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Functional Synergy and Physical Interactions of the Erythroid Transcription Factor GATA-1 with the Krüppel Family Proteins Sp1 and EKLF

MENIE MERIKA[1] AND STUART H. ORKIN[1,2]*

Division of Hematology-Oncology, Children's Hospital and Dana Farber Cancer Institute, Department of Pediatrics, Harvard Medical School,[1] Howard Hughes Medical Institute,[2] Boston, Massachusetts 02115

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An unresolved aspect of current understanding of erythroid cell-specific gene expression relates to how a limited number of transcriptional factors cooperate to direct high-level expression mediated by *cis*-regulatory elements separated over large distances within globin loci. In this report, we provide evidence that GATA-1, the major erythroid transcription factor, activates transcription in a synergistic fashion with two Krüppel family factors, the ubiquitous protein Sp1 and the erythroid-restricted factor EKLF (erythroid Krüppel-like factor), which recognize GC and/or GT/CACC motifs. Binding sites for both GATA-1 and these Krüppel proteins (especially Sp1) are found in close association in the promoters and enhancers of numerous erythroid cell-expressed genes and appear to cooperate in directing their expression. We have shown that GATA-1 interacts physically with Sp1 and EKLF and that interactions are mediated through their respective DNA-binding domains. Moreover, we show that GATA-1 and Sp1 synergize from a distance in constructs designed to mimic the architecture of globin locus control regions and downstream globin promoters. Finally, the formation of GATA-1–Sp1 complexes was demonstrated in vivo by the ability of Sp1 to recruit GATA-1 to a promoter in the absence of GATA-binding sites. These experiments provide the first evidence for functionally important protein-protein interactions involved in erythroid cell-specific expression and suggest a mechanism by which DNA loops between locus control regions and globin promoters (or enhancers) might be formed or stabilized.

Gene expression and development in eukaryotes are controlled in large part through transcriptional events. Central to these pathways are sequence-specific DNA-binding proteins that recognize short *cis*-regulatory motifs. Specificity in gene activation is achieved through complex protein-DNA and protein-protein interactions involving bound transcription factors, adaptor proteins, and components of the basal transcriptional machinery assembled at the initiation site (see reference 52 for a review).

Studies of globin gene expression during erythroid cell differentiation have served as excellent models for understanding mechanisms governing tissue- and developmental stage-specific gene regulation. High-level erythroid expression of globin genes requires regulatory elements located in gene promoters and enhancers as well as in upstream sequences, the locus control regions (LCRs) (for reviews, see references 3, 10, and 22). Three aspects of the control of globin gene expression are particularly noteworthy. First, core segments of the LCRs, minimal sequences that confer tissue-specific expression of a linked transgene in mice, display a relative simplicity of DNA-binding motifs. These include GATA, AP-1/NF-E2-like, and Sp1-like (GC or GT/CACC) motifs assembled in various configurations (reviewed in reference 10). Second, no single motif, even as a multimer, is competent to provide erythroid cell-specific expression of a transgene. Only paired combinations of motifs will create artificial erythroid elements (56). Moreover, within erythroid cell-expressed promoters, these *cis* elements often appear to cooperate with each other to direct tissue specificity and transcriptional activity (11, 16, 27, 35, 44, 61). Third, *cis*-regulatory elements (the LCRs) control globin gene transcription over large distances (~50 to 100 kb) (10, 19).

The cooperation between *cis* elements within promoters and between LCRs and promoters is best accounted for mechanistically by models that propose specific protein-protein interactions of regulatory factors bound to the critical motifs. In the instance of LCR-gene interactions, it is envisioned that the LCR is brought into the proximity of the downstream genes by chromosomal looping and that proteins bound to the LCR core regions as a complex interact with promoter-bound factors to activate globin gene transcription (reviewed in references 10 and 22). The nature of the protein-protein interactions that establish these functional connections is unknown. The studies described here are a first step toward defining the proteins and their associations that contribute to the cooperative effects noted above.

Sequence-specific DNA-binding proteins which recognize the three principal motifs—GATA, AP-1-like, and Sp1-like (GC or GT/CACC)—have been characterized. GATA and AP-1/NF-E2 motifs are targets for the cell-restricted transcription factors GATA-1 and NF-E2, respectively (reviewed in references 10 and 22). GATA-1, which is highly expressed in erythroid, mast, and megakaryocytic lineages, is the founding member of a family of transcription factors notable for a novel zinc finger structure (12, 53; see references 38 for a review). Its essential role in erythroid development has been established by gene targeting experiments (41). NF-E2 is a heterodimer of two basic region-leucine zipper polypeptides; the smaller (p18) is widely expressed, whereas the larger subunit (p45) is expressed in nearly the same hematopoietic lineages as GATA-1 (1, 2). Several factors bind in vitro to GC or GT/CACC elements (21, 43, 49, 50). These include ubiquitous proteins, including Sp1 (24) and Sp1-related polypeptides (26), and at least one cell-restricted protein, designated EKLF (erythroid

* Corresponding author. Mailing address: Division of Hematology, Children's Hospital Medical Center, 300 Longwood Ave., Boston, MA 02115.

Krüppel-like factor) (36). Recent evidence suggests that EKLF may act principally at an extended CACC element present in the adult β-globin gene promoter (39a).

Indirect data suggest that GATA-1 may cooperate with Sp1 protein(s) in erythroid cell-expressed promoters. For example, the erythropoietin receptor (EpoR), chicken α^{π}-globin, and stem cell leukemia (SCL/tal-1) promoters can be activated in cotransfection experiments by forced expression of GATA-1 in nonerythroid cells, but only if a nearby GC or GT/CACC motif is present (5, 27, 60, 61). Within LCR core elements, the occurrence of GATA and Sp1-like motifs at a relatively fixed distance (4, 11, 42, 43, 47–50) hints at functional cooperation of the bound proteins.

In this study, we have addressed how transcriptional cooperation for erythroid cell-specific expression might be mediated by considering potential interactions of the major erythroid factor GATA-1 with two Krüppel family factors, Sp1 and EKLF, which recognize GC and/or GT/CACC motifs. Here we provide evidence that GATA-1 synergizes with either protein at the transcriptional level. In addition, the zinc finger DNA-binding domain of GATA-1 mediates a physical interaction with the Krüppel zinc finger domains. Our experiments provide the first evidence for important protein-protein interactions that may serve to activate erythroid cell-specific gene expression and possibly aid in forming or stabilizing chromosomal loops between distant LCR and promoter elements.

MATERIALS AND METHODS

Cell culture and transfections. *Drosophila melanogaster* Schneider line 2 (SL2) cells were maintained in Schneider medium (Gibco) supplemented with 12% fetal calf serum and antibiotics at ambient temperature. Twenty-four hours prior to transfection, SL2 cells were plated in six-well dishes. Transfections were performed by the method of Chen and Okayama (7). Cells were harvested 48 h posttransfection, and chloramphenicol acetyltransferase (CAT) activity was determined from cell extracts as previously described (18). Normalization for transfection efficiency was performed in all experiments on the basis of β-galactosidase activity (45). The percentage conversion of ^{14}C-chloramphenicol was quantitated by PhosphorImager (Molecular Dynamics) analysis.

In most experiments, SL2 cells were cotransfected with 200 ng of reporter plasmid. In the experiments shown in Fig. 8, 1 μg of reporter plasmids was used. As an internal control for transfection efficiency, 100 ng of plasmid hsp82lacZ (provided by D. Thanos) was included in all cases. The amounts of transfected activators are indicated in the figure legends. Vector DNA was added as necessary to achieve a constant amount of transfected DNA.

Plasmid constructions. The reporter plasmids -33AdhCAT and -41h βglobinCAT have been previously described (13, 28). All oligonucleotides were synthesized with *Bam*HI ends and cloned either at the *Bgl*II or *Bam*HI sites of -33AdhCAT (28) and -41hβglobinCAT (13), respectively. To generate the construct in which Sp1 and GATA sites are separated by 300 bp, we first cloned the Sp1 oligonucleotide at the *Bam*HI site immediately upstream of the β-globin TATA box and then inserted the GATA oligonucleotide at the *Sma*I site located 300 bp upstream. To generate expression vectors for EKLF and GATA-1 derivatives, the respective entire open reading frames were cloned at the *Bam*HI site of the *Drosophila* expression vector pPac (28). The Sp1 expression vectors have been described previously (39). The disrupted c-finger GATA-1 (CfGATA-1) derivative was constructed by PCR, using appropriate primers for the introduction of Cys→Gly mutations. All constructs were verified by DNA sequencing.

In vitro transcription and translation. In vitro transcription was performed with linearized templates, using T7 or T3 RNA polymerases, and in vitro translation was carried out with nuclease-treated rabbit reticulocyte lysates as instructed by the supplier (Promega).

Expression and purification of recombinant proteins. The entire open reading frame of mouse GATA-1 (mGATA-1) was cloned at the pGEX-2T vector (Pharmacia) in frame with the glutathione *S*-transferase (GST) moiety. Similarly, a region of mGATA-1 containing only the N and C fingers (residues 177 to 333) was cloned in the pGEX-3X vector. GST–GATA-1(Δf) was generated by PCR and contains an internal deletion spanning residues 198 to 316. GST–GATA-1 derivatives containing deletions in either the N or C finger were generated from previously described constructs (33). The GST-EKLF Zn finger fusion protein contains residues 286 to 376 of EKLF (36) in the pGEX-2T vector. The GST-Sp1 fusion contains the three zinc fingers of Sp1, residues 600 to 700 (24, 25), in the pGEX-2T vector. Deletions of Sp1 were generated by PCR using appropriate primers and cloned in the pSP73 polylinker (Promega).

GST fusion proteins were prepared from 50-ml *Escherichia coli* DH5α cultures essentially as previously described previously (46) and estimated to be >90% pure by Coomassie blue staining. Approximately 1 μg of GST protein or fusion derivatives was immobilized on glutathione-agarose beads and incubated with in vitro-translated ^{35}S-labeled proteins for 2 h at 4°C in interaction buffer (150 mM NaCl, 10 mM Tris [pH 8], 0.3% Nonident P-40, 1 mm dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.25% bovine serum albumin [BSA]) followed by three washes with interaction buffer and a final wash with the same buffer lacking BSA. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was then used to analyze bound proteins.

RESULTS

Transcriptional synergy between GATA-1 and Krüppel family proteins Sp1 and EKLF. Previous studies have suggested that GATA and Sp1-like (GC or CACC) motifs cooperate in the function of several erythroid cell-expressed promoters (see the introduction). Sp1 recognizes both GC and CACC motifs in vitro (see the introduction and references 31 and 51), whereas EKLF binds to a subset of extended CACC sequences (14, 36). We first examined whether these Krüppel family proteins are able to activate an erythroid promoter through these elements and, specifically, whether GATA-1 synergizes with them to activate transcription. To address these aspects, we performed cotransfection experiments in *D. melanogaster* SL2 cells, which lack proteins highly related to mammalian Sp1 or EKLF (9).

First, SL2 cells were transfected with a reporter bearing a segment of the EpoR promoter containing a GATA site and an Sp1-like GC element in their natural configuration cloned immediately upstream of the *Drosophila* Adh TATA box (see Materials and Methods). Cotransfections were performed with expression vectors encoding GATA-1 and Sp1 or EKLF. As shown in Fig. 1A (lanes 2 and 3), increasing amounts of GATA-1 alone activated the reporter by approximately 12-fold. Upon cotransfection of GATA-1 with a fixed amount of the Sp1 expression plasmid (lane 4), the levels of transcription elicited greatly exceeded the sum of the activities obtained by each activator alone (lanes 5 and 6). For example, transfection of 2 μg of GATA-1 expression plasmid yielded about threefold activation (lane 2). Cotransfection with a fixed amount of Sp1 plasmid, which by itself activated 6-fold (lane 4), led to 35-fold activation (lane 5, black bar). This level of activation is approximately fourfold greater than that anticipated if GATA-1 and Sp1 were acting independently (gray bar). Thus, Sp1 is competent to activate transcription through the GC element of the EpoR promoter, and activation by GATA-1 and Sp1 is synergistic.

Synergistic activation of transcription may involve direct interactions between activators and/or independent activities of the factors along the same transcriptional pathway. Considering these possibilities, we presumed that if GATA-1 and Sp1 physically interact when bound to DNA, their relative position on the double DNA helix might be crucial. GATA-1 and Sp1 bound to the EpoR promoter are predicted to lie normally on the same face of the DNA helix, as the spacing between their cognate motifs spans two helical turns of DNA (20 bp) (60). Accordingly, when the relative position of the two elements was altered by 5-bp intervals, synergistic activation was lowered fourfold (data not shown).

As GATA sites are also found in close proximity with diverse CACC elements, in addition to GC motifs (see the introduction), we examined whether Sp1 or EKLF functions through these elements and also acts synergistically with GATA-1 in stimulation of transcription. We designed a reporter construct containing the GATA and CACC elements of the chicken α^{π}-globin promoter in their natural configuration. Previous experiments demonstrated that both elements are indispensable for promoter activity (27). SL2 cells were cotransfected

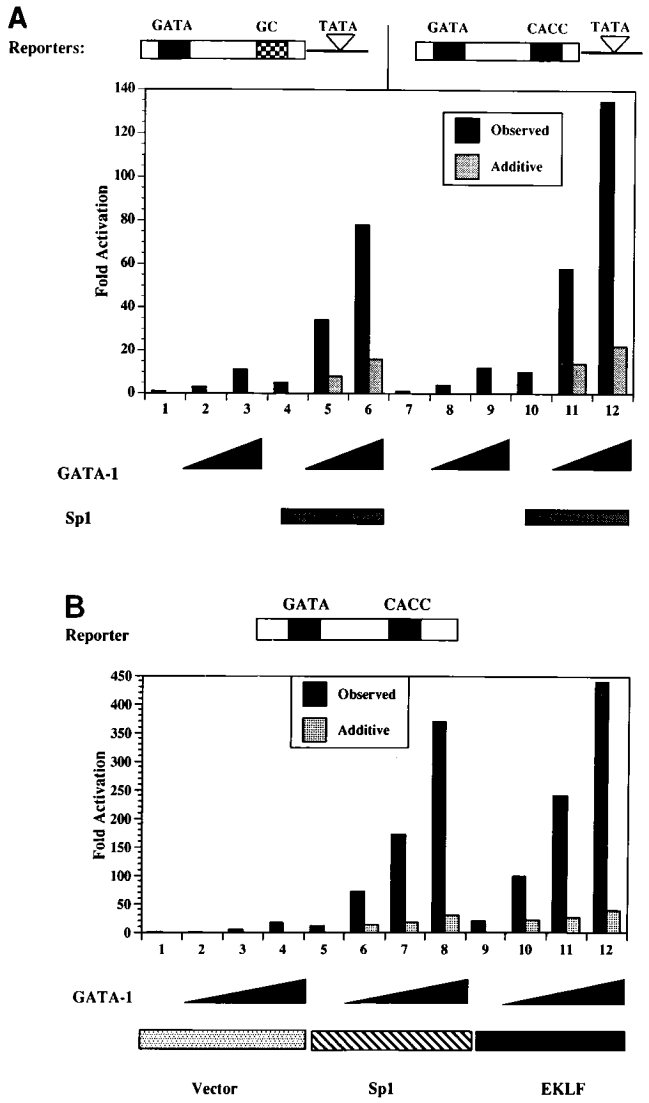

FIG. 1. Transcriptional synergy between GATA-1 and Sp1 or EKLF from GATA and GC/CACC elements. *Drosophila* SL2 cells were cotransfected with the indicated reporter constructs (shown at the top) along with increasing amounts of GATA-1-expressing plasmid in the absence or presence of a constant amount of Sp1 or EKLF expression vector. (A) In lanes 1 to 6, the reporter contained the EpoR promoter oligonucleotide, whereas in lanes 7 to 12, the reporter contained the chicken α^{π}-globin promoter oligonucleotide. Lanes 1 and 7, 8 μg of vector; lanes 2 and 8, 2 μg of GATA-1; lanes 3 and 9, 4 μg of GATA-1; lane 4, 200 ng of Sp1; lane 10, 1 μg of Sp1; lanes 5, 6, 11, and 12, both GATA-1 and Sp1. We used 1 μg of Sp1 expression plasmid in lane 10, versus 200 ng in lane 4, to compensate for a lower-affinity Sp1-binding site in the case of the chicken α^{π}-globin promoter. (B) Lane 1, 8 μg of vector; lanes 2 to 4, 1, 2, and 4 μg of GATA-1 expression vector; lane 5, 1 μg of Sp1; lanes 6 to 8, 1, 2, and 4 μg of GATA-1 plus 1 μg of Sp1 expression vectors; lane 9, 1 μg of EKLF expression vector; lanes 10 to 12, 1, 2, and 4 μg of GATA-1 plus 1 μg of EKLF expression vectors. The histograms represent average CAT activities from two independent experiments. The black bars represent the levels of CAT activity observed, whereas the gray bars represent the expected levels of activity if GATA-1 and Sp1 activated independently.

with the chicken α^{π}-globin reporter along with GATA-1 and Sp1 or EKLF expression vectors. As shown in Fig. 1A, lanes 7 to 12, cotransfected GATA-1 and Sp1 synergized on the chicken α^{π}-globin promoter reporter (lanes 7 to 12) to levels similar to those obtained with the EpoR reporter (lanes 1 to 6). In addition, cotransfected EKLF, like Sp1, strongly synergized

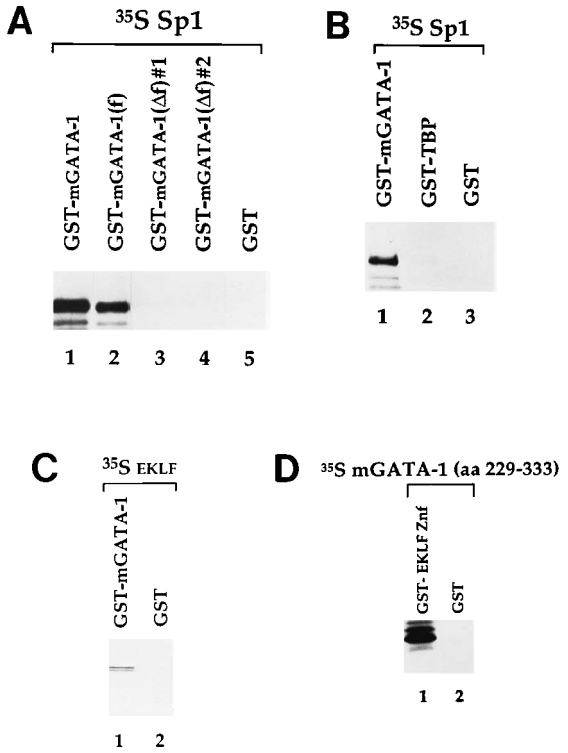

FIG. 2. Physical association between GATA-1 and Sp1 or EKLF in the absence of DNA. In vitro-translated ^{35}S-labeled Sp1, EKLF, or a GATA-1 peptide (residues 229 to 333) were incubated with the indicated *E. coli*-produced GST fusion proteins, immobilized on glutathione-agarose beads. Bound proteins were analyzed in SDS-polyacrylamide gels and visualized by autoradiography. (A) Interactions of ^{35}S-labeled Sp1 with GST alone (lane 5), GST–GATA-1 (lane 1), GST–GATA-1(f) (lane 2), or GST–GATA-1(Δf) (lanes 3 and 4; two independent clones tested) protein. Specific interactions were observed only with GST–GATA-1 or GST–GATA-1(f) protein. (B) Interactions of ^{35}S-labeled Sp1 with GST-GATA-1 (lane 1), GST–TATA-binding protein (TBP) (lane 2), or GST alone (lane 3). (C) ^{35}S-labeled EKLF incubated with GST-GATA-1 (lane 1) or GST (lane 2). The relatively weaker signal for labeled EKLF protein reflects its limited methionine content. (D) ^{35}S-labeled GATA-1 protein (amino acid [aa] residues 229 to 333) containing the C finger specifically interacts with GST-EKLF Zn fingers (lane 1) but not with GST (lane 2).

with GATA-1 on the chicken α^{π}-globin promoter reporter (Fig. 1B; compare lanes 10 to 12 with lanes 6 to 8).

Thus, two Krüppel family proteins, Sp1 and EKLF, bound to Sp1-like GC or CACC elements transcriptionally synergize with GATA-1 within intact cells. From these findings, we infer that the frequent occurrence of GATA and GC or GT/CACCC elements in *cis*-regulatory elements is of functional significance.

Protein-protein interactions between GATA-1 and Krüppel proteins Sp1 and EKLF in the absence of DNA. In light of functional cooperation described above, we next addressed if physical interactions between GATA-1 and Sp1 or EKLF might be detected. To do so, we performed in vitro binding experiments in which *E. coli*-expressed GST–GATA-1 fusion protein immobilized on glutathione-agarose beads was incubated with in vitro-translated, ^{35}S-labeled Sp1 or EKLF. As shown in Fig. 2A, ^{35}S-labeled Sp1 was specifically retained on the agarose column containing the GST–mGATA-1 fusion protein (lane 1) but not on glutathione beads containing only GST (lane 5). In vitro-translated Sp1 was also retained on a column containing the two-finger DNA-binding domain of GATA-1 [GST–GATA-1(f)] alone (lane 2). Thus, the DNA-binding domain of GATA-1 interacts with Sp1. Additional

regions of GATA-1 may stabilize this interaction, as suggested by the consistently stronger interaction of Sp1 with intact GATA-1 (compare lanes 1 and 2) and the failure of a fingerless GATA-1 [GST-GATA-1(Δf)] to bind Sp1 (lanes 3 and 4). As a further demonstration of the specificity of these interactions, we showed that in vitro-translated Sp1 fails to interact with a GST–TATA-binding protein fusion (Fig. 2B, lane 2), a result consistent with previous observations (23). In addition, under these conditions GATA-1 does not bind to the RNA-binding protein R17 or p45 NF-E2 (not shown). Moreover, the specific interaction between GATA-1 and Sp1 was observed in the presence of high concentrations (100 μg/ml) of ethidium bromide (data not shown), which has been shown to disrupt DNA-dependent protein-protein interactions (29).

The comparative transcriptional effects of EKLF–GATA-1 and Sp1–GATA-1 cooperation prompted us to consider whether GATA-1 and EKLF also interact in vitro. As shown in Fig. 2C, in vitro-translated EKLF was specifically retained on glutathione beads containing GST–GATA-1 fusion protein (lane 1) but not on GST alone (lane 2). This interaction was mediated through the zinc fingers of EKLF (Fig. 2D). Thus, both Krüppel family proteins, Sp1 and EKLF, specifically interact with GATA-1 in vitro, even in the absence of DNA.

To determine if the interaction of GATA-1 with Sp1 might lead to formation of a ternary complex on DNA, we examined the ability of purified recombinant proteins to bind cooperatively on the EpoR promoter. By combining electrophoretic mobility shift assays and DNase I footprinting, we were able to show that GATA-1 and Sp1 exhibit a low degree of cooperativity in DNA binding on the EpoR promoter oligonucleotide (data not shown).

Localization of the Sp1 domains mediating physical interaction and transcriptional synergism with GATA-1. To identify the region of Sp1 necessary for interaction with GATA-1, a series of N- and C-terminal deletions was constructed (Fig. 3). ^{35}S-labeled proteins were produced by in vitro transcription-translation and were incubated with GST or GST–GATA-1 proteins immobilized on glutathione-agarose beads. Analysis of the N-terminal deletions revealed that the region of Sp1 containing the previously identified activation domains A, B, and C (9, 24, 25) were dispensable for interaction with GATA-1 (Fig. 3A; compare lane 2 with lanes 6, 9, and 13). However, further deletion of the triple zinc finger (24, 25) prevented the interaction with GATA-1 (lane 17). Similarly, deletions from the C terminus demonstrated that an intact third zinc finger domain is required for interaction with GATA-1, since deletion of only 13 amino acids in the C-terminal third zinc finger abolished binding (compare lanes 19 and 3). Furthermore, an Sp1 derivative containing only the triple zinc finger DNA-binding domain interacted with GATA-1 (lane 15). In summary, our combined deletional analyses indicate that the zinc finger region of Sp1 is both necessary and sufficient for physical interaction with GATA-1 (Fig. 3B).

Prior studies revealed that different domains of Sp1 confer distinct functions (39). To define the domains of Sp1 required for transcriptional synergy with GATA-1, we assessed the abilities of different Sp1 deletion derivatives to transactivate the EpoR reporter synergistically with GATA-1. For this purpose, we performed cotransfection experiments in SL2 cells with the EpoR reporter and expression plasmids for GATA-1 and deletion derivatives of Sp1 (39). Deletion of either of the glutamine-rich activation domains A and B together with domain C (ΔA+ΔC or ΔB+ΔC), or deletion of the activation domain D (ΔD), did not significantly affect Sp1–GATA-1 synergism (Fig. 4; compare lanes 7 to 10 with lanes 12 to 15, 17 to 20, and 22 to 25). Similarly, an Sp1 derivative containing only the active carboxy terminus of the B domain (39), the zinc finger domain, as well as domain D (B-c) efficiently synergized with GATA-1 (lanes 27 to 39). By contrast, a derivative including the transcriptionally inactive N terminus of the B domain (39) in combination with the zinc fingers and domain D (B-n) failed to synergize (lanes 32 to 35).

In conclusion, these experiments suggest that any portion of Sp1 that contains the zinc finger domain and a transcriptional activation domain is competent for synergistic interactions with GATA-1. This conclusion is in agreement with our in vitro interaction experiments which reveal that Sp1 interacts with GATA-1 via its zinc finger region. In contrast, the ability of Sp1 to activate transcription synergistically from multiple Sp1 sites requires an intact D domain (39).

Other GATA family members also interact with Sp1. GATA-1 interacts with Sp1 through its finger DNA-binding domain. Members of the GATA family of transcription factors are related to GATA-1 exclusively by virtue of their similarity in this region (33; see reference 38 for a review). Hence, it was of particular interest to examine whether the interaction of Sp1 with GATA proteins might be general. To investigate this, we tested the in vitro binding of ^{35}S-labeled GATA-2 and GATA-3 polypeptides to the zinc finger region of Sp1 (Fig. 5). Both GATA-2 and GATA-3, in addition to GATA-1, are specifically retained on a GST-Sp1 column (lanes 1, 3, and 5, respectively) but not on GST alone (lanes 2, 4, and 6). Thus, interaction with Sp1 appears to be a general feature of GATA family proteins.

The C finger of GATA-1 is sufficient for interaction with Sp1. Members of the GATA family of proteins contact DNA through two highly conserved zinc fingers. The C-terminal finger is required for specific DNA binding, whereas the N-terminal finger contributes to establish full DNA-binding specificity (33, 34, 59). Above, we provide evidence that a GATA-1 derivative containing both fingers is sufficient for interaction with Sp1 (Fig. 2A). To further localize the domain of GATA-1 which mediates this interaction, we generated GST fusion GATA-1 derivatives in which either the N-terminal or the C-terminal finger was deleted [GST–mGATA-1(ΔNf) or GST–mGATA-1(ΔCf), respectively]. These proteins were tested for the ability to interact with Sp1. As shown in Fig. 6A, the GATA-1 protein lacking the N-terminal finger (lane 4) interacted with in vitro-translated Sp1 to a level comparable to that of the wild-type protein (lane 5). Deletion of the C-terminal finger, however, drastically reduced interaction (lane 3). The differential ability of the N- and C-terminal GATA-1 fingers to interact with Sp1 was confirmed by testing interaction of N- and C-finger constructs alone with Sp1 (Fig. 6B). A GATA-1 derivative including the C finger (amino acids 208 to 304) strongly interacted with Sp1 (lane 3), whereas the N finger associated with Sp1 to a lesser extent (lane 4). Thus, we conclude that the GATA-1 C-terminal finger is the major determinant for interaction with Sp1.

To test whether the interaction of Sp1 with the C finger of GATA-1 is sufficient to provide for transcriptional synergy, we cotransfected SL2 cells with the EpoR reporter construct along with Sp1 and either the intact GATA-1 or N- or C-finger-deleted GATA-1 expression vector. Figure 6C shows that the N-finger-deleted GATA-1 derivative strongly synergized with Sp1 (lane 15 to 18) to levels comparable to those of intact GATA-1 (lanes 7 to 10). The overall levels of activity were somewhat lower, consistent with previous transactivation experiments which demonstrated reduced transactivation by the single-finger GATA-1 derivative (33). As expected, the C-finger-deleted GATA-1 failed to activate transcription by itself (since it is not capable of DNA binding) and therefore did not

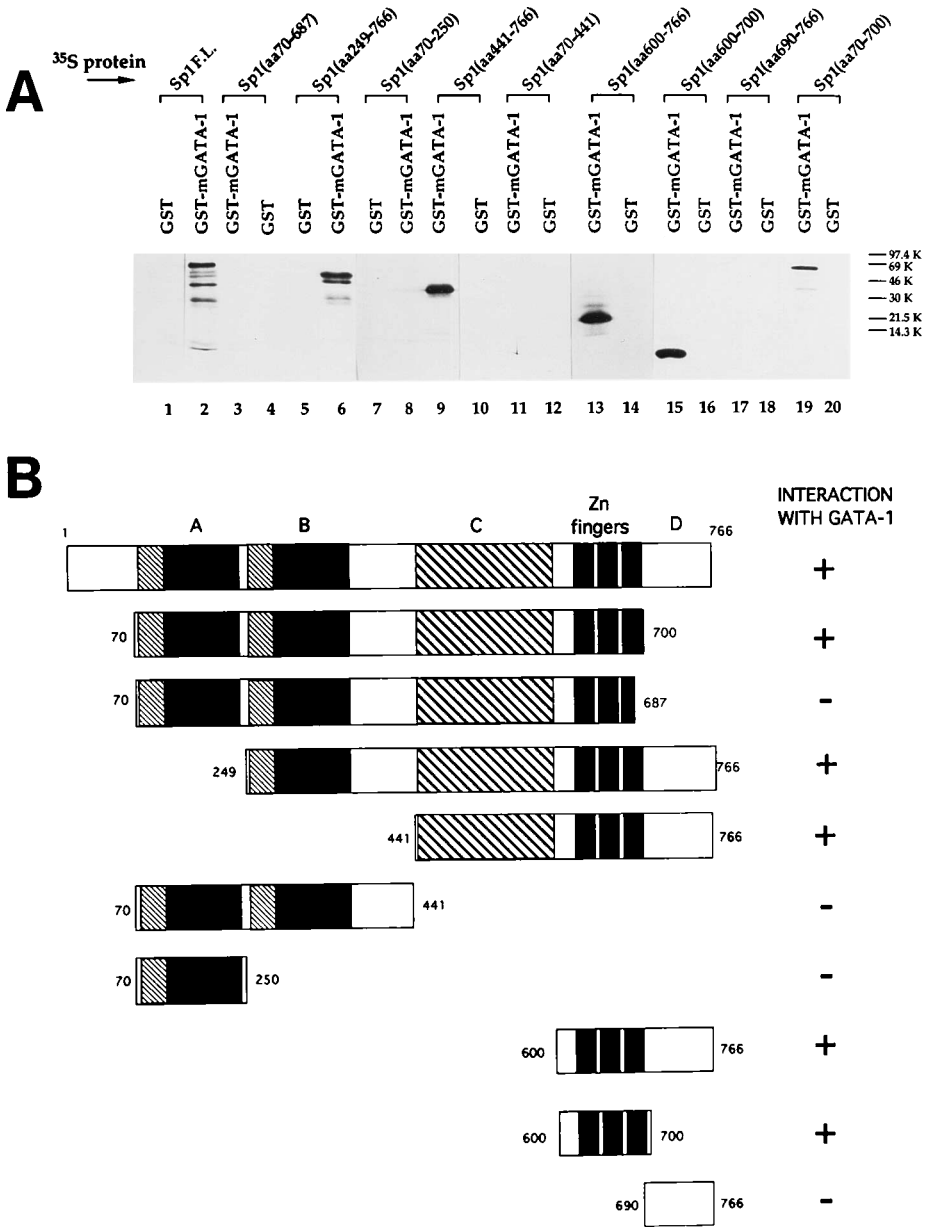

FIG. 3. Localization of the interaction domain of Sp1 with GATA-1. (A) ^{35}S-labeled N- and C-terminal deletions of Sp1 were produced by in vitro transcription-translation reactions and incubated with *E. coli*-produced GST or GST–mGATA-1 protein immobilized on glutathione-agarose beads. Bound proteins were analyzed in SDS-polyacrylamide gels and visualized by autoradiography. The coordinates of all Sp1 deletions tested are indicated. F.L., full length; aa, amino acids. (B) Schematic diagram of the Sp1 deletion derivatives used and summary of the interaction results obtained in panel A. +, specific interaction; −, no interaction.

synergize with Sp1 (lanes 23 to 26). Thus, the presence of only the C-terminal finger of GATA-1 in the otherwise intact body of the protein is sufficient to permit synergistic activation of transcription by GATA-1 and Sp1.

Sp1 and GATA-1 recruitment to a promoter via tethering. On the basis of the data presented above, we infer that GATA-1 and Sp1 are able to interact specifically within a complex. To provide functional evidence for the formation of such a complex in vivo, we initially examined the abilities of cotransfected GATA-1 and Sp1 to activate transcription synergistically from EpoR reporters in which the GATA or Sp1 sites were mutated. We found that when small amounts of both plasmids were cotransfected into cells, transcriptional synergy was dependent on the integrity of both binding sites (Fig. 1). In contrast, high-level expression of both GATA-1 and Sp1 led to strong transcriptional synergy, especially from reporters containing a single Sp1 site (data not shown). Such experiments suggested that under these conditions, DNA-bound Sp1 might recruit GATA-1 to the promoter. These experiments, however, did not preclude the possibility that transcriptional synergy results from the binding of GATA-1 to cryptic sites in the plasmid backbone.

To determine whether Sp1 might recruit a GATA-1 molecule into a transcriptional complex, we constructed a GATA-1 derivative which is unable to bind DNA but retains its capacity to interact with Sp1. Since DNA binding of GATA-1 requires the coordination of a zinc ion by four cysteine residues (37), mutations in these cysteines will disrupt the tertiary structure

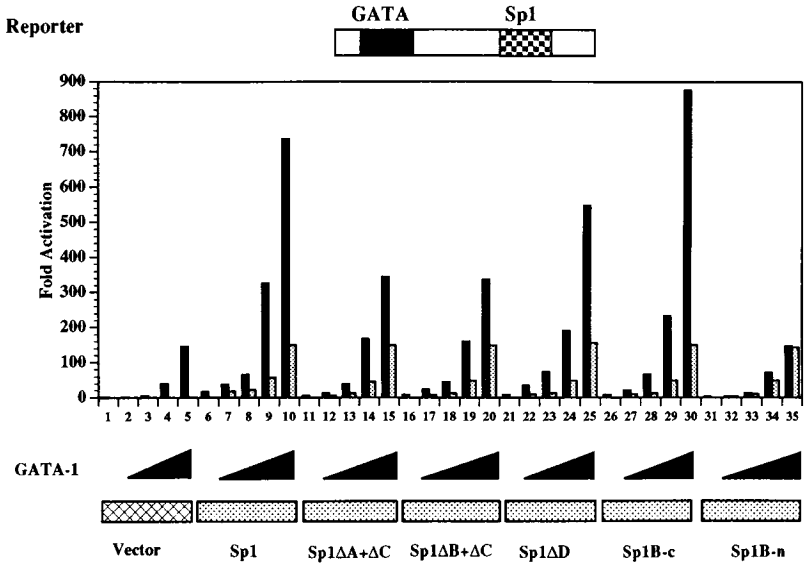

FIG. 4. Localization of Sp1 domains required for transcriptional synergy with GATA-1. SL2 cells were cotransfected with the EpoR reporter and increasing amounts of GATA-1-expressing plasmid in the absence or presence of a constant amount of plasmid encoding the indicated Sp1 derivative. Lanes: 1, 8 μg of vector; 2 to 5, 1, 2, 4, and 8 μg of GATA-1-expressing plasmid; 6, 200 ng of wild-type Sp1 expression vector; 7 to 10, 1, 2, 4, and 8 μg of GATA-1-expressing plasmid plus 200 ng of wild-type Sp1 expression plasmid; 11, 1 μg of Sp1ΔA+ΔC plasmid; 12 to 15, 1, 2, 4, and 8 μg of GATA-1 plus 1 μg of Sp1ΔA+ΔC expression plasmids; 16, 1 μg of Sp1ΔB+ΔC plasmid; 17 to 20, 1, 2, 4, and 8 μg of GATA-1 plus 1 μg of Sp1ΔB+ΔC expression vectors; 21, 1 μg of Sp1ΔD-expressing plasmid; 22 to 25, 1, 2, 4, and 8 μg of GATA-1 plus 1 μg of Sp1ΔD expression vectors; 26, 1 μg of Sp1B-c-expressing plasmid; 27 to 30, 1, 2, 4, and 8 μg of GATA-1 plus 1 μg of Sp1B-c expression vectors; 31, 1 μg of Sp1B-n-expressing plasmid; 32 to 35, 1, 2, 4, and 8 μg of GATA-1 plus 1 μg of Sp1B-n expression vectors. We used 1 μg of Sp1 deletion derivatives to compensate for their decreased abilities to activate transcription compared with the wild-type Sp1 expression plasmid, which was used at 200 ng. The histogram represents average CAT activities from two independent experiments. The black bars represent the levels of CAT activity observed, whereas the gray bars represent the expected levels of activity if GATA-1 and Sp1 activated separately.

and prevent DNA binding. Thus, we mutated the first two cysteines of the C-terminal finger (residues 258 and 261) to glycines, and the mutated protein (disrupted CfGATA-1) was tested for DNA binding. As expected from analyses of prior mutants (33), this protein failed to bind DNA (data not shown). Remarkably, ^{35}S-labeled in vitro-translated disrupted CfGATA-1 interacted specifically with a GST-Sp1 finger fusion protein (Fig. 7A). Therefore, although the tertiary structure of the GATA-1 C finger is a major determinant for DNA binding, it is not critical for the interaction with Sp1. Moreover, a GST derivative containing the disrupted C finger in isolation retains its ability to interact with Sp1 at levels similar to those for the wild-type C finger (data not shown). These experiments exclude the possibility that the full-length disrupted CfGATA-1 interacts with Sp1 via a previous unidentified cryptic interaction domain.

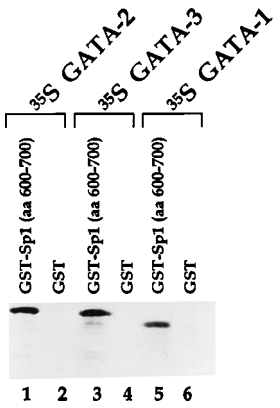

FIG. 5. Sp1 physically interacts with different GATA family members. In vitro-translated ^{35}S-labeled GATA-2, GATA-3, and GATA-1 proteins were incubated with a GST-Sp1 fusion protein containing only the zinc finger domain (amino acid [aa] residues 600 to 700) (lanes 1, 3, and 5) or GST alone (lanes 2, 4, and 6). Bound proteins were analyzed in SDS-polyacrylamide gels and visualized by autoradiography.

We then directly compared the ability of intact GATA-1 and disrupted CfGATA-1 to synergize with Sp1 on the EpoR promoter reporter. Consistent with experiments described above, transfection of increasing amounts of wild-type GATA-1 efficiently activated this reporter (Fig. 7B, lanes 3 to 6). On the other hand, transfection of disrupted CfGATA-1 did not activate the reporter (lanes 11 to 14), as the expressed protein fails to bind DNA. When various amounts of intact GATA-1 protein expression plasmid were cotransfected with a constant amount of Sp1 plasmid (lane 2), strong transcriptional synergy was observed at all concentrations tested (lanes 7 to 10). Of particular note, the disrupted CfGATA-1 derivative strongly synergized with Sp1, but only at high amounts of transfected expression vector (lanes 17 and 18). These experiments show persuasively that Sp1 can recruit GATA-1 to the promoter in the absence of GATA-1 binding as a result of protein-protein interactions and generate a ternary complex that synergistically activates transcription. As anticipated, these interactions take place only at high protein concentrations. By contrast, intact GATA-1 protein synergizes with Sp1 at both low and high protein concentrations. The experiments strongly suggest that transcriptional synergy seen at low GATA-1 input is due at least in part to the cooperative binding of GATA-1 and Sp1 on the EpoR promoter. However, synergy observed at high concentrations is due to both cooperative binding and recruitment of GATA-1 to the promoter by Sp1.

To rule out a promoter-specific effect, additional reporters containing the human β-globin promoter TATA box (see Ma-

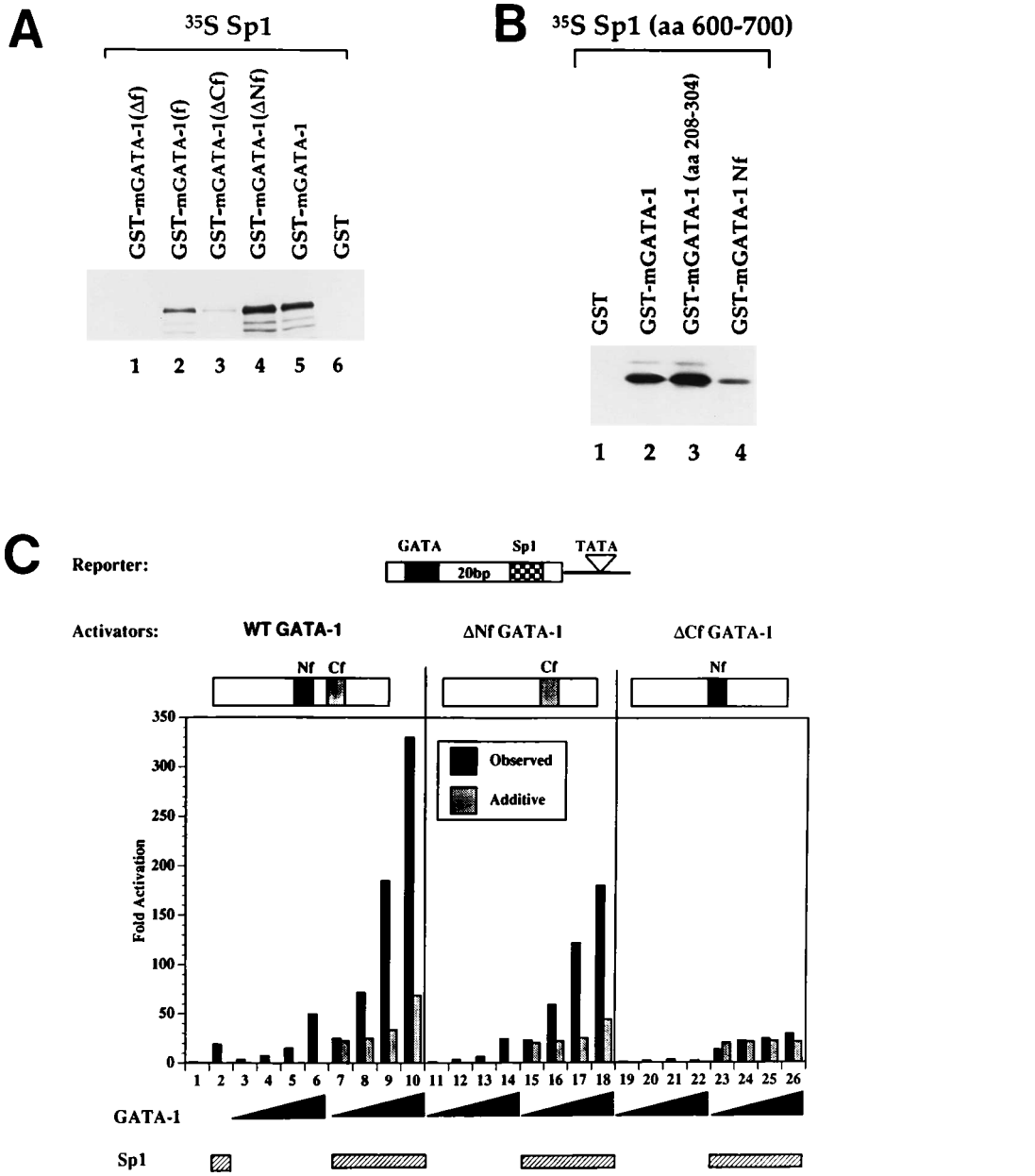

FIG. 6. The C finger of mGATA-1 is the major determinant for interaction with Sp1. (A) ^{35}S-labeled Sp1 protein was produced by in vitro transcription-translation and incubated with GST alone (lane 6), GST–GATA-1 (lane 5), GST–GATA-1(f) (lane 2), GST–GATA-1(ΔCf) (lane 3), GST–GATA-1(ΔNf) (lane 4), and GST–GATA-1(Δf) (lane 1) bacterial proteins immobilized on glutathione-agarose beads. (B) The zinc finger domain of Sp1 (residues 600 to 700) was ^{35}S labeled and incubated with GST (lane 1), GST–mGATA-1 (lane 2), GST–GATA-1 (amino acid [aa] residues 208 to 304, including the C-terminal finger; lane 3), and GST-GATA-1 N finger (Nf; residues 200 to 254; lane 4). Bound proteins were analyzed in SDS-polyacrylamide gels and visualized by autoradiography. (C) SL2 cells were transfected with the EpoR reporter plasmid. These transfections were performed with increasing amounts of wild-type (WT) GATA-1 (lanes 3 to 10), N-finger-deleted GATA-1 (ΔNf GATA-1; lanes 11 to 18), and C-finger-deleted GATA-1 (ΔCf GATA-1; lanes 19 to 26) activators in the absence or presence of a constant amount of Sp1 expression plasmid. Lane 1, 8 μg of expression vector; lane 2, 200 ng of Sp1-expressing plasmid; lanes 3 to 6, 500 ng, 1 μg, 2 μg, and 4 μg of wild-type GATA-1-expressing plasmid, respectively; lanes 7 to 10, 500 ng, 1 μg, 2 μg, and 4 μg of wild-type GATA-1 plus 200 ng of Sp1 expression vectors; lane 11 to 14, 500 ng, 1 μg, 2 μg, and 4 μg of ΔNf GATA-1 expression vector; lanes 15 to 18, 500 ng, 1 μg, 2 μg, and 4 μg of ΔNf GATA-1 plus 200 ng of Sp1 expression vectors; lanes 19 to 22, 500 ng, 1 μg, 2 μg, and 4 μg of ΔCf GATA-1 expression vector; lanes 23 to 26, 500 ng, 1 μg, 2 μg, and 4 μg of ΔCf GATA-1 plus 200 ng of Sp1 expression vectors. The histogram represents average CAT activities from two independent experiments.

terials and Methods) were used. Moreover, to test whether GATA-1 and Sp1 are able to synergize at a distance, we designed a reporter in which GATA and Sp1 elements were separated by 300 bp. Reporter constructs were transfected into SL2 cells along with the expression vectors encoding GATA-1 and Sp1, and we examined whether GATA-1 and Sp1 bound to their cognate binding sites synergistically activate transcription (Fig. 8). Transfection of either GATA-1 or Sp1 failed to activate transcription from the reporter containing only the β-globin TATA box (line 1). The reporter containing a GATA element immediately upstream of the TATA box (line 2) was activated eightfold by GATA-1, whereas no significant activation was obtained by Sp1. However, when GATA-1 and Sp1 expression plasmids were cotransfected, transcription was

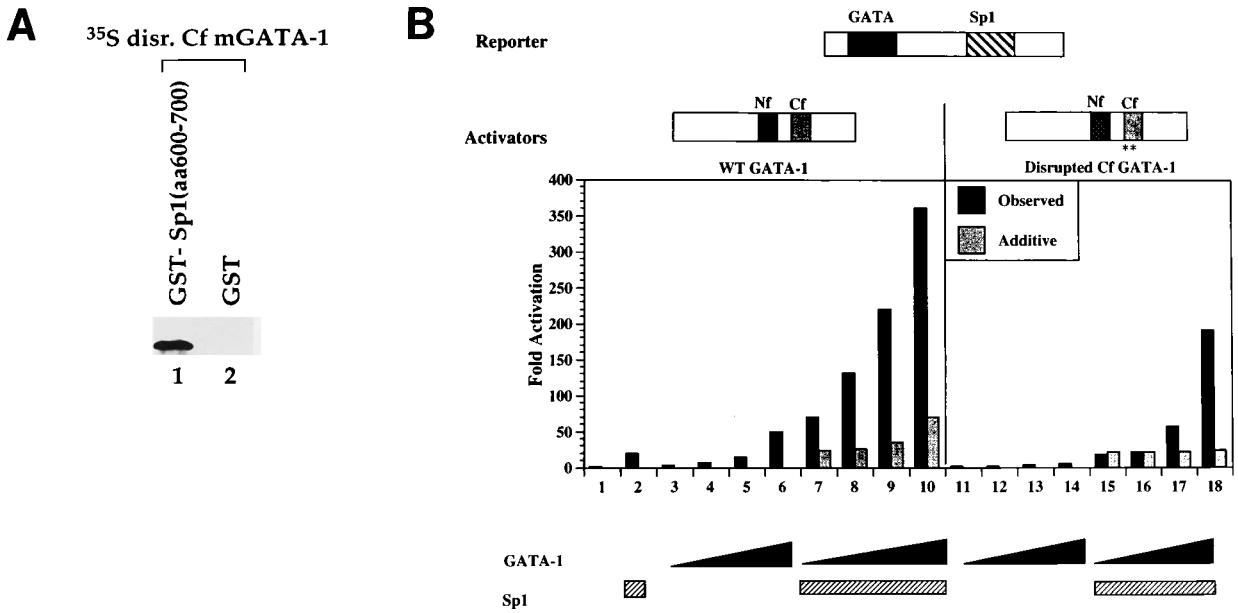

FIG. 7. The tertiary structure of the mGATA-1 C finger is not critical for interaction with Sp1. (A) ^{35}S-labeled disrupted CfGATA-1 protein (disr. Cf mGATA-1) was incubated with GST (lane 2) or GST-Sp1 finger protein (amino acid [aa] residues 600 to 700; lane 1). Bound proteins were analyzed in SDS-polyacrylamide gels and visualized by autoradiography. (B) SL2 cells were transfected with the EpoR reporter plasmid and increasing amounts of wild-type (WT) GATA-1 (lanes 3 to 10) or disrupted CfGATA-1 (lanes 11 to 18) expression plasmid. Lane 1, 10 μg of expression vector; lane 2, 200 ng of Sp1 expression plasmid; lanes 3 to 6, 1, 2, 4, and 8 μg of wild-type GATA-1 expression plasmid; lanes 7 to 10, 1, 2, 4, and 8 μg of wild-type GATA-1 plus 200 ng of Sp1 expression plasmids; lanes 11 to 14, 1, 2, 4, and 8 μg of disrupted CfGATA-1 expression vector; lanes 15 to 18, 1, 2, 4, and 8 μg of disrupted CfGATA-1 plus 200 ng of Sp1 expression vectors. The two asterisks in disrupted CfGATA-1 represent the Cys→Gly replacements. The histogram represents average values from four independent transfection experiments. Nf, N finger.

stimulated 20-fold. Thus, GATA-1 is able to recruit Sp1 to the promoter in the absence of Sp1-binding sites. As shown in line 3, Sp1 efficiently activated the reporter containing an Sp1 element, whereas GATA-1 had no effect. Remarkably, when GATA-1 and Sp1 plasmids were cotransfected, transcription was stimulated 130-fold. Binding of GATA-1 to cryptic GATA elements in the plasmid is not responsible for synergy, since similar results were obtained when disrupted CfGATA-1 was provided in place of intact GATA-1. The capacity of GATA and Sp1 to synergize from a distance was established with a reporter construct bearing a promoter-proximal Sp1 site and distant GATA elements. As shown in Fig. 8 (line 4), Sp1 and GATA-1 activated the reporter 30- and 5-fold, respectively. When GATA-1 and Sp1 were coexpressed, transcription was activated 600-fold. Again, the contribution of DNA-bound GATA-1 was established by cotransfection of the disrupted CfGATA-1 expression plasmid. As shown in line 4, Sp1 synergized with disrupted CfGATA-1 to levels approximating those obtained in the absence of GATA-binding sites (compare lines 3 and 4). Thus, the difference between transcriptional activity in the presence and the absence of GATA elements (~5-fold) approximates that observed between

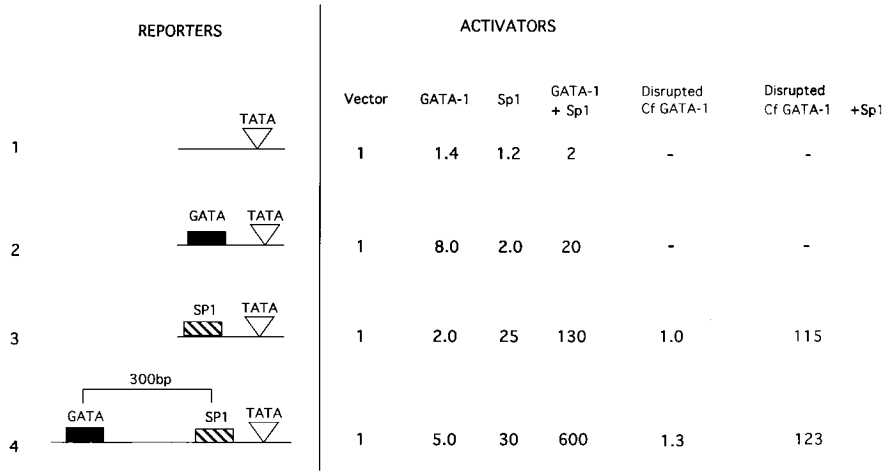

FIG. 8. Sp1 can recruit GATA-1 to a promoter in the absence of a GATA binding site (and vice versa). SL2 cells were transfected with the reporter plasmids (1 μg) shown on the left and the indicated expression plasmids (4 μg of each) encoding the activators shown at the top. The reporters used in these experiments are based on the -41 human β-globin TATA box (see Materials and Methods). The average of four independent experiments is presented.

expression of intact GATA-1 and disrupted CfGATA-1 proteins.

In conclusion, our data provide evidence that transcriptional synergy between GATA-1 and Sp1 is regulated at two levels. At a low activator concentration, DNA binding by both proteins is required and synergy is evident even when binding sites lie 300 bp apart. In this latter instance, GATA-1 and Sp1 most likely interact through a looping mechanism. At a high activator concentration, the interaction between these proteins occurs via tethering in the absence of binding sites, and as a consequence each protein may influence the other's transcriptional activity.

DISCUSSION

Studies of eukaryotic *cis*-regulatory regions have revealed a complex arrangement of both positive and negative elements. Synergistic interactions among the combinations of transcription factors bound to these elements are believed to underlie both high-level and cell-specific gene activation. The experiments reported here address physical interactions between GATA-1, the major erythroid transcription factor, and two Krüppel family proteins, Sp1 and EKLF, that recognize sequences (GC or GT/CACC) often found in association with GATA motifs in promoters, enhancers, and LCR cores of the globin loci in all vertebrate species (see the introduction). In our studies, we have assessed functional cooperation of GATA-1 and these Krüppel family proteins on erythroid promoters and then proceeded to document and map physical interactions between these protein classes.

Functional synergy between GATA-1 and Sp1 or EKLF in transcription of model erythroid promoters. Prior studies have provided indirect evidence for functional interactions between GATA and GC-rich or CACC motifs in erythroid promoters (see the introduction). For example, both types of binding sites in the promoters of the EpoR, chicken α^{π}-globin, and SCL genes are required for both erythroid cell-specific expression and activation by forced expression of GATA-1 in transfected cells (see the introduction). Moreover, GATA and CACC-like elements are both necessary for a minimal region of the human LCR HS3 core to direct expression of a transgene in vivo (42). To establish whether GATA-1 can function synergistically with proteins bound to GC or GT/CACC motifs, we performed cotransfection experiments in *Drosophila* SL2 cells lacking Sp1 like factors. We assessed functional cooperation with the two proteins thought to participate in transcriptional control through GC or GT/CACC elements in erythroid cells, Sp1, and EKLF. As Sp1 appears to bind in vitro to a broader array of sequences, including both GC-rich and GT/CACC elements, it is quite likely that it participates in control of numerous erythroid cell-expressed genes, whereas EKLF, which recognizes only a subset of extended CACC sequences, probably is involved in regulation of limited targets, such as the adult β-globin gene (39a).

Our results reveal marked synergy in transcription of promoters activated by GATA-1 and Sp1 or GATA-1 and EKLF (Fig. 1). Such cooperation is observed between GATA-1 and protein bound to either GC-rich or GT/CACC elements. As we retained the normal configuration of the GATA and GC or GT/CACC sequences in the two promoters used in these experiments, it is likely that the functional interactions that we have observed are representative of those occurring on the promoter in situ. In the context of a more complex promoter of the human γ-globin gene, others have described either synergistic or antagonistic functional interactions between GATA-1 and Sp1 (15). The relevance of their findings to other promoters is unclear.

Our studies also demonstrate that the spacing of GATA and Sp1-like elements markedly influences transcriptional synergy (data not shown), a finding consistent with the relatively fixed distance between GATA and GT/CACC binding sites in HS3 of the β-globin LCR and several erythroid cell-specific promoters, and also with the effects of altering spacing of GATA and CACC sequences in the porphobilinogen deaminase promoter (16). Maximal promoter activity is achieved when GATA-1 and Sp1 are positioned on the same face of the DNA helix. Taken together with the apparent cooperative binding of GATA-1 and Sp1 on the EpoR promoter fragment in vitro (data not shown), these findings suggest that the proteins contact each other when bound to DNA and this interaction is required for synergistic activation of transcription. How such interactions would lead to synergy is uncertain. It is plausible that interactions of the proteins bound to DNA induce a conformational change in either or both proteins, which then alters their ability to interact with components of the basal transcriptional machinery. Alternatively, interaction of GATA-1 and Sp1 might generate a new activation domain with increased affinity for the same or different targets in the basal complex. Previous studies have established that the interaction of Sp1 with $TAF_{II}110$ suffices for activation of transcription (17). Thus, we can envision that the GATA-1–Sp1 complex interacts more efficiently with $TAF_{II}110$ because GATA-1 causes a conformational change in Sp1 and/or directly contacts $TAF_{II}110$. Alternatively, the GATA-1–Sp1 complex may contact multiple targets of the basal complex, resulting in synergistic activation of transcription. Additional experimental approaches will be required to develop these models further.

Physical interaction between GATA and Krüppel factor families. The functional cooperation between GATA-1 and Sp1 or EKLF led us to investigate potential physical interactions. We observed that the zinc finger domains of GATA-1 and the two Krüppel factors, indeed, associate in vitro (Fig. 2A and D; Fig. 3A). Although either finger of GATA-1 associates with Sp1, the major activity appears to reside in the C finger (Fig. 6A and B). While we had not anticipated that the GATA-1 zinc finger domain would be involved in protein-protein interactions, the finding that a DNA-binding domain participates in physical associations of this kind is not unprecedented. For example, the zinc fingers of Sp1 directly interact with YY1 (a Krüppel-related factor) (30) and NF-κB (40), and YY1 and NF-κB interact with Sp1 through zinc finger and Rel homology domains, respectively. Furthermore, viral transactivators, such as E1A and Tax, exert their effects on cellular activators via direct protein-protein interactions with the DNA-binding regions of the activators (32, 55).

Our findings apply more generally to members of the GATA family of proteins, as GATA-2 and GATA-3 also associate with Sp1, ostensibly also through their finger domains. Perhaps this is not unexpected given the extensive similarity of GATA protein finger domains (60, 61; reviewed in reference 38). Nonetheless, these data suggest that functional interactions between GATA and GC- or GT/CACC-binding factors in nonerythroid cells are also likely to be relevant. In accord with this, we note, for example, the presence of GATA and CACC motifs in the T-cell receptor α gene, which is believed to be acted upon by GATA-3 (57). Thus, the functional interaction between GATA and Krüppel family proteins may be employed broadly.

Although the use of a DNA-binding domain as a protein interaction region might appear paradoxical, it may reflect evolutionary economy, in that protein-protein interaction in-

terfaces between different transcription factor families (e.g., GATA and Krüppel) would be conserved and the need for diverse interaction surfaces would be minimized. Our experiments also reveal that the tertiary structure of the C finger of GATA-1, although indispensable for DNA binding, is not required for physical interaction with Sp1. Thus, the protein-protein interaction domain is distinct from, but overlaps, the DNA-binding domain. We are led to propose, therefore, that the zinc finger region of GATA-1 is multifunctional, as it specifies not only DNA recognition but also interactions with other transcription factors (e.g., Sp1 and EKLF), self-association (9a), and possibly transcriptional activation (59).

The notion that the finger region of GATA-1 may confer functions beyond DNA binding alone is supported by recent findings in two different biological systems. First, partial rescue of the erythroid developmental block of GATA-1$^-$ embryonic stem cells following introduction of various GATA factor constructs correlates with the presence of a competent finger region rather than with a specific activation domain (6). Second, megakaryocytic differentiation seen in myeloid 416B cells upon forced expression of GATA factors minimally requires a DNA-binding domain, derived from either GATA-1 or GATA-2 (54).

Implications of protein-protein interactions with GATA-1 for globin gene control. Our results bear significance not only for the functional cooperation between GATA-1 and Sp1-like (or EKLF) factors but also for long-range transcriptional control. A central, unresolved aspect of globin gene regulation is how LCRs exert effects over more than 50 kb of DNA in the α- or β-globin loci. It is generally envisioned that chromosomal looping brings together two kinds of complexes: one of proteins assembled on the promoters (or enhancers) of the downstream genes and the other formed by binding of proteins to the LCR cores (see reference 10 for a review). The specificity and stability of such LCR-gene associations are likely to be mediated by protein-protein interactions of the type described herein, as numerous binding sites for GATA-1 and Sp1 exist in functionally relevant regions (see the introduction). Our observation that GATA-1–Sp1 synergistic transcriptional activation occurs even when binding sites are well separated on test plasmids (Fig. 8) provides an experimental basis for this model. In the accompanying report (9a), we have also shown that GATA-1 self-associates through its finger region. Thus, we propose that GATA-1–Sp1, Sp1-Sp1, and GATA-1–GATA-1 associations mediate or stabilize important long-range interactions. Given the fine-tuned developmental regulation of the genes in the β-globin locus, it is assumed that additional factors will also interact with GATA-1, Sp1, and their respective protein complexes. Within this context, one can envision how subtle changes in other nuclear proteins could alter relative affinities of LCR-gene promoter interactions and lead to hemoglobin switching in development in a manner consistent with the enhancer competition model of gene switching developed by Choi and Engel (8).

In this context, it is interesting to consider specific situations in which different GATA-1 protein interactions might be associated with developmental switching of globin gene expression. Silencing of the embryonic β-like ε-globin gene during fetal life appears to be mediated in part by a promoter region that binds both GATA-1 and the Krüppel-related protein YY1 (20). In light of GATA-Krüppel factor interactions, we speculate that physical interactions between GATA-1 and YY1 may be involved in stage-specific repression. The promoter of the human β- but not γ-globin gene contains a strong binding site for EKLF. As we have shown in other studies, the adult β-globin gene is likely to be a direct in vivo target of EKLF (39a). Thus, we raise the possibility that interactions of LCR-bound GATA-1 with β-globin promoter-bound EKLF serve to stabilize β-globin expression at the adult stage. Additional experiments are needed to address these speculations directly.

Some of the interactions and functional consequences reported herein appear to require high-level expression of GATA-1 and Sp1 or EKLF. For example, superactivation of promoter-bound Sp1 with a mutant GATA-1 defective in DNA binding (Fig. 7B) or tethering of GATA-1 or Sp1 to promoter-bound Sp1 or GATA-1, respectively (Fig. 8), is demonstrable only at a high input level of effector plasmids. Although it might be argued on this basis that physical interactions of these proteins would be of minor in vivo relevance, we doubt that this is the case. While the actual local concentrations of these proteins in complexes of LCRs and promoters cannot be determined, they are likely to be substantial. First, GATA-1 is an abundant protein in maturing erythroblasts (see reference 38 for a review). Second, the frequency of GATA- and Sp1-like-binding sites in critical *cis* elements is sufficiently high (see the introduction) as to provide a considerable concentration of each within the local environment. Finally, the ability of Sp1 to recruit GATA-1 to a promoter seems highly specific for GATA-1, since Sp1 does not recruit NF-κB although these two proteins physically interact (40). Thus, protein-protein interactions on and off DNA are likely to mediate LCR-promoter associations and then further stabilize complexes as local concentrations rise.

In summary, we have defined physical and functional interactions between the erythroid transcription factor GATA-1 and two Krüppel family proteins, Sp1 and EKLF, that provide a mechanistic basis for communication of *cis*-regulatory elements distributed over large distances of globin, or other, gene loci. As additional transcription factors which display gene- or stage-specific properties are identified, it will be possible to assess how they may modify these interactions and contribute to the switching of globin genes during development.

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