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Biochemistry

A small single-"finger" peptide from the erythroid transcription factor GATA-1 binds specifically to DNA as a zinc or iron complex

(zinc finger/globin gene regulation)

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ABSTRACT **Sequence-specific DNA binding has been demonstrated for a synthetic peptide comprising only one of the two "finger"-like domains of the erythroid transcription factor GATA-1 (also termed Eryf-1, NF-E1, or GF-1). Quantitative analysis of gel-retardation assays yields a specific association constant of 1.2×10^8 M, compared with values of about 10^9 M for the full-length natural GATA-1 protein. By the use of peptides of various lengths, it was possible to delineate the smallest region necessary for specific binding. A single C-terminal finger of the double-finger motif is necessary but not sufficient for sequence-specific interaction. Basic amino acids located C-terminal to the finger (some more than 20 amino acids away) are also essential for tight binding. In addition to demonstrating that zinc is important for the formation of an active binding complex, we show that other ions, notably Fe^{2+}, can fulfill this role. Our results make it clear that the GATA-1 metal binding motif is quite distinct from that found in the steroid hormone family and that GATA-1 is a member of a separate class of DNA binding proteins.**

The erythroid transcription factor GATA-1 was the first identified (1–4) member of a distinct family of "finger"-motif DNA binding proteins, which now includes regulatory proteins expressed in other cell lineages (GATA-2, -3, and -4; refs. 5 and 6 and T.E., unpublished data) and in a wide variety of organisms. The GATA-1 protein of chickens, mice, humans, and other vertebrates is found in erythroid-lineage cells (1–4) and in some other human and mouse hematopoietic lineages (7, 8). Binding sites for GATA-1 are found in the cis-regulatory elements of all globin genes (9–13) and most other erythroid-specific genes that have been examined (14–19). Chicken GATA-1 binds (1, 20) as a 35-kDa monomer to an asymmetric DNA target sequence, (A/T)GATA(A/G). The chicken α^D-globin (21), the human γ-globin (22, 23), and the mouse GATA-1 promoters (24) contain slightly higher-affinity sites consisting of two copies of the core consensus sequence, both contacted by a single molecule of GATA-1. The protein (1–4) has two related but nonidentical finger elements of the form Cys-Xaa-Xaa-Cys-$(Xaa)_{17}$-Cys-Xaa-Xaa-Cys, reminiscent of the pair of motifs found in the steroid hormone receptor superfamily (25), but quite different in amino acid sequence, in the number of residues in the motifs, and in the spacing between them. Unlike other such proteins, multiple fingers are not essential to binding; the binding properties of GATA-1 are only weakly affected by deletion or mutation of the N-terminal finger (23). Furthermore, certain fungi [*Saccharomyces cerevisiae* (26, 27), *Aspergillus nidulans* (28), *Neurospora crassa* (29)] contain trans-acting factors that have only a single finger, which is more closely related in sequence to the C-terminal finger of vertebrate GATA-1. It therefore appeared likely that this finger alone might exhibit specific binding behavior. We synthesized a 66-amino acid peptide containing the C-terminal finger domain and found that it binds tightly and specifically to the GATA target sequence. Quantitative binding studies have been carried out with this peptide and with truncated versions of the peptide that delineate the domain essential to binding. The DNA binding activity requires stoichiometric quantities of Zn^{2+}, but other heavy metals can be substituted. We find that the addition of Fe^{2+} in place of Zn^{2+} results in a peptide–metal complex that binds somewhat better than the zinc compound. This raises the tantalizing possibility that some transcription factors of the GATA-1 family may use iron to stabilize the active structure.

METHODS

Peptide Synthesis. Assembly of peptide chains was accomplished using solid-phase synthesis procedures on an Applied Biosystems model 430A automated synthesizer. The following side-chain protecting groups were used: tosyl (Arg), cyclohexyl (Glu, Asp), formyl (Trp), benzyl (Ser, Thr), 4-chlorocarbobenzoxy (Lys), 2-bromocarbobenzoxy (Tyr), *N*-benzyloxymethyl (His), and 4-methylbenzyl (Cys). Cleavage of the side-chain protecting groups and removal from the resin were accomplished by low–high HF cleavage (30). The cleaved peptide was extracted from the resin with 5% (vol/vol) aqueous acetic acid containing 1 mM dithiothreitol. The peptide solution was concentrated, applied to a Sephadex G-50 column, and eluted with the same solution. Fractions containing the peptide were pooled, concentrated, and further purified by reverse-phase HPLC on a Vydac C_8 column, with a water–acetonitrile solvent gradient in 0.05% trifluoroacetic acid. Purity of the peptides was determined by HPLC (93% or greater), amino acid analysis, and peptide sequencing.

Gel-Mobility-Shift Titrations of GATA Peptides. Peptides were dissolved in solvents containing 0.05% trifluoroacetic acid at 5–10 pmol/μl, as determined by ultraviolet absorption spectroscopy using an estimated molar extinction at 280 nm of 12,100. The solvent also contained various amounts of zinc acetate, ferrous chloride, ferric chloride, manganese chloride, cobalt chloride, or cadmium acetate, typically at molar ratios to peptide of 1.5:5. For Fe^{2+}, this solvent also contained 1 mM 2-mercaptoethanol. Although the yield of active (binding) peptide varied (see figures), there was no obvious correlation with this ratio. Samples of the peptide dissolved in 40 μl of the trifluoroacetic acid/metal ion solvent were adjusted in pH by the stepwise addition of four 5-μl aliquots of 75 mM Tris·HCl (pH 7.5). In some cases nitrogen was bubbled through the solutions before they were mixed.

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Abbreviation: GR, glucocorticoid receptor.

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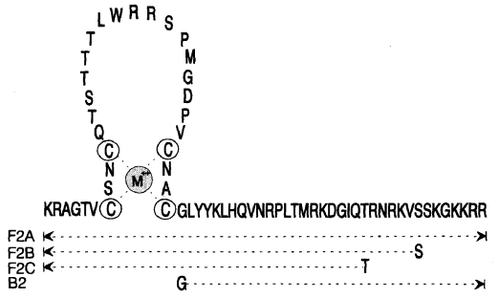

FIG. 1. Peptides used in these studies are derived from the C-terminal finger domain and adjacent basic region of the chicken GATA-1 (*Eryf1*) gene (1). M, metal.

All data including gel images shown in the figures were obtained with a Molecular Dynamics PhosphorImager; all numerical values were obtained by direct computer analysis of the stored images.

RESULTS

The properties of the C-terminal finger of GATA-1 were explored using the 66-amino acid peptide (F2A) containing the four cysteine residues and extending 35 amino acids beyond this cluster in the C-terminal direction (Fig. 1). Gel-mobility-shift assays were used to measure the interaction between this peptide and an oligonucleotide duplex containing a single GATA motif, derived from one of the GATA-1 binding sites in the chicken β/ε-globin enhancer (31). As shown in Fig. 2*A*, the peptide interacted strongly with its target DNA to produce a single complex. Quantitative analysis of the binding data revealed an apparent association constant of 1.2×10^8 M (Table 1). A typical Scatchard plot is shown in Fig. 3. In comparison, binding studies of crude nuclear extracts from 9-day embryonic erythrocytes gave values of about 10^9 M for the affinity of full-length chicken GATA-1 for this sequence (data not shown). To determine the binding affinity for DNA sequences from which the GATA motif is absent, we measured binding to the labeled GATA oligonucleotide in the presence of an excess of unlabeled nonspecific competitor carrying either the binding motif of the transcription factor Sp1 (32) or a mutated GATA-1 binding site (data not shown). We also directly measured the binding to a labeled oligomer carrying the Sp1 site. We estimate that the affinity of F2A for the nonspecific sequences is about three orders of magnitude weaker (moles of nonspecific site vs. moles of specific site) than its affinity for the GATA sequence.

Table 1. Association constants of F2A peptide with specific DNA as a function of heavy metal ion

Metal ion	Apparent association constant, M
Zn^{2+}	$1.20 \pm 0.25 \times 10^8$ (3)
Fe^{2+}	$2.18 \pm 0.48 \times 10^8$ (3)
Co^{2+}	$1.20 \pm 0.25 \times 10^8$ (2)
Cd^{2+}	$0.63 \pm 0.09 \times 10^8$ (2)
Mn^{2+}	—

Means ± standard deviations are shown for the number of titrations given in parentheses.

Provided that the peptide was prepared in the absence of Zn^{2+} and other heavy metals, it did not bind to DNA (Fig. 2*A*). Addition of Zn^{2+} resulted in formation of a peptide–metal complex that bound. CD and NMR studies (data not shown) with this peptide demonstrated that it existed as a random coil in the absence of Zn but formed a single ordered structure when one equivalent of Zn was added. Although we have reported (1) difficulty in demonstrating a similar metal ion dependence with full-length GATA-1, we have more recently been able to remove and add back (unpublished data) the metal ion in such a way (33) as to reconstitute some activity.

The gel-shift assay is easily adapted to the measurement of the Zn/peptide ratio. F2A was reconstituted in the presence of ^{65}Zn, and the labeled peptide was mixed either with unlabeled specific DNA probe or with the same concentration of probe containing ^{32}P label. Parallel gel-mobility-shift data were obtained with both probes. The results (Table 2) show that each mole of complex carries almost exactly one mole of zinc, as expected for an asymmetric site binding a single F2A molecule. This result is entirely consistent with the earlier observation (1) that GATA-1 itself binds as a monomer.

To determine whether other heavy metal ions could substitute for zinc to form a structure capable of specific binding,

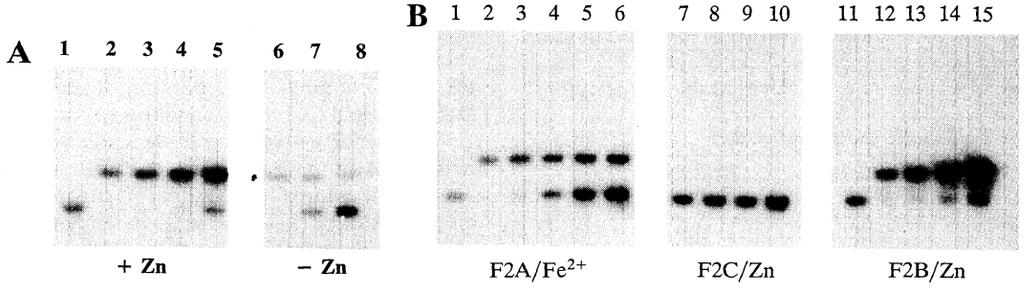

FIG. 2. Gel-mobility-shift titrations of GATA peptides. (*A*) Lanes 1–5 show titration of peptide F2A, reconstituted with Zn^{2+}, with a 5′-^{32}P-end-labeled synthetic oligonucleotide duplex containing the sequence of oligo A/B (see Fig. 4) derived from the chicken β/ε-globin enhancer. Of the two adjacent sites in the enhancer, this binds full-length GATA-1 more strongly. All titrations in this figure were carried out with this oligonucleotide. Lanes: 1, oligo A/B only; 2–5, each sample contained, in a volume of 10 μl, 2.7 pmol of peptide and 0.21, 0.42, 0.84, or 1.68 pmol of duplex, respectively. The solvent contained (final concentration) 50 mM Tris·HCl (pH 7), 10 mM NaCl, 3.7% (vol/vol) Ficoll, 0.0125% Triton X-100, and 50 ng of poly(dI-dC). This small amount of polynucleotide serves to prevent nonspecific binding to oligo A/B when peptide is in excess. It does not appear to have a significant effect on measured apparent binding constants. At this concentration, a nonspecific DNA would uniformly reduce apparent binding constants about 2-fold. Lanes 6–8 show peptide F2A treated identically to sample used in lanes 1–5, except that no metal ion was added; 2.7 pmol of peptide and 0.21, 0.42, or 0.84 pmol of DNA were present in lanes 6–8, respectively. The small amount of complex observed probably arises from unavoidable contamination with metal ions during preparation of peptide. (*B*) Gel-shift assays. Conditions were similar to those described in *A*. Lanes: 1, oligo A/B DNA only; 2–6, 2.7 pmol of F2A/Fe^{2+} with 0.22, 0.44, 0.87, 1.31, or 1.74 pmol, respectively, of oligo A/B; 7–10, peptide F2C/Zn, showing absence of binding for this truncated peptide; 7–9, 0.87 pmol of oligo A/B DNA and 2.7, 5.4, or 16.2 pmol, respectively, of F2C/Zn; 10, 1.74 pmol of oligo A/B and 5.4 pmol of F2C/Zn; 11, oligo A/B only; 12–15, 3.3 pmol of peptide F2B/Zn with 0.21, 0.43, 0.86, or 1.72 pmol of oligo A/B, respectively. Metal ion/peptide ratio, 4.9; reaction volume, 10 μl in all cases.

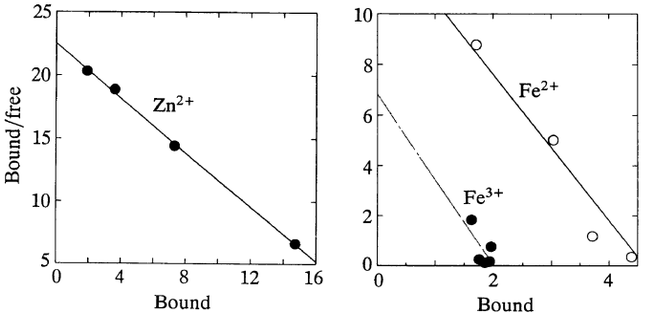

FIG. 3. Scatchard analysis of gel-shift binding data shown in Fig. 2*A*. Titrations were performed with a fixed amount of peptide and various amounts of probe. The ratio of bound to free DNA sequence A/B is plotted vs. the molar concentration of bound sequence A/B in the reaction mixture ($\times 10^8$). (*Left*) F2A/Zn peptide. (*Right*) F2A–Fe^{2+} and F2A–Fe^{3+} peptides. A summary of numerical results for several such experiments is given in Table 1. The concentration of peptide capable of binding, determined from such graphs, can be compared with the total peptide concentration measured by optical absorbance (see above). The efficiency of reconstitution was about 75% with Zn^{2+} and 17–47% with Fe^{2+}.

we introduced Co^{2+}, Cd^{2+}, Mn^{2+}, Fe^{2+}, and Fe^{3+} ions into peptide F2A with the same reconstitution methods employed to make the zinc complex. As shown in Table 1, Mn^{2+} was ineffective in generating binding activity, but addition of cobalt or cadmium ion produced actively binding peptide–metal ion complexes with affinity constants the same as or somewhat smaller than that obtained with the zinc complex. The reconstitutions with ferrous and ferric ion are of particular interest. As shown in Fig. 2*B*, addition of ferrous ion produced a peptide capable of binding to the specific DNA probe with an apparent affinity constant (Table 1) that was somewhat greater than that of the zinc or cobalt complexes and more than twice as great as that of the cadmium compound. Introduction of ferric ion also resulted in a species that bound with this affinity, but the amount of active complex formed was smaller (Fig. 3 *Right*). It is likely that the active complex binds ferrous ion. Under the acidic conditions used in the reconstitution, ferric ion solutions also contained some ferrous ion, but Fe^{3+} itself was probably inactive.

The ability of F2A to bind DNA specifically depends both upon the zinc-binding domain and on the highly basic C-terminal tail, as predicted by mutagenesis studies (34) with intact GATA-1. A peptide containing only the tail (peptide B2, Fig. 1) bound to the GATA-1 site to form only nonspecific

Table 2. Zn/DNA ratio in gel-shifted complexes

Zn, pmol	DNA duplex, pmol	Zn/DNA molar ratio
2.65	2.50	1.06
2.86	3.00	0.95

Gel-shift experiments like those in Fig. 2 were carried out as follows. Lanes: 1 and 2, 3.37 pmol of unlabeled oligo A/B DNA and 0.13 pmol of 5′-^{32}P-end-labeled oligo A/B; 3 and 4, DNA as in lanes 1 and 2, but 10.9 and 16.4 pmol of F2A/^{65}Zn peptide was added, respectively; 5 and 6, 3.5 pmol of unlabeled DNA in each and 10.9 and 16.4 pmol, respectively, of F2A/^{65}Zn. Peptide samples were prepared as in Fig. 2*A*, except that the zinc chloride/trifluoroacetic acid solution used to dissolve the peptide sample contained 9×10^{-4} mCi of ^{65}Zn (Amersham) lyophilized from a 0.1 M HCl solution. Conditions were similar to those in Fig. 2*A*, except that 200 ng of poly(dI·dC) was added to each reaction mixture. Zinc ion concentrations in the complex were obtained by comparison with standards. DNA concentration in the complex was determined from the fraction of all ^{32}P found in the complex and the known concentration of DNA. (In samples containing high ^{32}P and ^{65}Zn, the contribution of ^{65}Zn to the radioactive signal is only 2% of the total.)

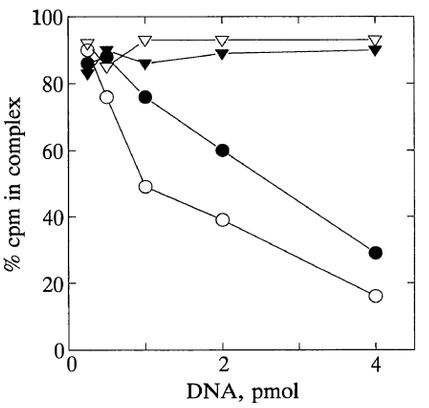

FIG. 4. Competition of oligo A/B with other oligomers for binding to F2A/Zn. ^{32}P-labeled oligo A/B was mixed with a 40-fold molar excess of unlabeled oligomer. Aliquots containing 0.5 pmol of peptide F2A/Zn (measured as binding activity rather than total concentration) were titrated with increasing amounts of the oligomer mixture. The percentage of labeled (specific) DNA in complex is plotted vs. total pmol of DNA present in a volume of 10 μl. The unlabeled oligomers used were as follows: ○, oligo A/B; ●, oligo C/D, containing the more weakly binding GATA sequence from the chicken β/ε-globin enhancer (see text); ▼, possible GATA-family binding site for the yeast Dal80 protein (36); ▽, oligo ABM, which is A/B that has been mutated at the GATA binding site. Oligomer duplexes used in these studies were as follows: the binding site motif is underlined and mutated bases are in lowercase type: A/B, 5′-AGCTTCGGTTGCAGATAAACATTGAATTCA; C/D, 5′-AGCTTCGAGTCTTGATAGCAAAAGAATTCA; ABM, 5′-AGCTTCGGTTGCActgAAACATTGAATTCA; Dal80, 5′-ATTTAAACTGAAAATGATAGTCTGCGCGGCA.

aggregates, as expected for electrostatic interactions at low ionic strength (data not shown). A second peptide, F2C (residues 1–53), from which 13 amino acids in the C-terminal tail were deleted (Fig. 1) showed no evidence of any binding (Fig. 2*B*), despite the fact that NMR and CD studies revealed that F2C was able to bind zinc and form a structure closely related to that observed with the F2A peptide (data not shown). However, a peptide slightly longer than F2C [F2B (residues 1–59)] bound to GATA sequences with an affinity comparable to that of the full-length peptide (Fig. 2*B*). These results suggest that the six additional residues (residues 54–59 of F2A) either contact DNA or are important for stabilization of residues nearby that contact DNA. The exceedingly basic region (KGKKRR) at the C terminus of F2A was not needed for binding; it has been suggested that this may be a nuclear localization signal (35).

We have also used the F2A peptide to study interactions with variant or mutated DNA binding sites. The chicken β/ε enhancer contains two binding sites for GATA-1, of unequal binding affinity (31). The sequence contained in the stronger of these (oligo A/B) was used in the experiments described above. Experiments that measure the competition of the two sites for binding to peptide F2A are shown in Fig. 4. The peptide retained the binding preference observed in the full-length protein; analysis of the data revealed that the stronger site (oligo A/B) bound somewhat more tightly than the weaker one (oligo C/D). We have used two other DNA sequences as competitors (Fig. 4). One of these (ABM) was a mutated version of the strong GATA-1 binding site. Under these conditions, as expected, no significant competition was detected. The second sequence was a possible binding site (36) for the yeast GATA-family protein Dal80. Both chicken GATA-1 and the F2A peptide bound to this sequence only weakly. The F2B peptide showed similar binding specificity to these oligonucleotides but appeared to have a relatively higher affinity than did F2A for nonspecific sequences (data

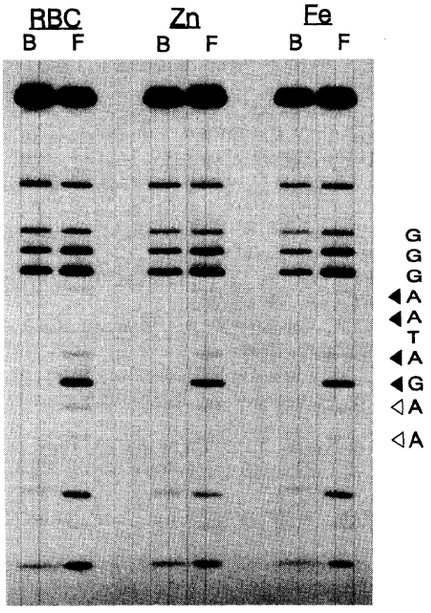

FIG. 5. Duplex probe (E/F) 5′-AGCTTCGCAGCAA-GATAAGGGCTGAATTCA was partially methylated with dimethyl sulfate (41) and used in a gel-mobility assay exactly as described above, except that the reaction was scaled up 5-fold. At the 50% titration point, bound (B) and free (F) complexes were eluted from the gel, and the DNA in each sample was purified and cleaved with piperidine (37) prior to electrophoresis on a 15% polyacrylamide gel. Arrows indicate strong (solid) and weaker (open) sites of interference. RBC, eryrothcytes.

not shown). Thus much of the information for sequence discrimination is retained in the 59-amino acid F2B peptide.

To examine further the specificity of the interaction between the GATA peptides and their binding site, we studied the effect of DNA methylation on binding. The methylation interference patterns of the complexes are shown in Fig. 5. The patterns are identical whether full-length GATA-1 or peptide F2A complexed with either zinc or iron was used. This shows that either metal ion is capable of organizing the peptide structure to give a DNA binding domain that makes the same contacts with DNA as the native erythroid protein.

DISCUSSION

It is instructive to compare the known properties of the glucocorticoid receptor (GR) (38) with those of the GATA peptides and GATA-1 itself. Marked differences are obvious. In the GR, both the regions containing the zinc fingers are required to stabilize and orient the DNA-binding α-helical region, permitting specific contacts within the recognition half-sites. Thus the two finger motifs of the GR are part of a single structural domain that binds DNA. High binding specificity is further achieved by dimerization of the protein, which brings each monomer into the proper orientation for recognition of its half-site on the DNA. The region containing the C-terminal finger of the GR has a predominant role in establishing the protein–protein contacts that stabilize the dimer.

In contrast (Figs. 2–4), the single-finger GATA-1 peptide F2B alone contains the information necessary for strong specific binding; our data suggest that this peptide is close to the minimum size necessary for the binding function. Mutagenesis of full-length mouse or chicken GATA-1, which contains two finger regions, has shown that the region containing the C-terminal finger is of central importance for strong binding to a single asymmetric GATA site. When appropriate double sites are present, methylation interference assays reveal that the N-terminal finger protects one of the binding motifs (23) but makes only a small contribution to binding properties detected by the effect of its deletion on the dissociation rate constant (23, 34). The data presented here show that both the metal-binding region and basic portions of the peptide are required for strong selective binding; we note that five of the six amino acids at the C terminus of peptide F2B are conserved in all vertebrate GATA proteins. As shown by our data (Fig. 2*B*), these residues (amino acids 54–59) distant from the metal binding motif are also crucial for DNA binding. The metal complex region of F2A or F2B may serve to stabilize and orient a domain that also carries residues that interact specifically with DNA.

The properties of Zn^{2+} complexes and the known structure of such proteins as the GR make it probable that the cysteine sulfhydryl groups of GATA-1 are arranged tetrahedrally about the central metal ion. The ability of GATA-1 and the single-finger peptide F2A to form an active complex in the presence of Fe^{2+} is consistent with the known physical properties of that ion. Fe^{2+} is able to form both octahedral low-spin, and tetrahedral high-spin complexes. It is reasonable to suppose that in this case the four sulfur atoms of the finger are disposed tetrahedrally around Fe^{2+}. An example of such a configuration is found in rubredoxin, an iron protein with the sequence Cys-Xaa-Xaa-Cys-$(Xaa)_{29}$-Cys-Xaa-Xaa-Cys; the cysteine residues are placed tetrahedrally around iron (39–41). Rubredoxin is not a DNA binding protein but is thought to function in bacterial electron transport. We do not yet know whether the members of the vertebrate GATA-1 family are zinc or iron proteins *in vivo*; definitive determination of the state of mouse, human, or chicken GATA-1 *in vivo* awaits the availability of large quantities of quite pure GATA-1. These proteins function in the iron-rich erythroid intracellular environment, which by itself is not necessarily an argument for the presence of iron in the vertebrate factors. On the other hand, it is quite possible that the GATA-1 family of proteins may have evolved from early iron binding factors. Some support for this proposal can be derived from the observation that globin-like genes of primitive organisms are often involved in electron transport functions, and it may be that the early regulatory factors controlling the expression of such proteins also contained iron. A vestige of this system might remain in the GATA-family regulatory factor URBS-1, found in *Ustilago*. URBS-1 is involved in the regulation of synthesis of siderophores (S. Leong, personal communication), compounds that tightly complex Fe^{3+} and are involved in iron transport and homeostasis. Whether these and other GATA-family proteins of primitive organisms ever form functional iron complexes must now be determined.

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