^{719} \rightarrow$ Ala, and assayed their ability to interact with GATA-1 (residues 200–254) in the yeast two-hybrid system. Neither mutant was able to interact with GATA-1 (Fig. 4). We also mutated the 4th cysteine to histidine, $Cys^{719} \rightarrow$ His, to mimic the CCHH configuration of a classical zinc finger. This mutant was also unable to interact with GATA-1 (Fig. 4), suggesting that the unusual CCHC configuration is critical for this protein-protein interaction.

To further assess the importance of metal ions, we carried out *in vitro* GST pull-down experiments in the presence and absence of EDTA. A ^{35}S-labeled *in vitro*-translated fragment of FOG containing finger 6 was assessed for its ability to interact with GST GATA-N-finger (residues 200–254) immobilized on agarose beads. This fragment of FOG was efficiently retained on the GATA N-finger-coated beads (Fig. 5, *lane 2*) but not on GST-coated beads (Fig. 5, *lane 4*). When the experiment was repeated in the presence of 5 mM EDTA, the FOG fragment was no longer retained (Fig. 5, *lane 3*). We have previously demonstrated that the GATA-1 zinc fingers bind zinc more tightly than classical fingers, and are not affected by the addition of EDTA under these conditions,[2] and we have also carried out additional controls indicating that EDTA does not affect the binding of GST GATA-1-N-finger to glutathione-coated beads (data not shown). Consequently, this particular experiment primarily addresses the importance of metal ions to the FOG finger.

The Integrity of the GATA-1 N-finger Is Required for Binding to FOG—We next carried out experiments to investigate whether zinc-binding by the GATA-1 N-finger (residues 200–254) was also required for the interaction. The N-finger coordinates zinc by means of four cysteines, Cys^{204}, Cys^{207}, Cys^{225}, and Cys^{228}. We prepared two cysteine to alanine substitution mutants, $Cys^{204} \rightarrow$ Ala and $Cys^{228} \rightarrow$ Ala, and tested their ability to interact with FOG-finger 6 (residues 678–760) in the

[2] J. P. Mackay, K. Kowalski, and M. Crossley, manuscript in preparation.

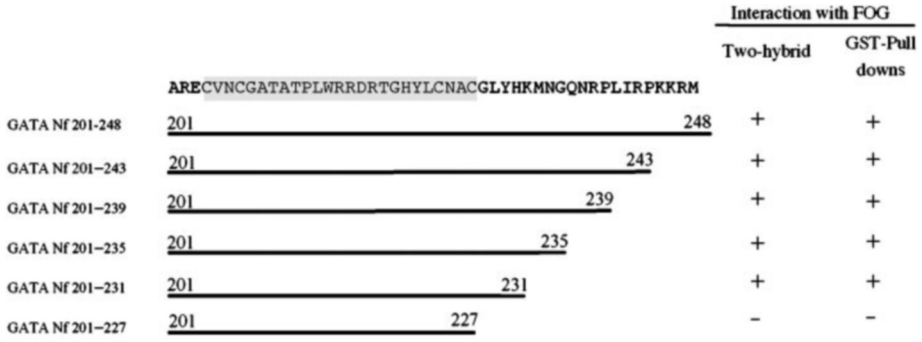

FIG. 2. **The core zinc-binding region of the N-terminal finger of GATA-1 interacts with FOG-finger 6.** Regions of GATA-1 were tested for their ability to interact with FOG (residues 678–760) using the yeast two-hybrid assay, as described in the legend of Fig. 1. The region between the first and fourth cysteines, is shaded in *gray*.

TABLE I

FOG-finger 6 is related to the zinc fingers of Sp1, EKLF, and TFIIIA

Shaded boxes indicate conserved residues, whilst the clear box indicates the zinc-coordinating residue in FOG-finger 6 which differs from the TFIIIA consensus.

FOG f6	C	E	A	.	.	C	N	I	R	F	S	R	H	E	T	Y	T	V	H	K	R	Y	Y	C
Sp1 f3	C	P	E	.	.	C	P	K	R	F	M	R	S	D	H	L	S	K	H	I	K	Y	.	H
EKLF f3	C	G	L	.	.	C	P	R	A	F	S	R	S	D	H	L	A	L	H	M	K	R	.	H
TFIIIA f3	C	D	S	D	G	C	D	L	R	F	T	T	K	A	N	M	K	K	H	F	N	R	F	H
Consensus for TFIIIA-like CCHH fingers	C	X	X	X	X	C	X	X	X	F/Y	X	X	X	X	X	L	X	X	H	X	X	X	X	H

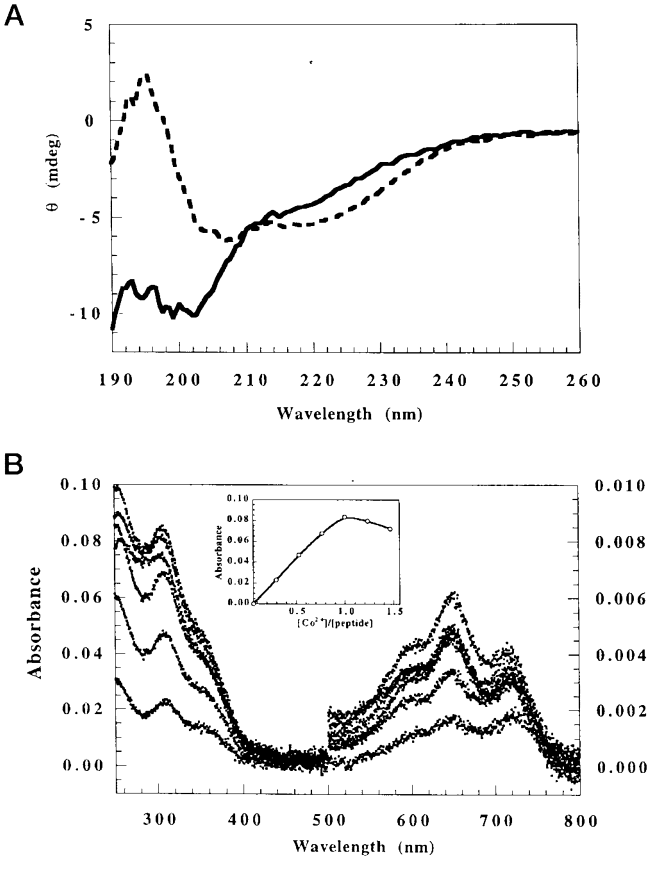

FIG. 3. **FOG-finger 6 folds around metal ions.** *A,* CD spectrum of FOG-finger 6 (694–723) in the absence of Zn^{2+} (*solid line*), and with 1 equivalent of Zn^{2+} added (*dashed line*). *B,* UV and visible absorption spectra recorded during titration of Co^{2+} into FOG-finger 6 (694–723). Spectra are shown at $[Co^{2+}]$/[peptide] ratios of 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50. A graph of absorbance at 310 nm *versus* $[Co^{2+}]$/[peptide] is shown in the *inset* to demonstrate the stoichiometry of metal binding.

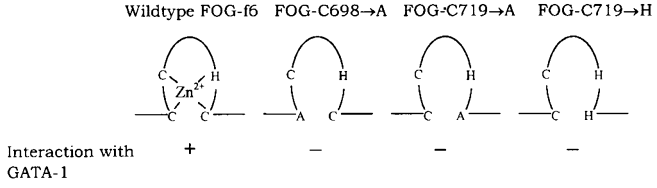

FIG. 4. **The integrity of the zinc-binding module in FOG is required for its interaction with GATA-1.** Mutations were made in the 6th zinc finger of FOG (residues 678–760) and its ability to bind the N-finger of GATA-1 (residues 200–254) was tested by the yeast two-hybrid system as described in the legend for Fig. 1.

yeast two-hybrid system. In each case the cysteine to alanine mutation abolished the interaction (Fig. 6*A*).

We confirmed this result *in vitro* using the GST pull-down assay. In this experiment, GST N-finger (residues 200–254) was able to efficiently sequester *in vitro* translated FOG (Fig. 6*B, lane 2*), while N-finger domains with mutations of either Cys^{204} or Cys^{228} could not (Fig. 6*B, lanes 3* and *4*). In a control reaction, GST-coated beads were similarly unable to bind to FOG (Fig. 6*B, lane 5*). Taken together, the results indicate that both FOG-finger 6 and the GATA N-finger must be intact for their interaction to occur; their association therefore represents a true finger-finger interaction.

Conserved Residues within the N-finger Bind FOG—The N- and C-terminal zinc finger domains of murine GATA-1 share about 50% amino acid sequence identity. The existing data suggest that FOG is capable of interacting only with the N-finger (7). There are a number of possible molecular explanations that might account for this observation. First, it is possible that FOG recognizes particular signature residues within the N-finger, which are not conserved in the C-finger sequence. Alternatively, one could argue that in the case of the C-finger, the protein-DNA contacts (or alternative protein-protein contacts, such as contacts between the C-finger and Sp1/EKLF (6)) may prevent it from interacting with FOG *in vivo*. We sought to investigate whether sequence differences between the two GATA fingers were sufficient to explain the specificity of FOG for the N-finger.

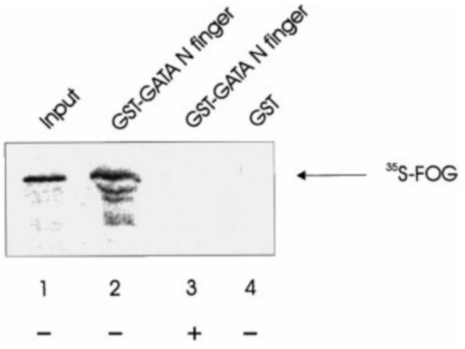

FIG. 5. **The addition of EDTA prevents FOG from binding to the N-finger of GATA-1, suggesting that the interaction depends on zinc binding by FOG.** *Lane 1* contains 10% of the input *in vitro* translated ^{35}S-labeled FOG. *Lanes 2* and *3* contain 1 μg of GST GATA-Nf (200–254) fusion protein incubated with FOG in the presence or absence of 5 mM EDTA as shown. *Lane 4* is a control containing 1 μg of GST alone incubated in the presence of FOG. After extensive washing, the GST or GST N-finger coated beads were boiled in loading buffer and subjected to electrophoresis, after which retained FOG was visualized by PhosphorImaging.

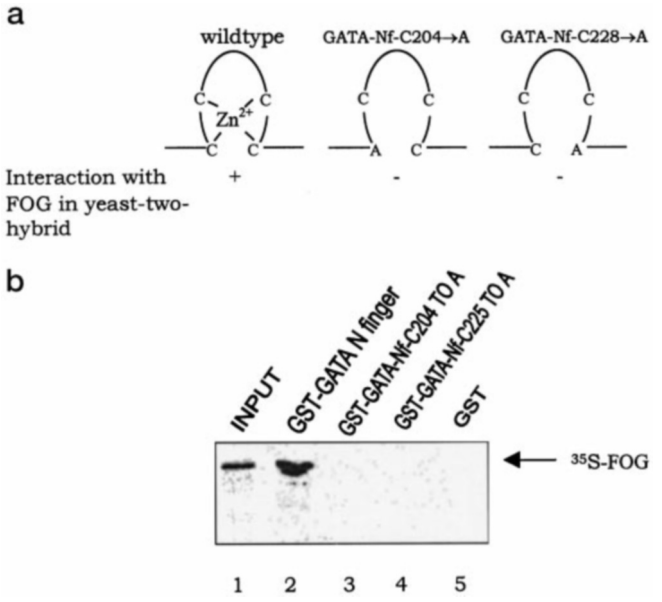

FIG. 6. **The integrity of the zinc-binding module in GATA-1 is essential for its interaction with FOG.** *A,* mutations were made in the N-terminal zinc finger of GATA-1 (residues 200–254) and tested with the sixth zinc finger of FOG (residues 678–760) in the yeast two-hybrid system, as described in the legend for Fig. 1. *B,* GATA-N-finger mutants were also tested in GST pull-down assays. *Lane 1* shows 10% input FOG; *lane 2* contains GST-GATA-N-finger; and *lanes 3* and *4* contain the indicated GST-N-finger mutant bound to beads. Each sample was incubated with FOG and treated as described in the legend to Fig. 5.

As shown above, the minimal region of the N-finger that is required for its interaction with FOG comprises only 32 residues, 17 of which are also found within the C-finger. We therefore focussed on the remaining residues in order to investigate whether FOG discriminated between the N- and C- fingers on the basis of these differences. We chose to prepare substitution mutants in which specific residues from the C-finger were introduced into the N-finger sequence. In essence, each of these new proteins is a microchimera, containing one (or in some cases two) residues from the C-finger within the N-finger sequence. We reasoned that since the N- and C- fingers are so similar in overall structure (58) (see Fig. 8, *A* and *B*) these substitutions would be unlikely to disrupt the general architecture of the finger, but would enable us to pinpoint key residues important for FOG binding.

The results of this analysis are shown in Fig. 7 and summarized in Table II. In Table II, the sequence of the minimal subdomain of the N-finger required for binding FOG is shown at the top of the table, with the corresponding region of the C-finger shown at the bottom. Microchimera mutants are shown in the body of the table. As expected, several of the mutations had little effect on FOG binding, and thus had disrupted neither critical contact points nor the overall structural integrity of the finger. Three separate mutations did, however, significantly affect the interaction with FOG. They were Val^{205} → Thr, Gly^{208}-Ala^{209} → Gln-Thr, and His^{222}-Tyr^{223} → Asp-Pro. A further mutation Glu^{203} → Val also impaired FOG binding, although the original conservative microchimera mutation Glu^{203} → Gln had had little effect (Val and Gln are found in the chicken and murine GATA-1 C-fingers, respectively). The binding results from the two-hybrid assay (Fig. 7*B*) were in agreement with those of the *in vitro* GST pull-down assay (Fig. 7*A*).

The FOG-binding Face of GATA-1—These results identify several key residues within the N-finger which are important for its interaction with FOG: *viz.* Glu^{203}, Val^{205}, Gly^{208}, Ala^{209}, His^{222}, and Tyr^{223}. As indicated by the boxes in Table III, these residues are conserved in the N-fingers of GATA-1, -2, and -3, all of which are known to bind to FOG, but are not present in any of the corresponding C-fingers. Thus it seems probable that the inability of FOG to recognize C-fingers is a result of these specific sequence features that distinguish C- and N-fingers.

The N-finger residues identified by these experiments are likely to form part of a conserved FOG-binding site. Although these residues are dispersed in the linear sequence of GATA-1 (Table III), they come together to form a contiguous surface when mapped onto the recently solved three-dimensional structure of the N-finger (58) (Fig. 8*A*). We propose that this face represents the FOG-binding site within GATA-1.

GATA-1 Mutants, Unable to Bind FOG, Cannot Activate the p45 NF-E2 Promoter—We next sought to determine whether the mutations that interfered with the physical interaction between GATA-1 and FOG *in vitro* also interfered with the functional interaction between full-length GATA-1 and FOG in mammalian cells. It has previously been shown that GATA-1 and FOG can synergistically activate the hematopoietic-specific p45 NF-E2 promoter (7). Deletion of the entire N-finger of GATA-1 abolishes synergistic activation, consistent with the view that FOG directly interacts with the GATA-1 N-finger *in vivo* (7). To assess the functional effect of the four GATA N-finger mutations that interfere with binding to FOG *in vitro* (Glu^{203} → Val, Val^{205} → Thr, Gly^{208}-Ala^{209} → Gln-Thr, and His^{222}-Tyr^{223} → Asp-Pro), we made these mutations in the context of full-length GATA-1 and tested the ability of the mutant proteins to cooperate with FOG. We also included full-length GATA-1 and GATA-1 ΔN-finger (containing a deletion of the entire N-finger 200–248), for comparison. No significant activation was seen in the absence of co-transfected FOG (data not shown). In the presence of FOG, wild type GATA-1 strongly activated the promoter, whereas the four mutant proteins and GATA-1 ΔN-finger gave only low levels of activation (Fig. 9*A*). Additional experiments confirmed that all of the mutant proteins retained the ability to bind DNA (Fig. 9*B*) and to transactivate the M1α promoter, a GATA-dependent promoter that does not depend on FOG (37) (although the level of transactivation was slightly lower than wild-type in the cases of E203V and GATA-1 ΔN-finger) (Fig. 9*C*). Taken together these results suggest that the mutations interfere with the functional interaction of GATA-1 and FOG and provide additional data indicating that physical interaction between the two proteins is required for synergistic transactivation.

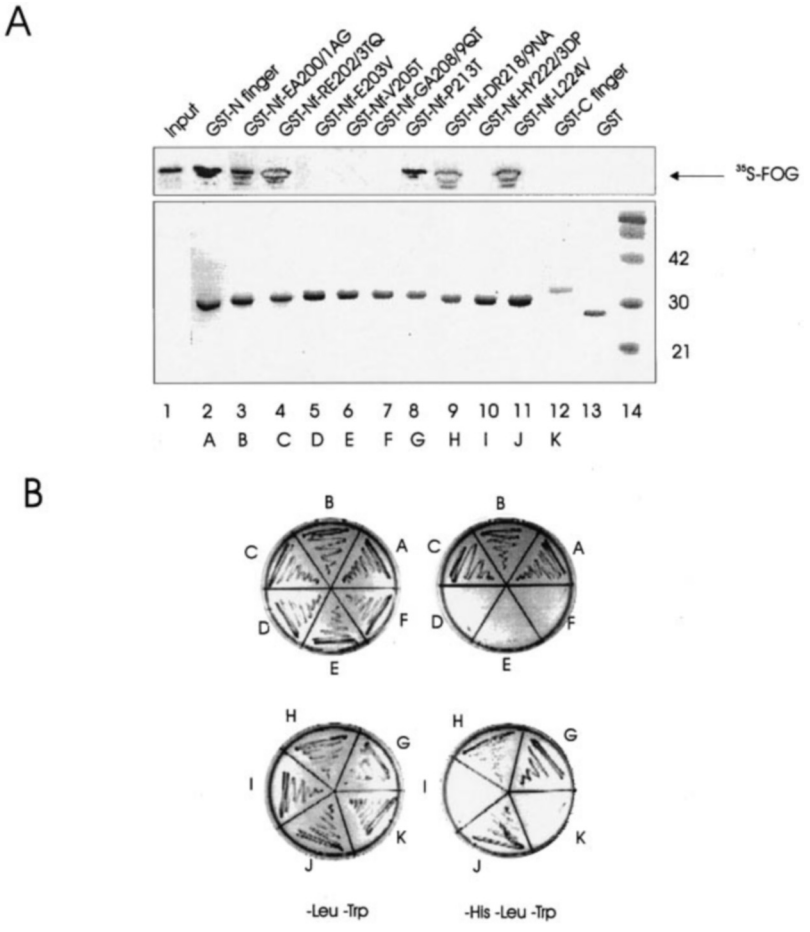

FIG. 7. **Interactions between mutant GATA-1 proteins and FOG.** *A,* GST pull-down interactions. *Lane 1* contains 10% of the input *in vitro* translated ^{35}S-labeled FOG (residues 279–760); *lane 2* contains GST-GATA-N-finger; *lanes 3–11* contain GST-GATA-N-finger mutants as shown. *Lane 12* contains GST-GATA-C-finger. *Lane 13* is a control containing 1 μg of GST alone. Each sample was incubated with FOG and treated as described in the legend to Fig. 5. *Lane 14* contains molecular weight markers with sizes (in kDa) indicated. The amount of FOG retained by each GST fusion protein is shown in the *top panel* and a sample of the GST fusion protein stained with Coomassie Blue in the *bottom panel*. The fusion protein GST-GATA-C-finger (*lane 12, lower panel*) was poorly expressed and additional quantities were utilized to ensure appropriate amounts of this protein were used in the experiment. *B,* HF7c yeast growth 48 h after streaking and incubation at 29 °C on the indicated minimal media. Each sector contains yeast harboring the FOG M10 pGAD10 plasmid (7) and various GATA.pGBT9 plasmids. These GATA fragments in pGBT9 are: *A,* N finger (residues 200–254); *B,* Nf EA200/1AG; *C,* Nf RE202/3TQ; *D,* Nf E203V; *E,* Nf V205T; *F,* Nf GA208/9QT; *G,* Nf P213T; *H,* Nf DR218/9NA; *I,* Nf HY222/3DP; *J,* Nf L224V; and *K,* C finger (residues 249–318).

TABLE II
Identification of key residues within the N-finger of GATA-1 that differ in the C-finger and are thus implicated in finger-specific recognition by FOG

		Interaction with FOG	
		Two hybrid[a]	GST pull downs[b]
GATA Nf 200 → 232	EARE**C**V**NC**GA**T**A**T**P**L**W**RR**DRT**G**HYL**CNACGLY**[c]**	+	+
GATA Nf EA200/201 → AG	AG----------	+	+
GATA Nf A201 → L	-L----------	+	+
GATA Nf RE202/203 → TQ	--TQ----------	+	+
GATA Nf E203 → V	---V----------	−	−
GATA Nf V205 → T	-----T----------	−	−
GATA Nf GA208/209 → QT	--------QT----------	−	−
GATA Nf P213 → T	----------T----------	+	+
GATA Nf DR218/219 → NA	----------NA----------	+	+
GATA Nf HY222/223 → DP	----------DP--------	−	−
GATA Nf L224 → V	----------V--------	+	+
GATA Cf 254 → 285	AGTQ**CTNC**QT**TTTTLWRR**NAS**G**DPV**CNACGLY**	−	−

[a] The yeast two-hybrid assay was used to test the interaction between these GATA-N-finger mutants and FOG (residues 563–859), + or − indicate growth on His$^-$Leu$^-$Trp$^-$ media as judged from Fig. 7*B*.

[b] Results of the GST pull-down assay, where interaction of GST N-finger fusions and in vitro translated FOG are indicated by either + signs or − signs reflecting the amount of FOG retained by the bead bound GATA-1 as judged from Fig. 7*A*.

[c] Residues shown in bold are common to both the N- and C-fingers.

DISCUSSION

Finger-Finger Interactions—The double zinc-finger domain of GATA-1 is critical for the protein's role in blood cell development (46, 47). The C-finger is known to be sufficient for binding to (A/T)GATA(A/G) motifs (10, 37), while the N-finger is thought to stabilize DNA-binding and to be important in the recognition of complex double GATA motifs (13, 37, 48). Recently, it has become apparent that the GATA-1 zinc finger domain is also involved in protein-protein interactions. It has been shown that the zinc fingers of GATA-1 can interact with zinc finger domains of Krüppel family proteins, such as Sp1 and EKLF (6). It is now also clear that the N-finger of GATA-1 is able to specifically recognize at least one zinc finger in FOG. This finger, FOG-finger 6, has some similarity to the Krüppel-like fingers but coordinates zinc in a CCHC rather than a CCHH configuration, and as a lone finger it lacks the TGEKP interfinger link characteristic of Krüppel subfamily members. Having localized the site within the N-finger of GATA-1 that is recognized by FOG, it will be interesting to see whether the fingers of Sp1 and EKLF bind to analogous faces within the C-finger of GATA-1. Experiments are also in progress to determine whether FOG-finger 6 is the only FOG finger that can

TABLE III

Sequences of the N- and C-terminal zinc fingers of GATA-1, -2, and -3 from different species

The shaded boxes indicate the key residues for FOG binding, conserved in N-fingers, but not C-fingers.

		Interaction with FOG
mGATA-1 Nf	E A R E C V N C G A T A T P L W R R D R T G H Y L C N A C G L Y	+
hGATA-2 Nf	E G R E C V N C G A T A T P L W R R D G T G H Y L C N A C G F Y	+
hGATA-3 Nf	E G R E C V N C G A T S T P L W R R D G T G H Y L C N A C G L Y	+
mGATA-1 Cf	A G T Q C T N C Q T T T T T L W R R N A S G D P V C N A C G L Y	
hGATA-2 Cf	A G T C C A N C Q T T T T T L W R R N A N G D P V C N A C G L Y	
hGATA-3 Cf	A G T S C A N C Q T T T T T L W R R N A N G D P V C N A C G L Y	

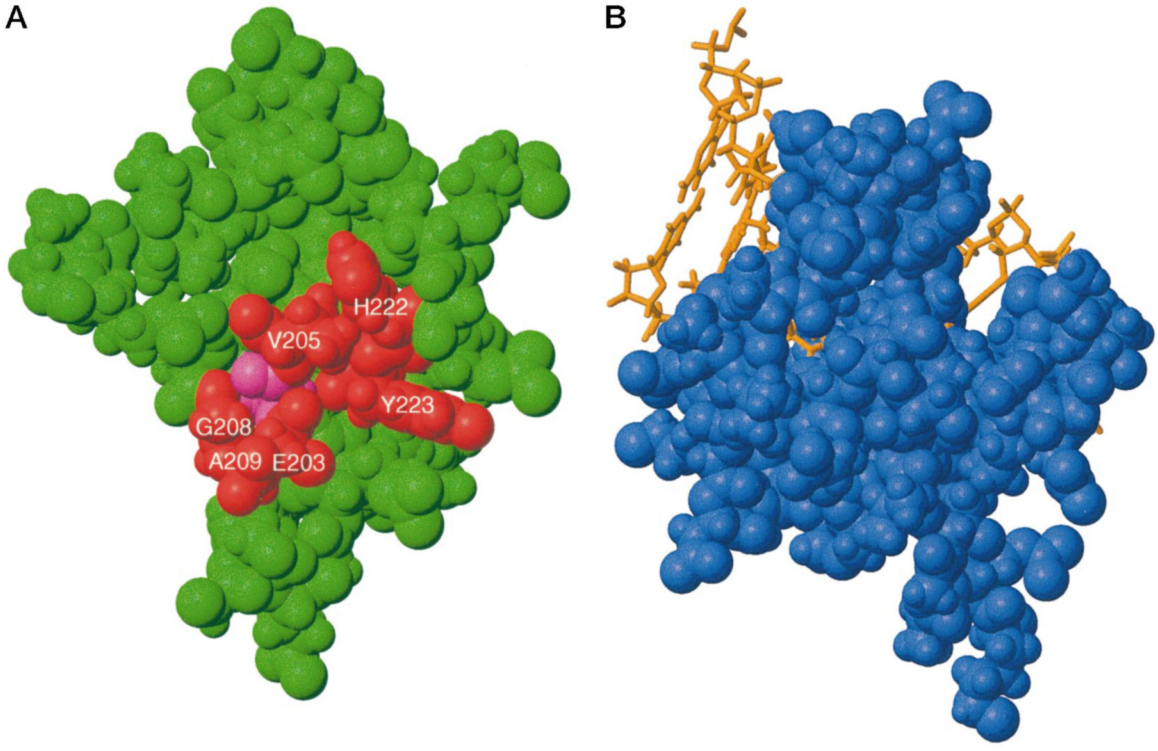

FIG. 8. **Critical residues in the N-finger of GATA-1 required for its interaction with FOG.** *A,* a space filling model of the N-finger of murine GATA-1 (including residues 200 to 245) with residues implicated in FOG binding (Glu^{203}, Val^{205}, Gly^{208}, Ala^{209}, His^{222}, and Tyr^{223}) shown in *red* and Cys^{204} shown in *pink*, other residues shown in *green*. *B,* a similarly oriented model of the corresponding region of the C-finger of chicken GATA-1 bound to DNA (10).

interact with GATA-1.

Implications for the Mechanism of GATA-1 Action—Although the N-finger domain of GATA-1 does not bind DNA in isolation, there is evidence that it makes contacts with DNA when presented at (A/T)GATA(A/G) motifs together with the C-finger (37, 49, 50). Moreover, the N-finger domains of the related proteins, GATA-2 and GATA-3, are able to bind (A/T)-GATA(A/G) motifs, even in the absence of their respective C-fingers (51). It is now known that the overall structure of the N-finger of GATA-1 is essentially identical to that of the C-finger (58) (Fig. 8, *A* and *B*) and it is reasonable to predict that the two fingers will contact DNA by means of analogous surfaces. It is therefore of interest to consider the relative locations of the FOG-binding face and the likely DNA-binding face of the N-finger structure. The recently solved structure of the N-finger of murine GATA-1 (residues 200–243) is shown in Fig. 8*A*, with the residues implicated in contacting FOG shown in *red*. In Fig. 8*B*, the DNA bound solution structure of the C-finger of chicken GATA-1 is shown in a similar orientation. The residues implicated in FOG binding clearly form an accessible surface opposite the DNA. This observation suggests that FOG is unlikely to inhibit DNA binding by GATA-1 and is consistent with the finding that FOG fusion proteins can activate DNA-bound GATA-1 in yeast one-hybrid experiments and that FOG and GATA-1 synergistically activate the p45 NF-E2 promoter (Ref. 7 and Fig. 9*A*). Interestingly, the FOG-related protein, U-shaped, appears to antagonize the activity of its partner, Pannier. From our results we would predict that it does this by contacting and repressing the action of DNA-bound Pannier, rather than by interfering with the DNA binding activity of Pannier. To date, however, attempts to directly visualize the GATA·FOG or Pannier·U-shaped complexes bound to DNA in gel retardation experiments have not been successful (33).

The proposal that FOG interacts with DNA-bound GATA-1 has important implications concerning the function of the complex. It has been suggested that FOG, like GATA-1, may also use its zinc fingers to bind DNA (7), although this remains uncertain (35). If FOG does bind DNA then there are a number of ways in which it could significantly influence GATA-1 activity. First, it might act to direct GATA-1 to bipartite FOG/GATA motifs within particular promoters or enhancers, and in this way the FOG/GATA combination might activate a subset of

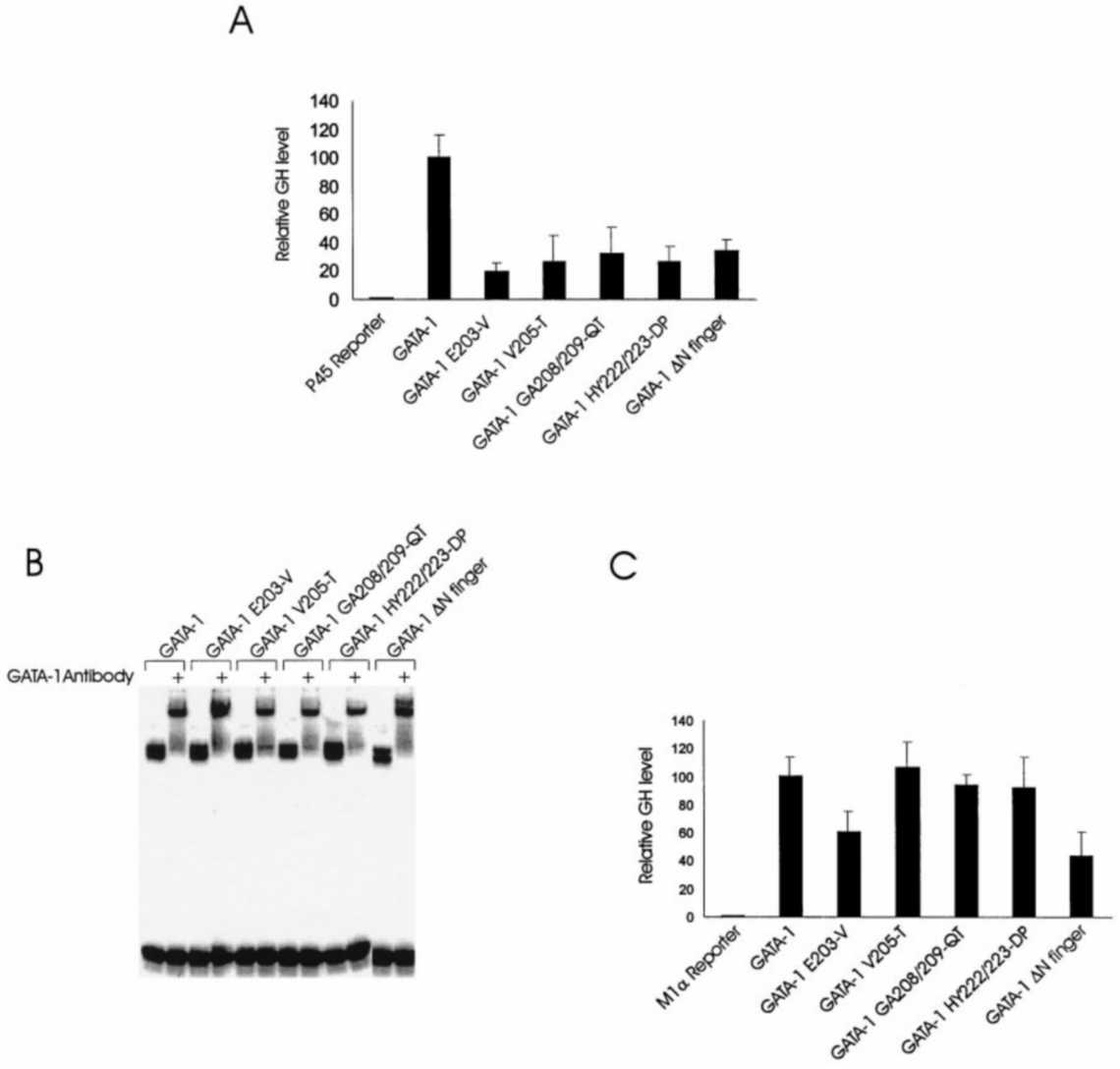

FIG. 9. **Specific mutations in the N finger of GATA-1 affect its capacity to cooperate with FOG to activate the p45 NF-E2 promoter but do not significantly impair its DNA-binding or transactivation ability.** *A,* NIH3T3 cells were transfected with the p45GH reporter alone (column 1), or together with expression plasmids encoding FOG and GATA-1, GATA-1 mutants, or GATA-1 ΔN-finger (columns 2–7). *B,* a gel shift assay was performed with a double-stranded oligonucleotide containing the mouse α-globin GATA site and nuclear extracts from COS cells expressing wild type GATA-1 protein, GATA-1 mutants, or GATA-1 ΔN-finger. Anti-GATA-1 antibody was added to the samples in each alternate lane as indicated. *C,* NIH3T3 cells were transfected with the M1αGH reporter plasmid alone (column 1), or together with expression plasmids encoding GATA-1, GATA-1 mutants, or GATA-1 ΔN-finger (columns 2–7).

genes containing both FOG and GATA-1-binding sites. Such a situation would be reminiscent of the complex formed between the E-box-binding proteins SCL/E12 and GATA-1, which recognizes composite E-box/GATA motifs (52), or the complex of Bob and Oct-1, which recognizes an extended octamer motif (53). Alternatively, it is possible that long range interactions between FOG and GATA-1 bound at widely spaced sites could mediate contact between enhancers and promoters or between separate enhancer elements. It has previously been suggested that protein-protein contacts between GATA-1 and Sp1/EKLF, and also GATA-1 self-association, may play similar roles (5, 6). Interestingly, it has been proposed that the four hypersensitive sites within the β-globin locus control region come together as a holocomplex and then loop directly to the globin gene promoters (54). It will be worthwhile to investigate whether FOG and GATA-1 contacts play a role in this process.

Different Fingers, Different Functions—Fungal GATA factors typically have only a single zinc finger, which appears analogous to the GATA-1 C-finger and mediates binding to (T/A)GATA(A/G) motifs in DNA (55). It is of interest that GATA factors from higher organisms typically have two fingers. Numerous explanations have been proposed for the purpose of the additional finger and it now appears likely that it plays important roles in both protein-DNA and protein-protein interactions. The duplication of the zinc finger domain may have increased the opportunities for GATA factors of complex organisms to participate in multiple simultaneous interactions, beyond the capacity of single-finger proteins.

Zinc finger genes are plentiful in organisms from yeast to man. It is currently thought that although classical CCHH fingers adopt similar overall structures, variations in their precise amino acid sequences result in the ability to recognize different DNA elements. Knowledge of the key amino acids involved in DNA recognition has enabled the prediction of the DNA motifs recognized by newly discovered finger proteins (56) and has allowed the selection and design of artificial zinc fingers with tailored specificity (57). The various GATA fingers are also likely to adopt related structures but to date all appear to recognize canonical GATA elements in DNA. The particular protein-protein contacts made by different fingers are likely to

be more difficult to predict, but it is again probable that they will reflect subtle differences in key amino acids, rather than major structural differences between fingers. As discussed here, both the N- and C-fingers of GATA-1 have similar structures, but by virtue of a small number of distinct amino acids they make very different protein contacts. Defining the exact topology of these finger-finger interactions will be a first step toward predicting which GATA fingers will interact with which FOG-like fingers, and ultimately to designing or selecting synthetic fingers with desired functions. An understanding of the fine molecular detail and structure of the complexes will also help in the generation of reagents to explore the mechanisms by which GATA-1 conspires with other proteins to regulate erythroid gene expression.

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