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A Palindromic Regulatory Site within Vertebrate GATA-1 Promoters Requires Both Zinc Fingers of the GATA-1 DNA-Binding Domain for High-Affinity Interaction

CECELIA D. TRAINOR,[1] JAMES G. OMICHINSKI,[2] THOMAS L. VANDERGON,[1]†
ANGELA M. GRONENBORN,[2] G. MARIUS CLORE,[2] AND GARY FELSENFELD[1]*

Laboratory of Molecular Biology[1] and Laboratory of Chemical Physics,[2] National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892

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GATA-1, a transcription factor essential for the development of the erythroid lineage, contains two adjacent highly conserved zinc finger motifs. The carboxy-terminal finger is necessary and sufficient for specific binding to the consensus GATA recognition sequence: mutant proteins containing only the amino-terminal finger do not bind. Here we identify a DNA sequence (GATApal) for which the GATA-1 amino-terminal finger makes a critical contribution to the strength of binding. The site occurs in the GATA-1 gene promoters of chickens, mice, and humans but occurs very infrequently in other vertebrate genes known to be regulated by GATA proteins. GATApal is a palindromic site composed of one complete [(A/T)GATA(A/G)] and one partial (GAT) canonical motif. Deletion of the partial motif changes the site to a normal GATA site and also reduces by as much as eightfold the activity of the GATA-1 promoter in an erythroid precursor cell. We propose that GATApal is important for positive regulation of GATA-1 expression in erythroid cells.

The erythroid transcription factor GATA-1 is the prototype of a family of DNA-binding proteins, the GATA factors. The protein was identified as a positive regulator of globin gene transcription. Later it was shown that GATA-1 is involved in the regulation of all erythroid cell-specific genes which have been examined; it stimulates transcription through GATA binding motifs that occur in the *cis*-regulatory regions of these genes. Six GATA family members have been identified in vertebrates (3, 11, 14, 33, 38). Three of these (GATA-1 to -3) are required for normal hematopoietic development in mice (24, 25, 31). These factors all have a zinc finger of the form Cys-X_2-Cys-X_{17}-Cys-X_2-Cys; most members bind to a recognition sequence of the form (A/T)GATA(A/G) (5). Vertebrate GATA factors contain two adjacent highly related fingers, while fungal proteins have a single finger (6, 13), more similar to the C-terminal vertebrate finger (C-f).

Mutagenesis studies of mouse GATA-1 (mGATA-1) and chicken GATA-1 (cGATA-1) showed that proteins lacking the amino-terminal finger (N-f) could bind to DNA and transactivate reporter genes containing GATA binding sites, while proteins lacking C-f were completely inactive (16, 39). Chimeric proteins containing GATA transactivation domains linked to C-f of GATA-1, or the single finger of a fungal GATA protein, were shown to be as effective as wild-type GATA-1 in rescuing erythroid development in an embryoid-body assay (27). No critical role for the N-terminal finger has been defined. However, N-f has been reported to stabilize binding to a group of promoters of erythroid cell-expressed genes which contain clusters of GATA sites.

To examine the role of N-f, we focused on these promoters. In the mGATA-1 promoter a double GATA element, consisting of a major and a minor site on opposite strands of DNA 3 bp apart and binding a single molecule of GATA-1, was found (Fig. 1). Methylation interference assays demonstrated protein-DNA contacts to both sites, with contacts in the 5′ site predominating (34). In the cGATA-1 promoter, three consensus motifs arranged as a palindromic site (referred to here as sites 1a and 1b) and a single site (site 2) 4 bp away were identified (Fig. 1) (8). While these sites bind only two molecules of GATA-1, methylation interference assays showed that bases in all three sites are important to the interaction (26). These studies established a role for N-f in DNA binding, but the distinct spacing and number of sites in the two promoters did not support a mode of interaction common to both promoters.

Other promoters which interact with N-f have also been identified. The chicken α-D-globin promoter (α D) (Fig. 1) has two overlapping GATA sites in the same orientation, which bind a single molecule of protein. A protein lacking N-f has an increased rate of dissociation from this site relative to the rate for the wild-type protein, suggesting a role for N-f in stabilizing binding (39). The human ^{A}γ-globin promoter (hγ) (Fig. 1) contains two consensus GATA sites 8 bp apart which have been defined as a bipartite site and reported to bind a single molecule of GATA-1 (16).

Thus, while N-f is not required, it can participate in DNA binding. Because of the diversity of these promoter elements and because the previous experiments were done with crude nuclear extracts or bacterial extracts containing recombinant proteins, it is not possible to define the requirements for N-f interaction from these studies. The sequence similarity between N-f and C-f strongly suggests that N-f should be able to recognize a GATA motif. In addition, the high degree of conservation of N-f throughout vertebrate evolution indicates that N-f likely plays some crucial role in GATA function.

Here we define GATApal as a site that is conserved in three GATA-1 promoters and that requires both N-f and C-f to achieve full stability of binding. GATA-1 interactions with this site are at least an order of magnitude stronger than with a single GATA-1 finger or single sites. GATApal consists of one

* Corresponding author. Phone: (301) 496-4173. Fax: (301) 496-0201.
† Present address: Natural Sciences Division, Pepperdine University, Malibu, CA 90263.

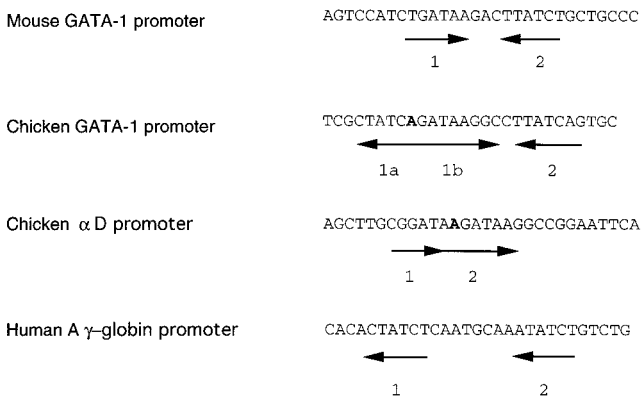

FIG. 1. GATA binding sites used in this study. Boldface letters indicate bases shared by two overlapping sites.

full and one partial GATA motif in a partially overlapping palindromic orientation and occurs very infrequently in vertebrate GATA sites. In the mGATA-1 promoter, reported to have two separate (5′ and 3′) GATA binding sequences, the 5′ copy is contiguous with the additional sequence that forms the partial palindrome. It is this extended motif, not the combination of the separate 5′ and 3′ sequences previously described as the double site (34), that is responsible for the high-affinity binding. The palindrome is also found within sites 1a and 1b of the cGATA-1 promoter, but we show that only part of site 1a is required. In addition, we have confirmed the observation that N-f is involved in binding to the GATA sites in the chicken α D promoter and the hγ promoter.

Thus, GATA-1 can discriminate between distinct GATA sites through the N-f domain and the stability of its protein-DNA interactions is site specific. We further demonstrate that the palindromic GATA binding site confers a markedly increased activity on the GATA-1 promoter in comparison with the activity conferred by a normal site. Our observations suggest a mechanism through which GATA-1 can differentially activate the many different genes that it regulates and suggest that N-f may have a special role in the autoregulation of the GATA-1 gene.

MATERIALS AND METHODS

Plasmid construction. The expression clone producing the two zinc fingers of human GATA-1 (hGATA-1) was made by PCR from an hGATA-1 cDNA clone (30). The 5′ primer had an *Nco*I site at the 5′ end, and this resulted in the addition of an extra alanine to the protein sequence. The 3′ primer had an in-frame stop codon and a *Bam*HI site at the 5′ end. The clone contains the coding sequence for amino acids 200 to 317 of hGATA-1. The sequences of the primers are as follows: 5′ primer, 5′ CCATGGCTGAGGCCAGGGAGTGTG TGAACTGC; 3′ primer, 5′ GGATCCTACCGTTTCTTTTTCCCTTTTCCAGA. The PCR product was cloned into the TA cloning vector from Invitrogen and sequenced from the vector primer sites. The plasmid was digested with *Nco*I and *Bam*HI, and the resulting fragment was inserted into the *Nco*I *Bam*HI sites of the pET 11D plasmid from Novagen for direct expression in bacteria.

The plasmid containing the cGATA-1 promoter with the mutant GATA site 1a was also made by PCR. The cGATA-1 promoter was inserted into M13 at the *Hin*dIII-to-*Xba*I sites. The primer listed in Fig. 3 as cGATA-1 M1a,2 and the M13 universal primer were used in the PCR. The PCR product was cloned into the TA cloning vector and sequenced. The plasmid was digested with *Hin*dIII and *Stu*I, and this fragment, which contains only GATA site 1a/1b of the cluster, was cloned into the *Hin*dIII-*Stu*I sites of GATP1. GATP1 is a plasmid containing the cGATA-1 promoter in pCAT basic at the *Hin*dIII-*Xba*I sites (8).

Protein purification. The expression and purification of SF (the C-terminal zinc finger of GATA-1) have been described previously (21), and similar methods were used for DF (double zinc finger). The PET 11D clone containing the coding sequence for DF was introduced into the BL21(DE3) strain of *Escherichia coli*. The bacteria were grown overnight at 37°C in Luria broth (LB) and induced with 5 mM isopropyl-β-D-thiogalactoside at 37°C for 4 h. Harvested cells were resuspended in 50 mM Tris (pH 8)–5 mM EDTA–5 mM benzamidine–5 mM dithiothreitol (DTT) and lysed with a French press. The lysate was centrifuged at 100,000 × *g* for 1 h, and the supernatant was applied to a DEAE-Sepharose Fast Flow (Pharmacia) column (bed volume, 200 ml) equilibrated with buffer A (50 mM Tris [pH 8], 5 mM EDTA, and 5 mM DTT). The flowthrough was applied to an S-Sepharose Fast Flow (Pharmacia) column. DF was eluted with a 0 to 1 M NaCl gradient in buffer A. The appropriate fractions were purified with a C_4 reversed-phase (Vydac) high-performance column with a 100% acetonitrile gradient in 0.05% (vol/vol) aqueous trifluoroacetic acid. DF was characterized by amino acid analysis and amino-terminal sequencing. DF was reconstituted with zinc by the same procedure previously used for SF.

The amino acid sequence of SF, in single-letter code, is KRAGTVCSNCQT STTTLWRRSPMGDPVCNACGLYYKLHQVNRPLTMRKDGIQTRNRKVS SKGKKRR. The amino acid sequence of DF is AEARECVNCGATATPLW RRDRTGHYLCNACGLYHKMNGQNRPLIRPKKRLIVSKRAGTQCTNC QTTTTLWRRNASGDPVCNACGLYYKLHQVNRPLTMRKDGIQTRNR KGSGKGKKRR.

Gel mobility shift assays. Assays with the peptides SF and DF were as follows. The sample buffer was 50 mM Tris (pH 7)–0.125% Nonidet P-40 (NP-40)–3.2% Ficoll–2 mM EDTA–20 μg of poly(dI-dC) per ml. Protein and DNA concentrations are given in the figure legends. Samples were electrophoresed on 8% polyacrylamide gels in 10 mM Tris–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–1 mM EDTA at 120 V.

Preparation of nuclear extract was as previously described (2). Gel mobility shift assays with nuclear extracts were as follows. Nuclear extracts and end-labeled DNA probe were mixed in 50 mM Tris (pH 7.5)–100 mM KCl–3 mM $MgCl_2$–1 mM $CaCl_2$–1 mM DTT–10 μg of bovine serum albumin (BSA) per ml–0.125% Triton–4% Ficoll–0.05% bromophenol blue–0.05% xylene cyanol–1 μg of poly(dI-dC) per sample. Samples were electrophoresed on 8% acrylamide gels in 10 mM HEPES–10 mM Tris–1 mM EDTA at 200 v. Gels were dried prior to autoradiography.

Oligonucleotides were either synthesized with an Applied Biosystems synthesizer or purchased from Operon Technologies Inc. All oligonucleotides were gel purified. The sequences of most oligonucleotides are given in Fig. 3. The sequence of the oligonucleotide containing the GATA site in the β/ε-globin enhancer element (β/εE) and a mutated version of it are as follows: β/εE, AGCT TCGGTTGCAGATAAACATTGAATTCA; β/εE M, AGCTTCGGTTGCAct gAAACATTGAATTCA. The consensus GATA site is underlined, and lowercase letters indicate altered bases.

Dissociation rate assays were quantitated with a Molecular Dynamics PhosphorImager. The percentage of the complex remaining relative to the amount seen immediately after the addition of competitor was calculated. Dissociation rate constants and dissociation half times were determined for the high-affinity interactions, but reactions with SF and mutant probes were usually completed by the first time point and consequently the 50% dissociation times indicated are actually upper limits. The values for DF and the GATApal motif ranged from 70 to 140 min, while values for DF and mutant probes or SF and all probes were always less than 8 min. The values for a representative experiment are presented in Fig. 3. The dissociation rate assays were not done under identical conditions; protein and DNA concentrations, temperatures, and times varied and are indicated in the figure legends.

Binding affinities were determined by quantitating free and bound probe on gels similar to those shown in Fig. 2B with a PhosphorImager. Saturation curves were calculated and Scatchard analysis was performed with these data.

Cell culture and transfections. HD24 cells were grown in blastoderm media (18) at 37°C in 5% CO_2. Transfections were performed with Lipofectamine (Bethesda Research Laboratories) as previously described (29). *cat* activity was determined by a liquid scintillation counting method (19).

BLAST sequence analysis. Database sequence searches were performed by using the BLAST network service from the National Center for Biotechnology Information (NCBI). Search templates were the 10-base site 1a/1b of the cGATA-1 promoter (8) with 1 or 3 bases flanking each side and the analogous region of the mGATA-1 promoter (34). BLAST network searches were performed on the nr (nonredundant) database (daily compilation of PDB, GBUpdate, GenBank, EMBLUpdate, and EMBL) by using a word size of 11, a single-pass file search strategy, and an HSP cutoff of 50 (1). Each file match was analyzed to determine that the match was in a known or potential regulatory region of a vertebrate gene.

BLAST network searches in the nr database resulted in 308 and 710 matches for the 12- and 16-base chicken site 1 templates, respectively. Perfect matches to the site ATCAGATAAG were found in potential regulatory regions of only one vertebrate gene, the mouse calbindin D28K promoter. The 12- and 16-base mouse double-site templates resulted in 306 and 817 matches, respectively. Perfect matches to the site ATCTGATAAG were identified in potential regulatory sequences of six vertebrate genes: the site was found in the upstream regions of five genes (the human and chimpanzee ζ-globin genes, the human CD40 gene, the human nerve growth factor β gene, and the human gap-1 gene) and in the rabbit β-globin locus control region (LCR).

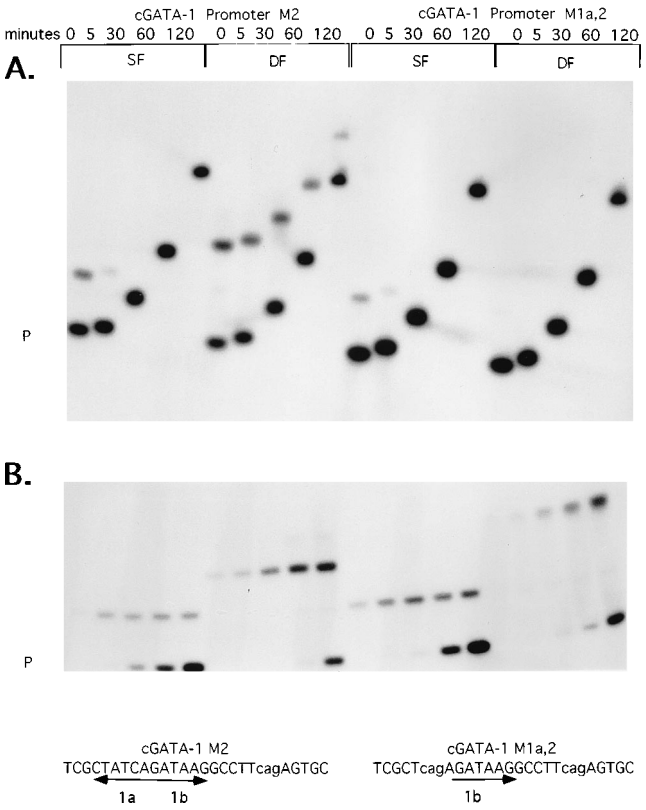

FIG. 2. GATA-1 SF and DF have distinct binding properties which are site dependent. (A) Dissociation rate assay. Complexes of SF and DF with either the M2 site from the cGATA-1 promoter or the M1a,2 mutation of this site were formed during a 15-min incubation at room temperature (0.2 pmol of peptide and 0.3 pmol of probe). A 100-fold excess of unlabeled oligonucleotide was added to the reaction mix. Time point samples were taken at 0, 5, 30, 60, and 120 min after addition of the competitor and electrophoresed immediately on the same gel; samples from the early time points were consequently electrophoresed much longer than were those from the later time points. The DF-M1a,2 complex is not visible in panel A but is shown in panel B. P indicates the position of the free probe in the 0 time point sample. (B) Increasing amounts of labeled M2 or M1a,2 probe (0.12 to 2 pmol) were added to 1.5 pmol of SF or DF. P indicates the position of the free probe. In other similar experiments used for Scatchard analysis, probe quantities varied from 0.12 to 8 pmol and protein quantities varied from 1 to 16 pmol in 10-μl reaction volumes.

RESULTS

To analyze the participation of N-f in DNA binding, we compared the binding properties of the previously described single-finger peptide (SF) which represents the C-terminal finger of cGATA-1 (22), a double-finger peptide (DF), and full-length GATA-1 on a series of well-characterized GATA binding sites. A clone expressing both finger domains of hGATA-1 was constructed in pET 11D and used to generate DF for these studies. Nuclear extracts from chicken embryonic erythrocytes or K562 cells were used as a source of full-length c- and hGATA-1.

GATA-1 N-f interacts with the cGATA-1 promoter. The cGATA-1 promoter contains multiple GATA binding sites which have been shown to be critical to the expression of the gene in erythroid cells. Among these is the cluster of three sites mentioned above which occurs at positions −155 to −124 relative to the transcription initiation site. A truncated promoter lacking these sites is completely inactive in erythroid cells (although active in fibroblasts) (26). Oligonucleotides containing the wild-type sites or mutations were used as probes in gel mobility shift assays to compare the binding properties of SF and DF. Figure 2A shows the dissociation rates of SF and DF complexes on cGATA-1 promoter sites 1a and 1b (cGATA-1 M2) and on a probe mutated to contain only site 1b (cGATA-1 M1a,2). (The sequences of these probes are presented in Fig. 2 and 3). An excess of unlabeled competitor was added to assembled complexes; aliquots of the reaction mixture were sampled at various times after this addition and analyzed with a gel mobility shift assay. While the dissociation rates of SF are similar with these two probes, DF dissociates greater than an order of magnitude more slowly than SF from the M2 probe. The DF complex was 50% dissociated at 70 min, while both SF complexes were more than 50% dissociated at 5 min (Fig. 3 gives a summary of these data). Mutation of site 1a increases the rate of dissociation of DF but not of SF. Dissociation of DF from this mutated site (M1a,2) is so fast that no complex is visible at the first time point, but the complex can be seen in the titration in Fig. 2B. Thus, two consensus binding sites and two fingers generate a very stable DF-DNA complex on the cGATA-1 promoter, which implicates N-f in complex formation. Multiple titrations of a fixed amount of SF or DF with these probes (similar to those shown in Fig. 2B) were performed to estimate the relative binding constants of these reactions. Scatchard analysis of such data indicates that while SF has similar (two- to threefold variation) affinities for the two probes, the binding affinity of DF with the M2 probe was about 20-fold higher than it was with the M1a,2 probe (data not shown). In addition, the mobility of the M2-DF complex was faster than that seen with the identically sized M1a,2 probe (Fig. 2B), suggesting that the complexes have different conformations. Complexes with slow mobility similar to that of DF-M1a,2 form between DF and other probes containing a single GATA site (data not shown). Such mobility changes can be the result of protein binding in distinct positions on the probe, or of the protein interacting with the DNA in distinct manners. The mobility of the SF complex was the same with both probes. We conclude that the faster-mobility complex with DF on cGATA-1 M2 reflects binding of N-f to site 1a, while C-f interacts with site 1b. Sites 1a and 1b are not equivalent, since no complexes form between DF and a probe in which only site 1b is mutated (data not shown).

To assess the relevance of these observations, the dissociation rate of full-length GATA-1 with these two probes was examined (Fig. 4). K562 and chicken embryonic erythroid cell nuclear extracts were used as sources of hGATA-1 and cGATA-1. Full-length GATA-1 proteins from both species are similar to DF in that both form more stable complexes with site 1a/1b (M2) than with a probe in which site 1a is mutated (M1a,2). All complexes with the M2 probe are visible at 90 min. In contrast, all M1a,2-DF complexes are reduced 90% after 5 min. In addition, recombinant GATA-1 expressed in avian fibroblasts and cGATA-1 in erythroid precursor cell extracts show dissociation rates which differ 10-fold with these two probes (data not shown). These data implicate N-f as the cause of the stabilization on site 1a/1b and indicate that N-f plays a role in the interaction of GATA-1 with its own promoter.

The m- and hGATA-1 promoters can also interact with N-f. If this interaction between N-f and the cGATA-1 promoter is critical in the regulation of the gene, it would likely be conserved in the promoters of the gene in other species, but no similar site was noted in previous studies. The mGATA-1 promoter was reported to contain two GATA sites, a major site (5′) and a minor site (3′) 3 bp apart (Fig. 1); mutation of the major site reduced promoter activity in MEL cells by 75%, while minor-site mutation reduced the activity 25% (34). No

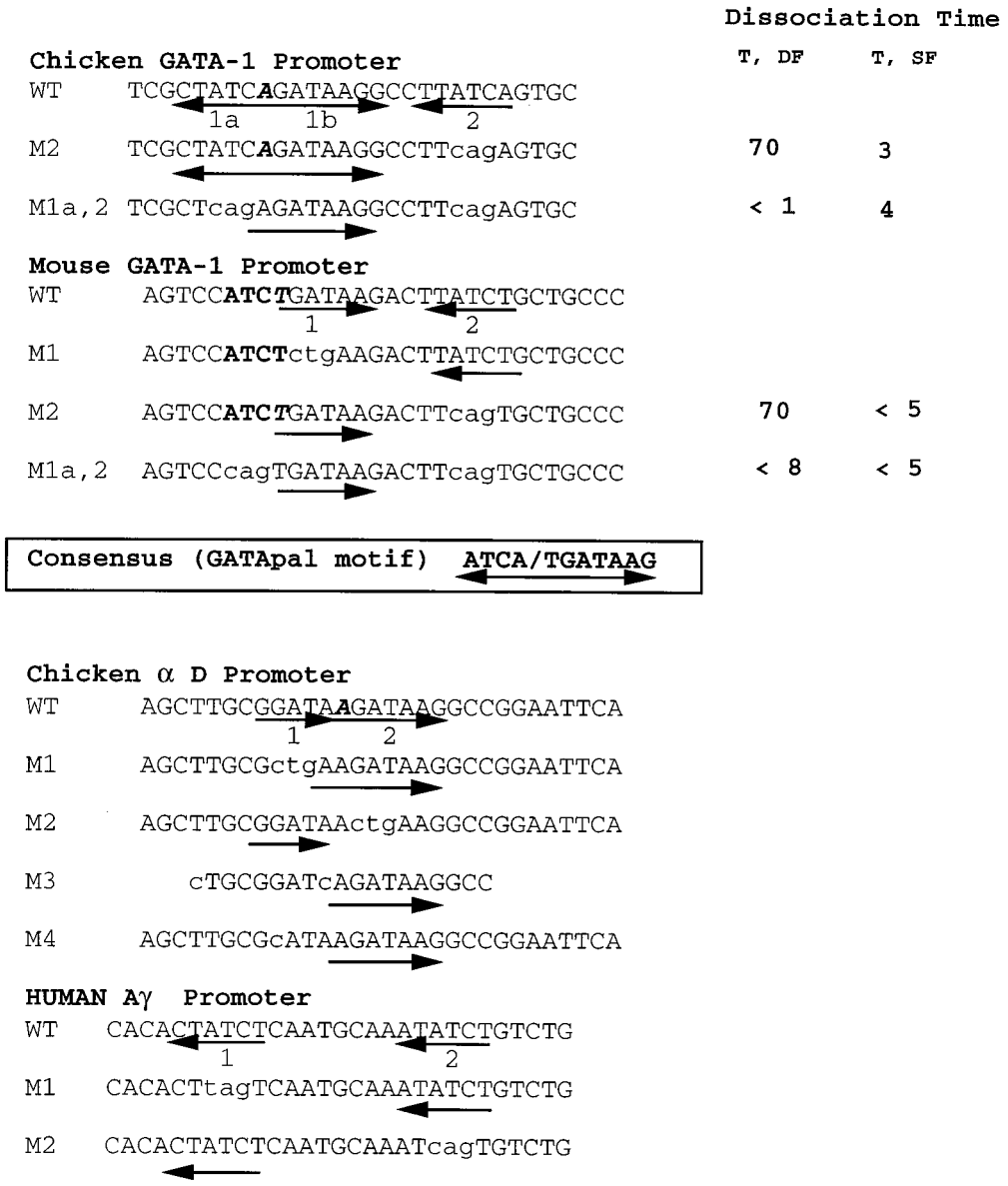

FIG. 3. Dissociation rates of SF and DF with GATA binding sites used in this study. Arrows indicate consensus GATA recognition sequences, (A/T)GATA(A/G). Individual sites are numbered. Mutations are indicated with an M, followed by the number of the site which has been changed. Additional mutations to the same sites are designated M and given an arbitrary number. Italicized, boldface letters indicate bases which are shared by two sites. Boldface letters indicate the partial site discovered in the mouse promoter. T refers to the time in minutes at which 50% of the indicated complex has dissociated.

palindromic sequences analogous to chicken site 1a/1b were identified in these studies. We noted, however, that a partial palindromic site does exist in the mouse promoter at the 5′ major site. It is similar to site 1a/1b of the chicken promoter, except that the 5′ nucleotide of chicken site 1 is not conserved (Fig. 3, boldface nucleotides). This suggested that the interaction of N-f with chicken site 1 might require not a complete site 1a but only the bases conserved between it and the mouse promoter. To test this hypothesis and to confirm the specificity of these interactions, we used a competition assay to assess the relative affinities of DF with the mGATA-1 sites. Oligonucleotide probes containing the mouse promoter wild-type sites or only the 5′ partial palindromic site (M2) were used in binding assays with SF and DF in the presence of various cold competitors. SF binding to the wild-type site (Fig. 5A, first four lanes) was completely inhibited by competition with an excess of an oligonucleotide containing a single GATA motif from the chicken β/ε-globin enhancer element (lane 2) (5) and by the 5′ site from the mGATA-1 promoter (lane 3) but was not inhibited by competition with an excess of a β/εE oligonucleotide in which the GATA site was mutated (lane 4), showing that, as expected, SF interacts specifically with the GATA sites in the mGATA-1 promoter. However, when DF was tested with the same probe and competitors (Fig. 5A, first series of DF), the β/εE single site (lane 2) was a less effective competitor than mGATA-1 M2 (lane 3), indicating a higher-affinity interaction of DF with mGATA-1 M2 than with the β/εE single site. As we predicted, the same results were obtained with DF when mGATA-1 M2 was used as the probe (Fig. 5A, last four lanes); the minor site previously identified as part of the double

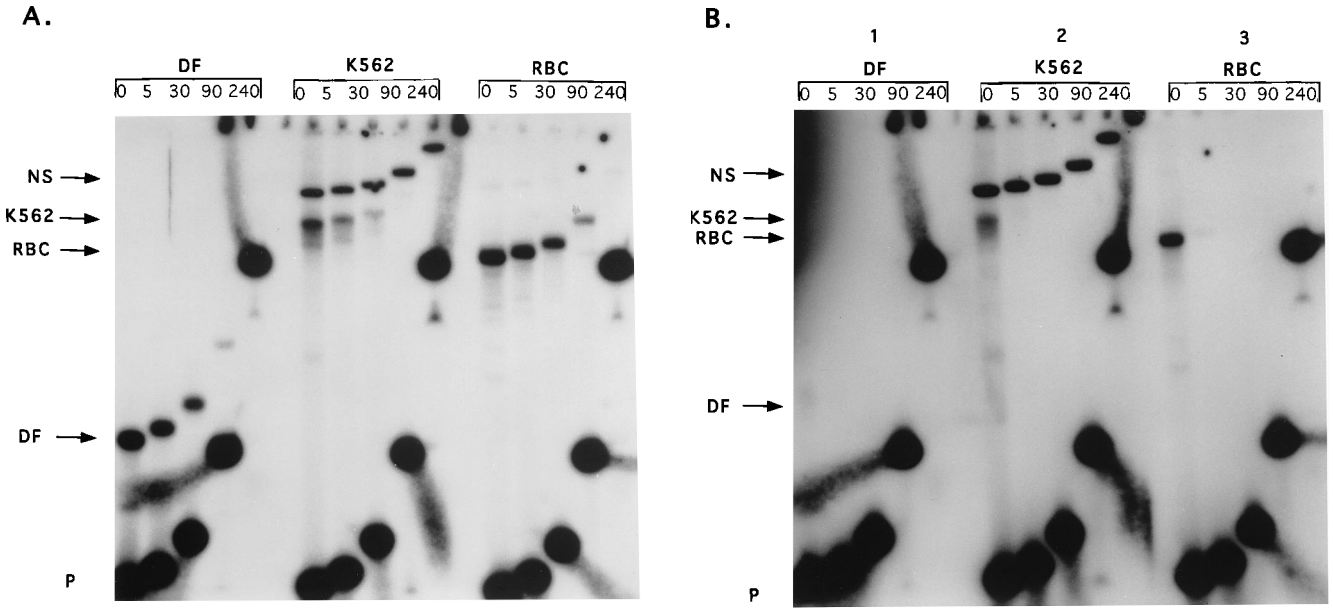

FIG. 4. Full-length GATA-1 also dissociates more slowly from the cGATA-1 M2 probe. (A) Complexes were formed with 1 pmol of cGATA-1 M2 probe and either DF (0.2 pmol) or nuclear extract from K562 cells (which contain hGATA-1) or 9-day chicken embryonic erythrocytes (RBC) (containing cGATA-1) during a 15-min incubation on ice. A 100-fold excess of unlabeled competitor was added, and samples were taken at 0, 5, 30, 90, and 240 min after this addition (as indicated above the lanes) and immediately electrophoresed on the same gel. (B) The cGATA-1 M1a,2 probe was used in an identical experiment. The arrows mark the positions of the protein-DNA complexes at time 0. NS indicates a nonspecific complex which forms with both of these probes and K562 nuclear extract. P marks the position of the free probe at time 0. Large amounts of labeled probe were used to ensure probe excess, and this led to smearing of the probe in several places.

site is not involved in this interaction. Under these conditions, there is no interaction between DF and a probe in which site 1 is mutated (mGATA-1 M1), indicating that the 5′ partial site (ATC) and the 3′ minor site are not sufficient for high-affinity DF interaction (data not shown).

The distinct behaviors of SF and DF with these competitors establish a role for N-f in binding to the mGATA-1 probe, and this N-f interaction requires only the 5′ partial palindromic site 1, not the 3′ minor site. We then compared directly the dissociation rates of SF and DF complexes with the β/εE and mGATA-1 M2 sites (Fig. 5B). Little (<5%) dissociation of a DF-M2 complex had occurred by 50 min, while SF-M2 was 50% dissociated by 5 min. SF and DF have similar fast rates of dissociation (more than 50% dissociation in 5 min) from the β/εE probe.

To confirm that the stable DF-DNA complex was dependent on an interaction between N-f and the inverted partial GATA site, we mutated the analog of chicken promoter site 1a in the mouse promoter (ATCTGATAAG to cagTGATAAG [lowercase letters indicate mutated nucleotides]) (Fig. 3, M1a,2) and tested SF and DF binding. Results were similar to those shown in Fig. 2A: all complexes were more than 50% dissociated after 5 min (data not shown).

Thus, N-f participates in the binding of GATA-1 to the mouse as well as the chicken promoter and, by analogy, to the hGATA-1 promoter in which these overlapping full and partial sites are also completely conserved (20, 42). The predicted consensus site for DF binding to the GATA-1 promoters is ATC(A/T)GATAAG. This consensus sequence will be referred to as the GATApal motif.

Most GATA sites do not contain the GATApal motif. To determine the distribution of the GATApal motif, we performed BLAST sequence analysis. For the vertebrate genome, the search located the GATApal motif in the three GATA-1 promoters mentioned above and did not find this element in the promoters of the other vertebrate GATA genes (GATA-2 and -3, for which sequences are available). GATApal was not found in the *cis*-regulatory elements of most erythroid cell-expressed genes or nonerythroid genes thought to be regulated by GATA proteins. It does occur in the promoter of the human ζ-globin gene in a consensus GATA site shown to be important for gene expression (36, 40), and it is conserved in the chimpanzee ζ-globin gene. The GATApal sequence was found in hypersensitive site 2 (HS2) of the rabbit β-globin LCR, in an area not shown to bind GATA-1, and the motif is not conserved in HS2 of other species (9). It was also found in the *Galago crassicaudatus* β-globin locus, between the epsilon and gamma genes in a region not known to have regulatory function. It was located in four other promoters or upstream regions of vertebrate genes not known to be regulated by GATA factors (human CD40, human gap-1, mouse calbindin D28K, and the human nerve growth factor β gene).

A very similar motif (ATCAGATATG) was found in the promoters of two GATA family genes of *Caenorhabditis elegans*, *elt-1* and *elt-2*; a regulatory role for these sites has not been established (10, 28). This restricted distribution suggests a special role for N-f and GATApal in the regulation of the GATA-1 gene, and perhaps in the ζ-globin gene as well. Both of these genes are expressed early in the erythroid lineage.

The chicken α D promoter also interacts with N-f, but through a different DNA motif. We next examined the interaction of DF with the double site in the α-D-globin promoter. This is composed of a 3′ major site required for binding and an overlapping direct-repeat minor site which increases the affinity of GATA-1 for this DNA; mutation of the 5′ site reduces the response of the promoter to coexpressed GATA-1 (4). Four concentrations of DF were tested with the wild-type α D probe or a probe in which the minor site was mutated (α D M1) (Fig. 3). As shown in Fig. 6A, two distinct complexes form on the wild-type probe but only the more slowly migrating

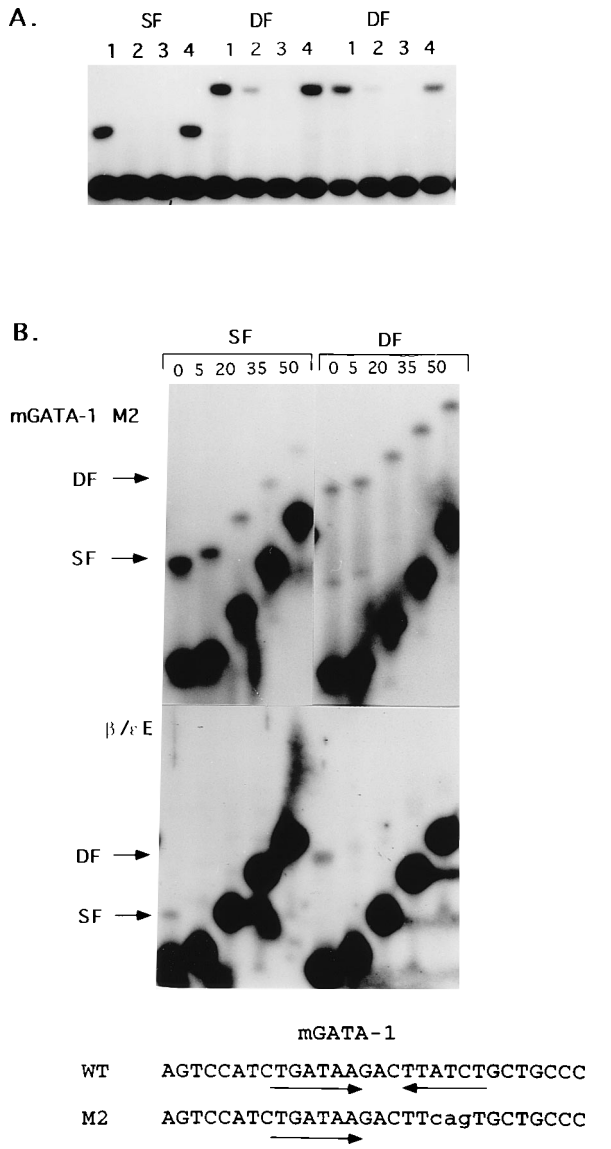

FIG. 5. N-f interacts with an analogous GATA binding site in the mGATA-1 and hGATA-1 promoters. (A) Competition assay. The first eight lanes contain 0.05 pmol of oligonucleotide containing the wild-type mGATA-1 double sites and 0.2 pmol of SF (first four lanes) or DF (next four lanes). In the last four lanes, 0.2 pmol of DF and 0.05 pmol of probe with only the 5′ site (M2) were used (second series of DF). In all cases, lane 1 has no competitor. A 50-fold excess of an oligonucleotide containing a GATA site from the chicken β/ε enhancer element (β/εE, lanes 2), the major site from the mGATA-1 promoter (M2, lanes 3), or the β/ε enhancer element in which the GATA site is mutated (β/εEM, lanes 4) was added. It appears that there is slight competition between the β/εEM competitor and the M2 probe, but additional experiments with this competitor and other nonspecific competitors indicate that this is because less protein was inadvertantly added to that sample. Sequences of the β/εE-related oligonucleotides are given in Materials and Methods. (B) Dissociation rate assay. SF and DF (0.2 pmol) were complexed on ice to 0.5 pmol of probe containing the 5′ GATA site from the mGATA-1 promoter (M2) or 1 pmol of the β/ε enhancer element GATA site. A 20-fold excess of unlabeled homologous competitor was added after complex formation, and aliquots of the reaction mixture were electrophoresed at 0, 5, 20, 35, and 50 min (as indicated above the lanes) after competitor addition on the same gel. Smearing of the probe occurred because large amounts were used to ensure probe excess during complex formation. Arrows indicate the positions of the complexes at the 0 time point. WT, wild type.

complex forms with the single-site α D probe (M1). Only one specific complex is visible with SF and either of these probes (data not shown), as with all overlapping-site probes used in this study. Thus it appears that DF can associate with the α D GATA site in two different ways. The 5′ GATA site and N-f are required for formation of the additional complex. No complex between DF and a probe in which site 2 is mutated is observed (Fig. 3, α D M2, and data not shown).

To further characterize the nature of these interactions, dissociation rate analysis was performed with the α D-DF complexes and shows that the faster-mobility N-f-requiring complex is the more stable one (Fig. 6B); the more slowly migrating complex is the result of DF association with the probe through C-f and site 2. The full-length GATA-1 appears to form only a single complex with the wild-type α D probe (data not shown). This suggests that regions of the protein not present in DF may restrict the movement of N-f and keep it in a position such that only the more stable interaction occurs. Titration experiments were performed, and Scatchard analysis of these shows that DF has eightfold higher affinity for GATApal than for the α D motif (data not shown). Thus, we have confirmed that N-f also interacts with the chicken α D promoter but does so through a DNA motif distinct from GATApal and with reduced binding energy relative to that for the GATApal motif.

Mutation of the α D GATA site confirms the consensus for the GATApal motif. The α D site is an overlapping direct repeat, while the GATApal motif is a palindrome. Comparison of these sites reveals that alteration of a single base converts the α D site to the GATApal motif defined above (Fig. 3, M3). When an α D-derived oligonucleotide containing this substitution was tested in the dissociation rate assay, a single fast-mobility stable complex was formed with DF while a complex which dissociates more than 50% by 1 min formed with SF (Fig. 7). This result confirms the GATApal motif: restoration of the palindromic GATA site results in a more stable interaction with DF than occurs with the directly repeated sites.

Orientations of N-f and C-f complexed to DNA. The three-dimensional structure of C-f on a single GATA motif has been determined by nuclear magnetic resonance (NMR) spectroscopy (21). Residues in the tip of C-f contact the G, while the adjacent helix 3′ contacts the ATA. If N-f binds to GATA in a similar manner, then N-f must be able to contact DNA in two different orientations relative to that of C-f, since DF binds to direct and palindromic repeats. The α D M3 mutation described above shows that the C in the GATApal motif is critical to high-affinity complex formation, strongly suggesting that the palindrome is critical. If the lower-affinity interaction of N-f with the α D direct repeat requires the entire 5′ GATA motif, this would suggest a binding orientation of N-f different from that seen with GATApal. Alternatively, if N-f binding requires only the ATA sequence at the center of the 5′ GATA site, either orientation of N-f would be possible on the direct repeat. To test this, we mutated the G in the 5′ site (Fig. 3, α D M4) to see if it was required for stable complex formation. Two complexes still form with the M4 site, as with the wild-type site (Fig. 8), but they are both less stable than the fast-mobility complex on the wild-type α D site (data not shown). The N-f-requiring α D complexes migrate in the same position as do DF and GATApal (Fig. 8). Titrations of DF with the α D and α D M4 probes show that the M4 mutation causes a fourfold drop in the binding affinity. In contrast to results with the wild-type α D probe, equivalent amounts of the two complexes form on the M4 probe at high DNA concentrations (Fig. 8); the fast-migrating complex is no longer so highly favored. Thus, the G of the 5′ GATA site is involved in the N-f inter-

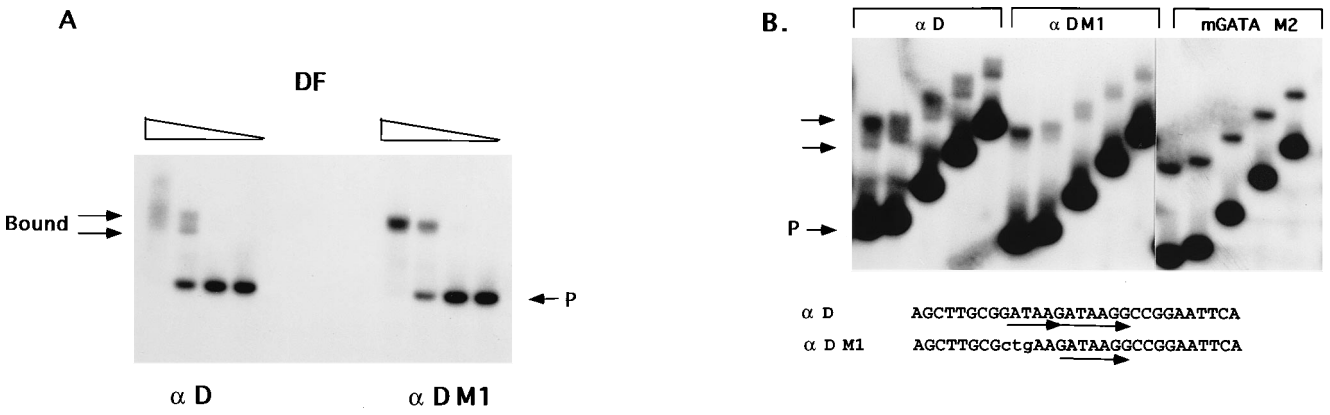

FIG. 6. N-f interacts with the GATA site in the chicken α D promoter. (A) End-labeled oligonucleotides containing the double site in the α D promoter or a probe in which the minor site was mutated (α D M1) were incubated with 20, 2, 0.2, and 0.02 pmol of DF at room temperature and electrophoresed on a native acrylamide gel. Bound and free (P) counts are indicated. (B) Dissociation rate assay. Complexes between 0.5 pmol of the three probes indicated and 0.2 pmol of DF were formed during a 15-min incubation on ice. Homologous unlabeled competitors were added, and samples were electrophoresed on the same gel at 0.5, 20, 35, and 50 min after this addition (second through fifth lanes in each set, respectively). Arrows indicate the positions of the complexes formed with DF on the α D probe. P indicates the free probe.

action. The α D M1 mutation (Fig. 6) showed that bases within the ATA are also involved. This makes it quite likely that N-f recognizes GATA as does C-f and implies that N-f can be oriented in two directions relative to that of C-f, depending on the orientation of the binding site.

The interaction of DF with the human ^{A}γ-globin promoter shows N-f-dependent binding to a third DNA motif. The bipartite site from the human ^{A}γ-globin promoter (16) also forms a high-affinity fast-migrating complex with DF implying N-f involvement (data not shown), and Scatchard analysis shows that this interaction is only slightly weaker (twofold) than the DF-GATApal interaction (Fig. 9). Unlike what was observed with GATApal and α D, there are no overlapping GATA sites in the hγ probe and both independent binding sites are required for the high-affinity interaction (Fig. 9). The hγ promoter represents a third motif to which N-f can bind. The 8-bp separation between the sites requires that a structure different from that formed with GATApal and α D form. Only DF (not other proteins or other parts of GATA-1) is required to generate this complex.

Mutation of the GATApal motif in the cGATA-1 promoter reduces promoter activity. To determine whether the interaction of N-f with the GATA-1 promoter can influence promoter activity, the cGATA-1 promoter was modified by site-directed mutagenesis to destroy site 1a by converting it to the sequence in the oligonucleotide cGATA-1 M1a,2 (see Materials and Methods). A restriction fragment containing only the mutated site 1a and normal 1b was then introduced into the promoter. Thus, only site 1a was altered. Plasmids containing the wild-type (GATP1) and mutant (GATP M1a) promoters driving the *cat* gene were used in transient-transfection assays in an immature hematopoietic cell line, HD24 (18). These cells produce GATA-1 and GATA-2 and represent precursors of the erythroid and myeloid lineages (7). Titrations were performed to test the promoters at different protein-to-DNA ratios. Since

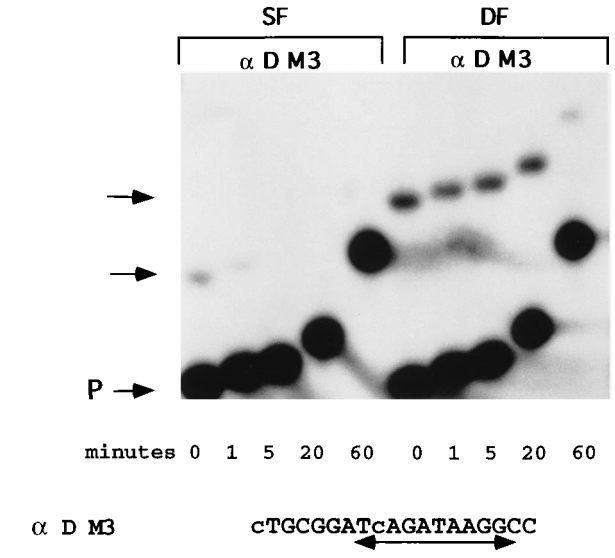

FIG. 7. A single base change in the α D promoter converts it to the GATApal sequence, and it now forms a single stable complex with DF. The α D GATA site was altered by substituting a C for an A at the position indicated by the lowercase letter in the figure. This new probe, α D M3 (1.5 pmol), was used to form complexes with SF and DF (0.2 pmol). Samples were electrophoresed at 0, 1, 5, 20, and 60 min after the addition of a 100-fold excess of unlabeled competitor DNA. DF now forms a single stable complex still visible after 60 min, while the SF complex is not detectable 5 min after the addition of competitor. The 5′ base of the oligonucleotide has been altered from T to C to increase the stability of the small oligonucleotide probe. Smearing from the probe has occurred in the last SF lane and extends to the DF lanes, just below the DF-DNA complex, and this signal has no bearing on the experiment. Arrows indicate bound counts, and P indicates the free probe at the 0 time point.

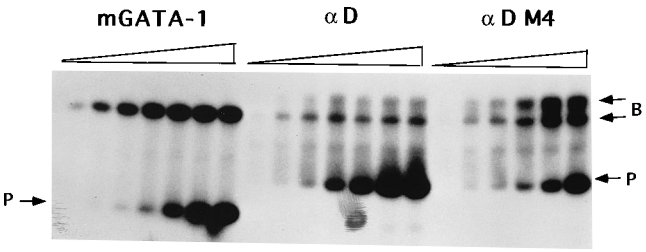

FIG. 8. N-f requires the 5′ GATA motif for strong interaction with the α-D-globin promoter. Titrations and mobility shift assays were performed with a fixed amount of DF and various amounts of the probes indicated. For mGATA-1, 0.25 to 2 pmol of probe was used, and for α D and α D M4, 1 to 8 pmol of probe was used. B indicates bound counts, while P indicates the position of the free probe.

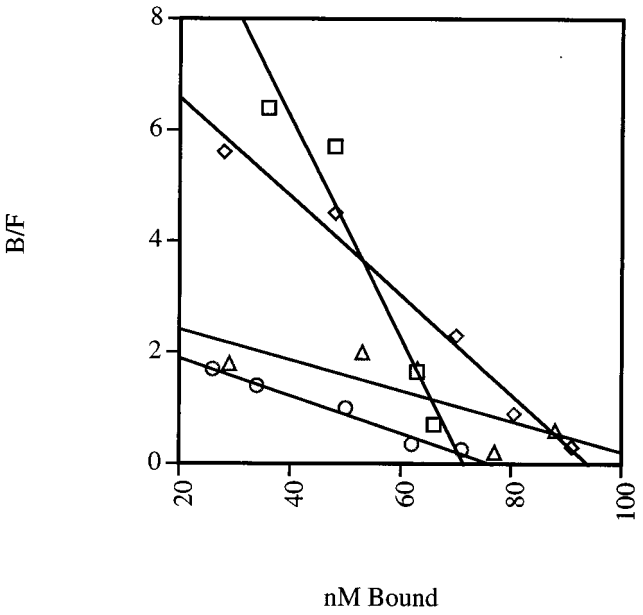

FIG. 9. Scatchard analysis of DF interaction with the human $^{A}\gamma$-globin promoter. One hundred picomoles of DF was incubated with various amounts of several labeled oligonucleotides and analyzed by gel mobility shift assay. Quantitation of bound (B) and free (F) fractions was performed with a PhosphorImager. Probes are 25 to 200 pmol of mouse GATApal (□), 50 to 400 pmol of hγ wild type (◇), and 100 to 800 pmol of hγ M1 (○) and hγ M2 (△). A representative Scatchard plot is presented. In this experiment, GATApal and the wild type are twofold different in affinity, while hγ M1 and hγ M2 have a binding affinity threefold lower than that of the hγ wild type. A twofold difference between GATApal and hγ wild type was observed in five independent experiments. The average difference between hγ wild type and hγ M1 and hγ M2 from three experiments is fivefold.

we were unable to alter the concentration of GATA-1 or -2 in this assay, we attempted to achieve a similar effect by varying the amount of DNA. Results were normalized to the activity of a cotransfected plasmid and are reported as the ratio of wild-type promoter activity to mutant promoter activity. At small amounts of DNA, the wild-type promoter was 8-fold more active than the mutant, and with high DNA concentrations (with which all GATA sites should be saturated) it was 3.5-fold more active (Table 1). This reduction in activity is similar in magnitude to the 75% reduction previously seen with the mutation of site 1b in the mouse promoter (34).

TABLE 1. Transient-transfection assay with erythroid precursor cells[a]

DNA (μg)	WT/mutant *cat* activity ratio[b]
1	8.8 (8)
2	7.0 (4)
3	3.5 (0.2)
9	3.5

[a] Results are from transient transfections into HD24 cells done by using Lipofectamine, the indicated amount of each test plasmid, and 0.1 μg of a plasmid containing a Rous sarcoma virus long terminal repeat-driven luciferase gene. *cat* activity was measured by a liquid scintillation assay.

[b] Results are reported as the ratio of *cat* activity of the wild-type (WT) GATA-1 promoter versus that of the mutant promoter. Results have been normalized to luciferase activity and are the average for at least four experiments, except for the 9-μg sample, for which two experiments were done. Standard deviations are given in parentheses. The actual *cat* activity increased in a linear fashion in response to increases in amounts of added DNA until the 9-μg sample, when some saturation was evident.

DISCUSSION

We have defined a consensus GATA binding site, ATC(A/T)GATAAG (GATApal), which requires both zinc finger motifs of GATA-1 for high-affinity binding. This motif is conserved in the GATA-1 promoters of chickens, mice, and humans. High-affinity stable binding requires one complete and one partial GATA motif in palindromic orientation. Mutation of the partial site leads to a decreased thermodynamic stability and an increase in the dissociation rate of the complex, as well as a reduction in its electrophoretic mobility. Mutation of the full site, 1b, abolishes DF binding. Dissociation half times for the high-affinity complexes are of the order of 70 to 140 min, while those for SF or mutant sites are 3 to 8 min. There are corresponding differences in the equilibrium binding constants.

We conclude that the amino-terminal finger (N-f) of GATA-1 is required for strong and specific binding to the GATA-1 promoter. It has previously been proposed that interactions with the multiple GATA elements in the GATA-1 promoters are stabilized by contributions from N-f (26, 34), but because the chicken and mammalian binding motifs were reported to be distinct from one another, it was not clear which of the sites was required for the N-f-specific interaction. We conclude from our results that N-f-dependent complex formation, a low complex dissociation rate, and greater complex mobility require only the GATApal motif.

Random binding-site selection studies with GATA-1, -2, and -3 have been performed by investigators from two other laboratories, and the data are consistent with the results reported here. In one of the studies, a number of probes containing double GATA sites were selected by all three proteins but were not further characterized. These sites were among the highest-affinity sites selected and contained overlapping motifs on opposite DNA strands (similar to the situation with the GATApal motif), as well as direct repeats (12). In the other study, a site very similar to the GATApal motif (cgATCAG ATAGgctgcctcgg) was selected by human GATA-2 (17), but the existence of the partial palindrome adjacent to the full GATA site was not noted. We have identified the first of the naturally occurring functional sites predicted to exist from both of these studies. Several double sites were also identified in a third random site selection study, although these were independent rather than overlapping sites (37).

The GATApal motif is the strongest N-f-dependent binding site, but others are also observed. The human $^{A}\gamma$-globin promoter displays characteristics of N-f-dependent binding, but unlike the situation with other sites, strong interaction requires two nonoverlapping direct repeats, consistent with previous data (16). A weaker interaction occurs with DF and the chicken α D promoter, which contains a complete GATA site (site 2) and a second overlapping partial direct repeat (site 1). Functional sites analogous to those of the α D and hγ promoters have not been found in any additional locations. GATA-1 is thus unusually versatile in its DNA binding capabilities, since a single molecule of DF can interact with at least three distinct motifs containing GATA repeats. Most Cys-Cys zinc finger proteins (such as the steroid hormone receptor superfamily) that recognize sites containing repeated elements bind to DNA as dimers (32).

Our results with two mutant α D probes (M3 and M4) suggest that N-f binds in distinct orientations relative to that of C-f, depending on the orientation of the GATA sites (palindromic or direct). Other data also support this view; NMR studies with a C-f–DNA complex have shown that the amino acids which contact the G of the GATA core consensus are in the tip of the finger (21); these residues are conserved between

N-f and C-f. The helix on the C-terminal side of C-f contacts the ATA of the GATA site; N-f has a highly homologous helical region in the analogous position. NMR analysis of DF in the free state has shown that, as expected, the secondary structure of each finger is very similar to that of C-f alone (unpublished data). Preliminary NMR analysis of a complex between DF and the GATApal sequence reveals that there is little change in the interaction between C-f and DNA from that seen in the published structure; C-f binds to GATApal in the same manner that it binds to a single GATA site. The conserved amino acid residues in the tip of N-f also appear to contact the DNA, but the bases have not yet been identified (unpublished data). This makes it extremely likely that N-f and C-f bind to GATA in very similar manners, and it is consequently probable that N-f does bind in two distinct orientations relative to that of C-f, depending on the orientation of GATA sites. C-f has a C-terminal region 16 amino acids downstream of the finger and helix which is required for DNA binding. N-f lacks this region, and this most likely explains why it does not bind to DNA independently, even though it can recognize a GATA motif. Our data raise the possibility that the two fingers of GATA-1 can interact with each other intramolecularly to stabilize binding to double sites in a manner analogous to the way in which the steroid hormone receptor fingers stabilize binding to repeating sites through dimerization of DNA-binding domains: this sort of stabilization could explain the higher binding affinity of DF to palindromic than to direct-repeat sequence (32). The NMR studies with DF and GATApal which are in progress should clarify these issues.

Most GATA sites are single sites which engage only C-f. These single sites sometimes occur in groups, but each is bound independently by a single molecule of protein and N-f is not required for the interactions with DNA. On the other hand, certain sites are capable of engaging N-f, and these are likely to form complexes with conformations distinct from those of complexes formed with single sites; these special GATA-1–DNA conformations may alter the extent and nature of GATA-1 interactions with other *trans*-acting factors and the degree to which GATA-1 bends DNA (21, 26). GATA-1 is believed to regulate genes which are expressed in quite different temporal patterns during hematopoiesis (23); differential interactions with certain DNA binding sites may be part of the basis for this selectivity. Site-specific binding affinities may also contribute.

The BLAST sequence analysis has allowed us to conclude that the GATApal motif is rare among the *cis*-regulatory regions of vertebrate genes. This observation is significant because the sequence database contains many genes which have authentic GATA binding sites in their *cis*-regulatory regions; GATApal occurs very infrequently among these elements. We were also able to conclude that the regulatory regions of most nonerythroid vertebrate genes do not contain this sequence, but we expect that GATApal or similar sequences will be found in the regulatory regions of genes which are regulated by other GATA family members. The observations that the *C. elegans elt-1* and *-2* promoters have a sequence which differs from GATApal by only one base (ATCAGATAtG) (10, 28) and that a site which differs at the same base was selected by GATA-2 (17) suggest that some variability in bases at the 3′ end of GATApal is allowed, but further studies will be required to confirm this. The conservation of GATApal in three vertebrate GATA-1 promoters suggests that it may have a special function in GATA-1 gene regulation. Support for this idea comes from our expression studies (Table 1). The same mutation that causes loss of the N-f binding site also reduces the activity of the cGATA-1 promoter in a transient assay with erythroid precursor cells. Thus, the partial palindromic site 1a/1b confers not only a higher level of stability but also greater potential for activation of expression on the GATA-1 promoter where it resides.

Because all GATA proteins have highly conserved DNA-binding domains, other family members probably interact similarly with GATApal. The mechanism controlling the initial activation of the GATA-1 gene is unknown, but the gene is thought to be positively autoregulated once it is activated (8, 34). GATA-2 is expressed slightly before GATA-1 in development (15, 41), and it has been suggested that GATA-2 may activate the GATA-1 gene. GATApal could be involved in the initial activation of the GATA-1 gene, since a high-affinity site among lower-affinity sites would be expected to make more difference at low GATA concentrations, and the lowest concentrations occur at early stages of development. In support of this theory, it has recently been shown that transcription of the endogenous GATA-1 gene can be stimulated in a myeloid cell line which expresses only very low levels of GATA-1 by the expression of full-length or truncated forms of GATA-2; more efficient activation of the endogenous gene occurs with a form of GATA-2 that contains both finger motifs than with a GATA-2 protein containing only C-f (35). We speculate that the GATApal motif in the GATA-1 promoter may be the target of this enhanced induction. The ζ-globin gene promoter, the other erythroid cell-specific promoter in which GATApal occurs, is also expressed relatively early in erythroid development; we do not know if this site is functionally equivalent to the GATA-1 promoter GATApal motif.

We have identified a new high-affinity GATA DNA binding motif (GATApal) which interacts with both zinc fingers of GATA-1. GATApal is so far unique among the naturally occurring N-f-requiring GATA sites in that it is exactly conserved in the same location in three species. We suggest that GATApal may play a special role in the activation of GATA-1 at stages of development at which GATA-1 or other GATA family members are not yet abundant and that it may contribute to the continuous efficient expression of GATA-1, which is a hallmark of the erythroid lineage.

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