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Enhancer-Dependent Transcription of the ε-Globin Promoter Requires Promoter-Bound GATA-1 and Enhancer-Bound AP-1/NF-E2

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We analyzed ε-globin transcription in erythroid cells and in erythroid extracts to determine the requirements for enhancer-dependent expression of this gene. Mutations that abolished GATA-1 binding at a single position in the promoter prevented interaction with enhancers, whereas elimination of a second more distal promoter GATA-1 site had no effect. Deletion or mutation of the GATA-1 sites in either the human β-globin locus control region DNase-hypersensitive site II enhancer or the chicken β^A/ε-globin enhancer did not diminish the ability of the enhancers to interact with the promoter. In contrast, mutation of the AP-1/NF-E2 sites in these enhancers resulted in elimination of enhancement. In vitro transcription of these constructs was promoter dependent and was not sensitive to abolition of GATA-1 binding in the promoter, consistent with the role of GATA-1 solely as a mediator of the enhancer effect. Thus, GATA-1 regulates the response of the ε-globin gene to enhancers through a specific site in the promoter and requires enhancer AP-1/NF-E2 binding to transduce the enhancer effect on transcription.

Differentiation in erythroid cells involves activation of the β-globin locus, followed by sequential expression of individual globin genes during development (6, 39). The β-globin locus control region (LCR), a region upstream of the β-globin cluster that contains a series of erythroid cell-specific and developmentally stable DNase-hypersensitive sites (11, 43), is normally required for activation of all of the genes. The LCR confers position-independent, copy number-dependent expression on linked human β-globin genes in transgenic mice (15). DNase-hypersensitive site II (HS II) appears to be necessary and sufficient for full LCR activity in transgenic mice (22, 27). This region of the LCR contains multiple protein binding sites (41) and can act as an enhancer in transient expression studies (29, 44). How elements of the LCR interact with individual globin promoters in the context of chromatin in the nucleus is still a matter of speculation.

Two lineage-specific proteins, GATA-1 and NF-E2, have been implicated in control of the expression of erythroid globin and nonglobin genes (30). GATA-1 is an early participant in regulation of the β-globin locus; the GATA-1 gene is required in vivo for development of cells of the erythroid lineage (31). The promoters or enhancers of virtually all erythroid cell-specific globin and nonglobin genes contain GATA-1 sites (6). NF-E2 is a less well characterized erythroid member of the AP-1 family of proteins (25). It is thought to interact with AP-1 sites in the human α- and β-globin LCRs, in the chicken β^A/ε-globin enhancer, and in the promoter of the nonglobin erythroid porphobilinogen deaminase gene (25, 28, 35, 40).

The ε-globin gene is the first of the human β-like globin genes to be expressed during development. Appropriate, stage-specific expression of the gene in transgenic mice was dependent on the presence of the β-globin LCR enhancer (32, 38). We have shown that a GATA-1 site in the human ε-globin promoter is required to interact with the chicken β^A/ε-globin enhancer (14). This enhancer contains two positive-acting domains consisting of duplicated GATA-1 sites and an AP-1 motif (35). The requirement for both a promoter and an enhancer GATA-1 site implies that GATA-1 could mediate the interaction, perhaps through dimerization. However, the human β-globin LCR HS II enhancer may possess only one positive-acting domain consisting of the duplicated AP-1/NF-E2 motifs (1a, 21, 26, 29). In this study, we sought to determine specifically what enhancer motifs were required by this promoter to establish high-level, erythroid cell-specific transcription.

In transient assays, mutation of the GATA-1 site at −165 in the ε-globin promoter abolished the effect of the human β-globin LCR HS II enhancer and of the chicken β^A/ε-globin 3′ enhancer. Mutations introduced into the enhancers showed that the binding of AP-1 (or an AP-1-like transcription factor, such as NF-E2) is necessary for enhancement, while the binding of GATA-1 is not. These requirements to achieve GATA-1 regulation of the response of the ε-globin promoter to enhancers suggest a role for multimeric interaction of transcription factors involving members of different regulatory subgroups.

MATERIALS AND METHODS

Plasmid constructions. The construction of pεCAT has been described elsewhere (14). It contains a 292-bp ε-globin promoter fragment (to position −274) fused to chloramphenicol acetyltransferase (CAT) gene coding sequences and includes the simian virus 40 splice and polyadenylation signal. Clustered point mutations were created at either the −165 or −213 GATA-1 site as described previously (18). Unique restriction enzyme sites from the pUC19 multiple cloning site (*Xba*I to *Hin*dIII) remained 3′ to the CAT gene and were used to insert enhancer fragments, placing them approximately equidistant from the promoter in either the 5′ or 3′ direction. The 585-bp *Bst*NI LCR HS II fragment (GenBank coordinates 8571 to 9156) was filled in with

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Klenow enzyme and blunt-end ligated into a similarly filled *Acc*I site in pεCAT. The *Hin*dIII-*Xba*I 374-bp LCR HS II fragment and mutants thereof were ligated between the *Hin*dIII and *Xba*I sites. The chicken β^A/ε-globin 3′ enhancer fragment and mutants thereof (a kind gift of M. Reitman) were 137-bp synthetic fragments with *Xba*I termini (35) and were ligated into the pεCAT *Xba*I site.

Transient expression assays. All conditions for growth and electroporation of K562 cells were as described previously (14). Cells were transfected with 10 μg of supercoiled plasmid and were cotransfected with 3 μg of a plasmid expressing β-galactosidase as a control for efficiency of transfection. Cell extracts were assayed after 48 h for CAT and β-galactosidase activities by standard methods (36).

Protein extracts. Extracts used for transcription in vitro were prepared from K562 cells according to the procedure of Dignam et al. (2). All extracts contained between 5 and 15 μg of protein per ml (determined by the Bradford assay) and were stored at −70°C in 20 mM N-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–0.1 M KCl–0.2 mM EDTA–0.5 mM dithiothreitol–20% (vol/vol) glycerol.

In vitro transcription. Reaction mixtures contained 500 ng of template DNA and were incubated with various amounts of transcription extract (see text) in a buffer containing (final concentrations) 12.5 mM HEPES (pH 7.6), 100 mM potassium glutamate, 6.7 mM $MgCl_2$, 0.03 mM EDTA, 2.5 mM dithiothreitol, 5% glycerol, and 0.5 mM ribonucleoside triphosphates (rNTPs) at 21°C for 60 min. For inhibition experiments, the oligonucleotide competitors were allowed to preincubate with the extract before transcription was initiated by addition of rNTPs. Reactions were purified by phenol-chloroform extraction and ethanol precipitation before analysis by primer extension. Human ε-globin promoter-initiated transcripts were hybridized to a ^{32}P-labeled oligonucleotide primer complementary to an RNA sequence in the body of the CAT gene. Primer extension was performed essentially as described in technical literature from Promega (Madison, Wis.). The products were separated on 8% acrylamide–7 M urea gels (16 by 8 cm) at 250 V for 2 h and exposed to film overnight at −70°C with an intensifying screen.

Oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems 380B synthesizer, purified, and annealed to the double-stranded form as described previously (14). ε-Globin promoter oligonucleotides contained the genomic sequences indicated in Results. The sequence of competitor oligonucleotide GATA (upper strand) is TAAC CACTAGTGAGATTGCTGGATTGTATGATAGTTCTGTT TTTA. This sequence appears 3′ to the ε-globin gene and is a strong in vitro binding site for GATA-1 (13a). Oligonucleotides containing Sp1 and AP-1 binding sites were as previously described (14).

RESULTS

GATA-1 binding at −165 but not at −213 mediates enhancer responsiveness of the ε-globin promoter. In earlier studies, we observed that the chicken β^A/ε-globin 3′ enhancer, which activates both the β^A- and ε-globin genes at different times in chick development, could activate the human ε-globin promoter in transient expression assays (14). A GATA-1 binding site in the ε-globin promoter at position −165 was involved in this response. To further elucidate the role of GATA-1 in high level erythroid cell-specific transcription of the ε-globin gene, we studied the effect of the human LCR HS II enhancer on reporter gene activity.

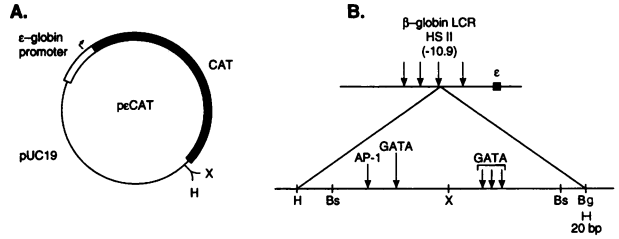

FIG. 1. (A) Expression vector pεCAT. Enhancer fragments were subcloned into restriction sites between the *Hin*dIII and *Xba*I sites of the pUC19 multiple cloning site at the 3′ end of the CAT gene sequences. (B) Positions of the four DNase I-hypersensitive sites present in the β-globin LCR. HS II is 10.9 kb upstream of the ε-globin coding sequences (filled box); this region is expanded to show the locations of restriction enzyme sites used to construct the expression vectors and the positions of AP-1 and GATA consensus sequences. H, *Hin*dIII; Bs, *Bst*NI; X, *Xba*I; Bg, *Bgl*II.

The abilities of the two enhancers to stimulate transcription from the ε-globin promoter were compared in vectors containing the promoter fused to the CAT gene, after transfection into human K562 erythroid cells that normally express the ε-globin gene. The parent vector, pεCAT, is illustrated in Fig. 1A. Enhancers were inserted into *Xba*I and/or *Hin*dIII sites at the 3′ end of the CAT gene.

The *Hin*dIII-to-*Xba*I HS II fragment (Fig. 1B) of the human β-globin LCR stimulated reporter gene expression about 100-fold over the enhancerless construct (Fig. 2A),

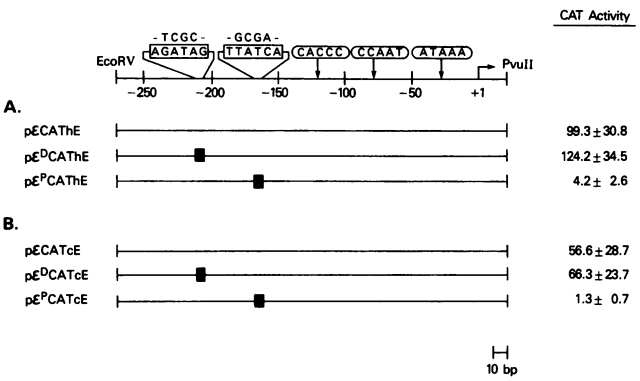

FIG. 2. Effects of promoter mutations on enhancer-driven CAT activity. The promoter of the ε-globin gene is diagrammed at the top. The positions of CACCC, CCAAT, and ATAAA sequence motifs are indicated by arrows. The GATA-1 consensus sequences at −165 and −213 are expanded, and shown above them are the clustered point mutations introduced that abolish binding of GATA-1 (14). K562 cells were transfected with plasmids, and CAT activity was determined. CAT activities indicated are relative to the activity given by pεCAT (set at 1), the enhancerless parent vector. The values represent the mean and standard error of at least three separate transfections done in triplicate within each experiment. (A) CAT activity of a construct containing the human LCR HS II enhancer *Hin*dIII-to-*Xba*I fragment (pεCAThE), and the values obtained when either the proximal (−165; $p\varepsilon^P$CAThE) or distal (−213; $p\varepsilon^D$CAThE) GATA-1 binding site was mutated (indicated by filled boxes). (B) CAT activity of pεCATcE containing the chicken β^A/ε-globin 3′ enhancer, and the effect on CAT activity of mutations abolishing each of the promoter GATA-1 sites as in panel A (indicated by filled boxes).

about twice the stimulation observed with the chicken β^A/ε-globin enhancer (Fig. 2B) (14). The chicken enhancer was not active in nonerythroid HeLa cells (1.5 ± 1.1 compared with the enhancerless plasmid [14]). The human LCR HS II enhancer increased CAT activity by 6.3 ± 2.1-fold over the enhancerless construct in HeLa cells (data not shown). A low level of activity of the β-globin LCR HS II enhancer in nonerythroid cells has been observed by others (29).

Clustered point mutations that eliminate the binding of GATA-1 (14) to each of its ε-globin promoter sites (indicated by filled boxes in Fig. 2) were studied in the presence of the enhancers. Elimination of the promoter-proximal GATA-1 site (−165; pε^{P}CAThE and pε^{P}CATcE) caused a greater than 90% decrease in CAT activity produced by either enhancer-containing plasmid. In contrast, transcription from this promoter was not diminished by the corresponding mutation at the more distal GATA-1 site (−213; pε^{D}CAThE and pε^{D}CATcE). Thus, GATA-1 binding uniquely to the −165 site in the ε-globin promoter mediates promoter-enhancer interaction.

This result is interesting since both sites are perfect consensus sequences for GATA-1 and both bind the factor in vitro (14). Oligonucleotides containing the sites can compete for the binding of GATA-1 to themselves as well as to the other site. Our direct binding and competition studies suggest that the affinities of the two sites for GATA-1 in vitro do not differ significantly (data not shown).

Enhancer binding of AP-1/NF-E2, but not GATA-1, is required for enhancer-dependent transcription. The enhancer activity of the β-globin LCR HS II resides on a fragment spanning *Hin*dIII and *Bgl*II restriction sites (29, 44). We found that a 585-bp *Bst*NI fragment, comprising most of the *Hin*dIII-to-*Bgl*II sequences (Fig. 1B), increased CAT activity from pεCAT 107.1 ± 47.8 times relative to the enhancerless plasmid in K562 cells. This activity does not differ significantly from the activity of the *Hin*dIII-to-*Xba*I fragment studied in the experiments reported in Fig. 2 (99.3 ± 30.8). Thus, a substantial 3′ deletion of the HS II enhancer fragment, including the loss of three potential GATA-1 binding sites (see Fig. 1B), had virtually no effect. Ney et al. made a similar observation in experiments in which the γ-globin promoter was driving a reporter gene (29).

The *Hin*dIII-to-*Xba*I HS II enhancer fragment contains duplicated AP-1/NF-E2 motifs and a single GATA-1 consensus site that binds GATA-1 in vitro (not shown). We mutated these sites independently and studied the effects on reporter gene activity driven by the ε-globin promoter. Figure 3 summarizes the results. Clustered point mutations at the GATA-1 site in the enhancer did not diminish reporter gene activity. In contrast, we found that the AP-1 mutations resulted in elimination of enhancement. The AP-1 sites were necessary for enhancer activity in other studies in which the human γ-globin promoter was driving the reporter gene (29) and in transgenic mice (1a). The results suggest that the ε-globin promoter does not require an enhancer GATA-1 site in order to interact with the enhancer.

The chicken β^A/ε-globin 3′ enhancer also contains an AP-1 site as well as tandem and inverted GATA-1 sites. Reitman and Felsenfeld (35) previously showed that in avian erythrocytes, the two regions of the enhancer (DNase footprints II and IV) which contained these motifs, each mediated a part of the positive-acting effect increasing CAT activity driven by the chicken β-globin promoter. Mutations at both sites II and IV were required to eliminate enhancer activity, whereas mutations at sites I and III had little effect. To investigate which elements of this enhancer were involved in

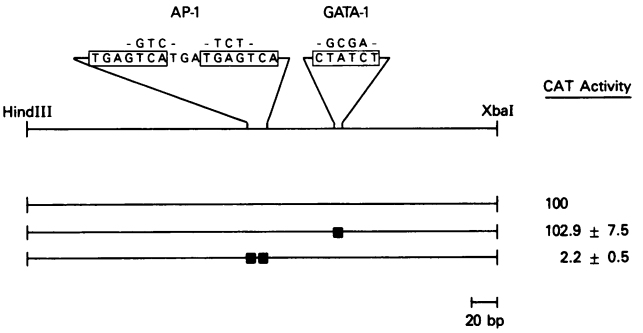

FIG. 3. Effects on CAT activity of mutations in the human β-globin LCR HS II enhancer. The *Hin*dIII-to-*Xba*I fragment of the enhancer is shown at the top. Sequences at the AP-1/NF-E2 and GATA-1 sites are expanded, and the point mutations used to abolish binding at these sites are given above the sequences (14, 29). K562 cells were transfected with plasmids, and CAT activity was determined. CAT activities obtained with the mutant enhancers are given relative to the activity of the unmutated enhancer, which was given a value of 100. Mutations are indicated by filled boxes. Other details are as in the legend to Fig. 2.

stimulating transcription from the ε-globin promoter, we used a series of mutated enhancers (Fig. 4). Mutations in sites I and III, as well as site IV, the duplicated GATA-1 sites, had virtually no effect upon CAT transcription from the human ε-globin promoter in K562 cells. However, mutations in region II that eliminated the AP-1 consensus site reduced activity to a very low level (13 ± 8.9, with the activity of the unmutated enhancer set at 100). We conclude that both human and chicken erythroid enhancers depend on AP-1 sites to effect enhanced transcription from the ε-globin promoter.

Mutation of the proximal promoter GATA-1 site does not affect in vitro promoter function. GATA-1 binding at −165 appears to mediate enhancer activation of the ε-globin promoter in vivo. We had previously observed in transient expression assays that in the absence of an enhancer,

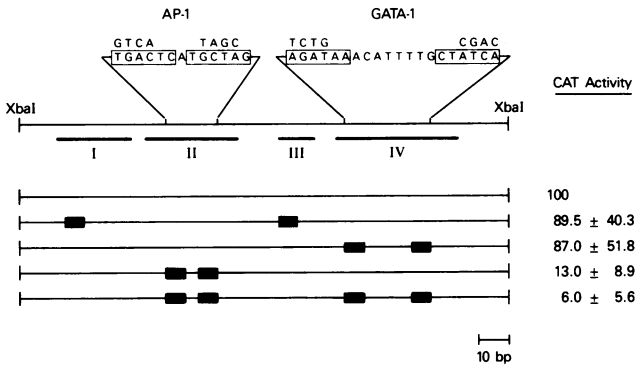

FIG. 4. Effects on CAT activities of mutations in the chicken β^A/ε-globin 3′ enhancer. The enhancer is diagrammed at the top. The roman numerals indicate regions containing DNase footprints as determined previously (3). Reitman and Felsenfeld (35) created mutations at the sites indicated by filled boxes. The sequences at regions II and IV are expanded at the top, and the mutations at these sites which eliminate the binding of GATA-1 and AP-1 (or a related factor such as NF-E2) are indicated. CAT activities of the mutant enhancers are given relative to the activity of the unmutated enhancer, which was set at 100. Other details are given in the legend to Fig. 2.

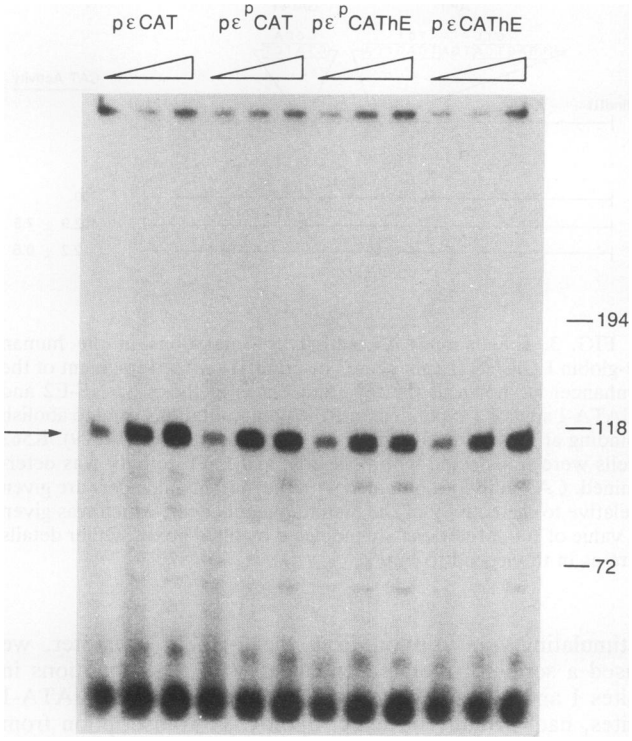

FIG. 5. In vitro transcription of ε-globin promoter templates. Reaction mixtures contained 500 ng of the DNA templates indicated above the lanes. Transcription was performed with increasing amounts of K562 nuclear extract as indicated by the wedge over each group of lanes (1, 2.5, or 4 μl of K562 transcription extract at a protein concentration of 8.6 μg/μl; see Materials and Methods). Primer extension was performed with a ^{32}P-labeled primer, and the products were analyzed on a denaturing acrylamide gel. The arrow indicates the position of primer extended to the correct ε-globin cap site (120 nucleotides). Positions of migration of DNA size markers are shown on the right in nucleotides.

mutation of the −165 promoter GATA-1 site did not affect reporter gene activity (14). Since expression in vivo was low in the absence of an enhancer, we used in vitro transcription of the templates constructed for expression studies to further explore this question. Transcription was performed with cell extracts from human erythroid K562 cells. Primer extension products terminating at the correct ε-globin transcription initiation site have an expected length of 120 nucleotides.

Abundant transcription was directed by the pεCAT template in various amounts of K562 cell extract (Fig. 5). The presence of the human β-globin LCR HS II enhancer (pεCAThE) did not further increase transcription over that obtained with the enhancerless template. Under these conditions, abolition of the −165 GATA-1 site in the promoter did not diminish transcription, either in the absence or in the presence of the enhancer (pε^{P}CAT and pε^{P}CAThE). The result supports the idea that GATA-1 binding at −165 is required for promoter-enhancer interaction but that GATA-1 does not act as a transcription factor when transcription is entirely promoter dependent. This observation is consistent with our earlier finding that expression from the enhancerless promoter in vivo is not affected by mutations at −165 eliminating GATA-1 binding (14).

Core promoter elements control in vitro transcription of the ε-globin gene. We used inhibition of transcription by oligonucleotides containing transcription factor binding sites to further study the requirements for in vitro transcription of the ε-globin templates. Increasing amounts of oligonucleotides containing either a GATA-1 site, an Sp1 site, or the CCAAT site from the human ε-globin gene promoter were incubated in the K562 cell transcription extract prior to addition of the template. The amounts of competitor used represented a 20- to 60-fold molar excess over template for the GATA oligonucleotide and the CCAAT oligonucleotide and a 50- to 150-fold excess for the Sp1 oligonucleotide.

In Fig. 6A, transcription of pεCAThE in the absence of added oligonucleotides is shown in the far left lane. Correctly initiated transcripts originating from the ε-globin promoter yield a primer extension product 120 nucleotides in length (arrow). Oligonucleotides containing GATA-1 and Sp1 binding sites did not inhibit transcription. However, transcription from the correct initiation site was inhibited by an oligonucleotide containing the ε-globin CCAAT site. A shorter transcript, initiated in plasmid sequences of the vector, results under these conditions (Fig. 6A, lanes at the right).

As shown in Fig. 6B, each region of the ε-globin promoter, from position +3 to position −274, was tested for its ability to inhibit in vitro transcription. The primary motif contained in the promoter oligonucleotide added is shown at the top of each lane of Fig. 6B; −213 and −165 indicate oligonucleotides containing the distal and proximal promoter GATA-1 sites, respectively. The protein-DNA interactions at these motifs were previously determined by DNase I footprinting and gel mobility shift experiments (14). The amount of oligonucleotide used (300 ng) represented a 60- to 150-fold molar excess over the amount of template, depending on the size of the oligonucleotide.

Transcription of pεCAThE in a K562 extract, in the absence of added oligonucleotides, is shown in the lane on the left in Fig. 6B. Oligonucleotides containing the core promoter elements, CACCC, CCAAT, and ATA, inhibited transcription from the correct initiation site, presumably by sequestering critical components of the transcription extract. The oligonucleotides containing the upstream GATA-1 binding sites (−213 and −165) did not inhibit transcription. The concentration of GATA-1 in the transcription extract is unknown and may be in excess of the concentration of the components interacting with the core promoter elements during transcription. However, in other experiments (not shown), we have used more than a 300-fold excess of the upstream GATA-1-containing oligonucleotides over template and have seen no inhibition.

An oligonucleotide containing the sequences at the start site for transcription (labeled +3) inhibited transcription from this site severely. This region contains a weak in vitro GATA-1 site (14). However, mutation of the central GATA nucleotides of the motif to TGCG (which abolishes GATA-1 binding) did not alter the inhibition, as shown in the lane marked +3M in Fig. 6B. The basis for inhibition by this oligonucleotide is not clear, since the only other protein-DNA complex that we observed on this region of the promoter was with Sp1 (14), and we see from Fig. 6A that an oligonucleotide containing an Sp1 site does not affect transcription. The latter result also suggests that the inhibition seen with the CACCC oligonucleotide is not due to the binding of Sp1, one of the two proteins that interact at this site in the promoter (14, 46).

Similarly, sequestering of AP-1 did not diminish transcription (Fig. 6B). AP-1 has a consensus site in the ε-globin promoter that overlaps with the CCAAT site, and we have

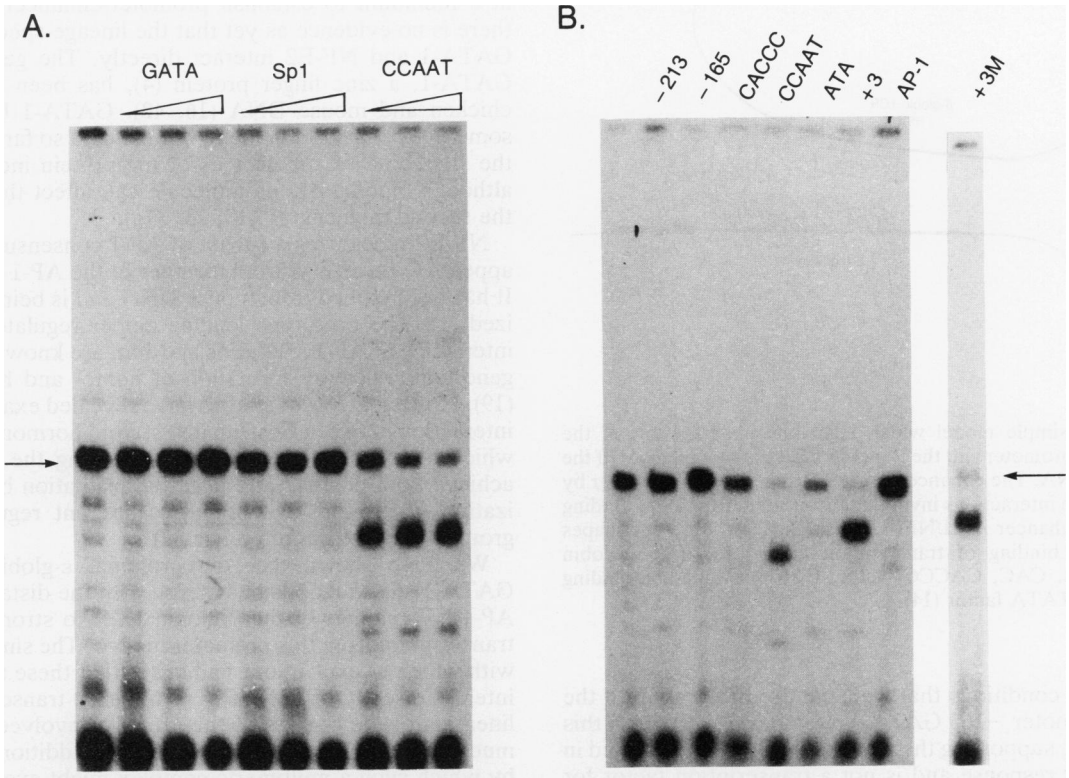

FIG. 6. Analysis of regions of the ε-globin promoter important for in vitro transcription of the promoter. (A) Transcription reaction mixtures contained 500 ng of pεCAThE template DNA and 2.5 μl (21 μg of total protein) of K562 transcription extract. Reaction mixtures were incubated with increasing amounts (100, 200, or 300 ng) of the oligonucleotide competitors indicated above the lanes before transcription was initiated. The lane at the left contained a sample with no added competitor. Primer extension was performed with a ^{32}P-labeled primer, and the products were analyzed on a denaturing acrylamide gel. The sequence of the GATA oligonucleotide (45-mer) is given in Materials and Methods. The CCAAT oligonucleotide contained ε-globin promoter sequences between −50 and −93. The Sp1 oligonucleotide was a 22-mer (14). (B) Transcription reactions under the same conditions as in panel A. The lane at the left represents no added competitor. Other samples contained 300 ng of oligonucleotide competitors as indicated above the lanes by the major motif present in each. ε-Globin promoter oligonucleotides: −213, sequence from −179 to −225 containing the distal GATA-1 binding site; −165, sequence from −135 to −178 containing the proximal GATA-1 binding site; CACCC, sequence from −94 to −134; CCAAT, sequence from −50 to −93; ATA, sequence from −9 to −49; +3, sequence from +26 to −8; AP-1, a 16-mer AP-1 consensus site (14); +3M, ε-globin promoter sequence from +26 to −8 with GATA nucleotides mutated to TGCG (see text).

previously shown that AP-1 can bind to this site if the CCAAT-box-binding protein is not present (14). Thus, promoter-dependent transcription was not mediated by GATA-1 binding at the promoter; it involved the CACCC, CCAAT, and ATA elements of the ε-globin promoter but required neither Sp1 nor AP-1, even though these transactivators can bind to the promoter in vitro (CACCC and CCAAT sites respectively [14]).

DISCUSSION

For the ε-globin promoter to functionally interact with its enhancer, it appears that only one of the two GATA-1 binding sites in the ε-globin promoter is required. The precise sequence of the recognition site or flanking DNA or the directionality of the site may give the −165 GATA-1 site an attribute not possessed by the −213 site. Alternatively, the position of the GATA-1 site in relation to other promoter elements may be important for its function. The GATA-1 site at position −165 has been preserved in the promoters of all the mammalian embryonic globin gene sequences available in GenBank (14).

In some cases, the functional role of GATA-1 sites in transcription of erythroid genes has been demonstrated by mutations abolishing GATA-1 binding (14, 23, 24, 35, 42, 45). However, similar to what we have observed, not all GATA-1 sites in regulatory regions of erythroid genes are functional. For example, in the promoter of the GATA-1 gene itself, at least one of three GATA-1 sites appears to be unimportant for transcription of the gene (37a). Likewise, four GATA-1 sites in the β-globin LCR enhancer do not seem to contribute to the positive-acting effect of the enhancer in transgenic animals (21), even though these enhancer GATA-1 sites are footprinted in vivo and thus presumably are bound by the factor (17, 33).

In vitro transcription from the ε-globin promoter was not responsive to the effect of an added enhancer. A similar observation was made for the chick β^A-globin promoter (10). In these experiments the template was naked DNA, whereas evidence has been provided that transfected DNA can be assembled in the nucleus into nucleosome-containing minichromosomes (34). The LCR enhancer effect may be manifest only in the context of chromatin, consistent with the recent proposal that the LCR acts to keep promoters free of nucleosomes (8). Nevertheless, the requirements for promoter-dependent transcription can be delineated under the

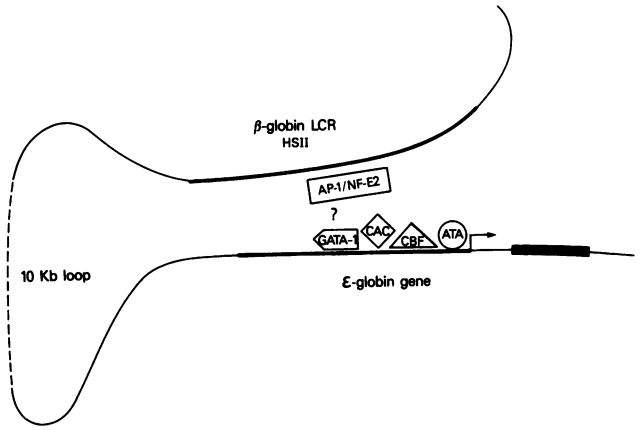

FIG. 7. A simple model which envisions juxtaposition of the ε-globin gene promoter and the β-globin LCR with looping out of the intervening DNA. The enhancer may be targeted to the promoter by protein-protein interactions involving the promoter GATA-1 binding site and the enhancer AP-1/NF-E2 sites. Boxes of different shapes represent the binding of transcription factors to these ε-globin promoter sites. CAC, CACCC factor; CBF, CCAAT-box-binding factor; ATA, TATA factor (14).

transcription conditions that we have used. Mutation of the ε-globin promoter −165 GATA-1 site does not diminish this transcription, supporting the idea that GATA-1 is involved in the enhancer response and is not a transcription factor for the promoter alone. This view is consistent with the observation that high levels of transactivation of the chicken α^D-globin promoter in erythroid cells by GATA-1 required the addition of an enhancer or multiple GATA-1 sites (5).

A study of chicken β^A-globin gene transcription has shown that this gene may interact with its enhancer via a promoter GATA-1 site as well. The promoter contains a specialized TATA motif which binds GATA-1, and the binding of GATA-1 to this site is necessary for enhancer-dependent transcription (10). In this case, functional GATA-1 binding sites in the enhancer are also required to mediate promoter-enhancer interaction, since it has been shown that enhancer GATA-1 sites contribute fully half of the positive-acting effect of the chicken β^A/ε-globin enhancer (35). These same GATA-1 sites were not required for the chicken enhancer to exert its effect on transcription from the human ε-globin promoter. Instead, for the ε-globin promoter, the positive-acting regions of both the human and chicken globin enhancers consisted solely of AP-1/NF-E2 motifs. Thus, distinct patterns of interaction of regulatory proteins may be utilized by different globin promoter-enhancer combinations.

Do enhancer-binding proteins mediate the enhancer effect by interaction with proteins that bind near the transcription start site? Figure 7 illustrates a simple mechanism by which the enhancer- and promoter-binding proteins might interact, directly or indirectly, with looping out of the intervening DNA of more than 10 kb in length. Such a hypothetical interaction could be tested by using the model plasmids derived for the experiments described above. Loops formed by the interaction of nonhomologous promoter- and enhancer-binding proteins have been demonstrated in viral systems (20), and DNA looping is likely to be involved as a means of promoter-enhancer interaction in globin gene regulation (9, 10, 12).

Although the GATA-1 and AP-1/NF-E2 sites are required at a minimum to establish promoter-enhancer interaction, there is no evidence as yet that the lineage-specific proteins GATA-1 and NF-E2 interact directly. The gene encoding GATA-1, a zinc finger protein (4), has been cloned from chicken and mouse DNA (16, 42). GATA-1 binding sites sometimes appear in tandem, but the data so far suggest that the sites bind two molecules of the protein independently, although binding of one molecule can affect the binding of the second molecule (7, 13, 23, 37a).

NF-E2 recognizes a subset of AP-1 consensus motifs and appears to be an erythroid member of the AP-1 gene family. It has been cloned from mouse DNA and is being characterized (1). The prototype leucine zipper regulatory proteins interacting at AP-1 sites, Fos and Jun, are known to regulate gene expression by formation of homo- and heterodimers (19). However, recent studies have revealed examples of the interaction between Fos/Jun and steroid hormone receptors, which are zinc finger proteins, indicating the potential to achieve both positive and negative regulation by multimerization of proteins belonging to different regulatory subgroups (reviewed in reference 37).

We have shown that the proximal ε-globin promoter GATA-1 site functions in synergy with the distantly located AP-1/NF-E2 sites of the β-globin LCR to strongly activate transcription from this promoter in vivo. The simplest model with which to explain our findings is that these two proteins interact directly or indirectly to increase transcription. Delineation of the regions of the proteins involved will reveal much about the putative association. In addition, the means by which such a multimeric complex might exert a positive effect upon the transcriptional machinery remains to be explored.

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