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Transcriptional activation of human adult α-globin genes by hypersensitive site-40 enhancer: Function of nuclear factor-binding motifs occupied in erythroid cells

(human α-globin promoter/genomic footprinting/site-directed mutagenesis/transient expression)

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ABSTRACT **The developmental stage- and erythroid lineage-specific activation of the human embryonic ζ- and fetal/adult α-globin genes is controlled by an upstream regulatory element [hypersensitive site (HS)-40] with locus control region properties, a process mediated by multiple nuclear factor–DNA complexes. *In vitro* DNase I protection experiments of the two G+C-rich, adult α-globin promoters have revealed a number of binding sites for nuclear factors that are common to HeLa and K-562 extracts. However, genomic footprinting analysis has demonstrated that only a subset of these sites, clustered between −130 and +1, is occupied in an erythroid tissue-specific manner. The function of these *in vivo*-occupied motifs of the α-globin promoters, as well as those previously mapped in the HS-40 region, is assayed by site-directed mutagenesis and transient expression in embryonic/fetal erythroid K-562 cells. These studies, together with our expression data on the human embryonic ζ-globin promoter, provide a comprehensive view of the functional roles of individual nuclear factor–DNA complexes in the final stages of transcriptional activation of the human α-like globin promoters by the HS-40 element.**

Transcriptional regulation of single genes and multigene families in eukaryotes is mediated by the interaction of trans-acting factors with cis-acting DNA sequences present in enhancers and promoters (ref. 1 and the references therein). In the vertebrate globin genes, a group comprising two chromosomally distinct, yet coordinately expressed, gene families, α-like and β-like, this regulatory process reflects a remarkable degree of complexity in that expression is not only tissue-specific but also occurs in a developmental stage-specific manner (for review, see refs. 2–4). This pattern of expression is mediated by diffusible trans-acting factors (refs. 5 and 6 and the references therein), as well as by positive regulatory, enhancer-like elements located upstream of each globin gene cluster, termed locus control regions (LCRs) (for review, see refs. 7 and 8). Analysis of cis-acting regions located in the LCRs and in the promoters of various globin genes suggests that ubiquitous transcription factors may be acting in concert with a smaller array of tissue-specific and stage-specific proteins to effect transcriptional control (refs. 4, 9, and 10 and the references therein).

The human α-like globin gene cluster comprises an embryonic globin gene (ζ2), two fetal/adult genes (α1 and α2), and a gene of undetermined function (θ1), arranged over ≈30 kb in the order 5′-ζ2–α2–α1–θ1-3′ (for review, see ref. 11). An element located 40 kb upstream of the ζ2 gene is associated with a prominent DNase I hypersensitive site (HS) and acts as the major erythroid tissue-specific regulator of α-globin-like gene expression (12, 13). This element, termed HS-40, exhibited LCR-like characteristics in stably integrated MEL cells and transgenic mice (12, 14–16). The HS-40 also behaves as a classical enhancer in transient expression assays (17–19). *In vivo* and *in vitro* footprinting analyses indicated that several motifs in HS-40 bind the erythroid-enriched transcription factors GATA-1 and NF-E2, as well as several ubiquitous transcription factors such as Sp1 (13, 19, 20).

In contrast to the picture we have of the nuclear factor–DNA interactions that exist within the human HS-40 element, as described above, and in the embryonic ζ2 promoter (19, 21), our knowledge of such interactions occurring within the human α1 and α2 globin promoters is relatively sparse. In this regard, more is known about two other mammalian α-globin genes at the promoter level—namely, the mouse and rabbit α-globin promoters (22–25). To gain further insight into the mechanisms by which the fetal/adult expression of the human α-globin genes in erythroid cells is regulated by HS-40, we have carried out genomic footprinting of the human α-globin promoter. Based on this information and the previously obtained genomic footprints of HS-40, we then used site-directed mutagenesis and transient expression assay to determine the functional contribution of the individual nuclear factor-binding motifs to the transcriptional activation of human α-globin genes by HS-40 in the embryonic/fetal erythroid K-562 cell line.

MATERIALS AND METHODS

Genomic Footprinting Analysis. The binding of nuclear factors *in vivo* at the human α-globin promoters was analyzed by dimethyl sulfate (DMS) footprinting. The maintenance of different cell lines, the isolation of cells from human tissues, and the purification of adult human erythroblasts were done by using described procedures (10). Cells were then treated with DMS, and the genomic DNA was purified and subjected to piperidine cleavage (26). These cleavage sites were then mapped by the ligation-mediated PCR (LMPCR) (27, 28), using the same procedures as described (10, 29). However, due to the high G+C content (96%) of the human α-globin promoter DNA, dimethyl sulfoxide was included in some of the

Abbreviations: DMS, dimethyl sulfate; LCR, locus control region; LMPCR, ligation-mediated PCR; GH, growth hormone; hGH, human GH; HS, hypersensitive site; AF1, α-globin factor 1; CBF, CCAAT box-binding factor; NFI, nuclear factor I; α-IRP, α inverted repeat protein.

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buffers, and higher temperatures of denaturation/renaturation were used, as described elsewhere (30).

DNase I Footprinting by LMPCR. Analysis of nuclear factor binding to the human α-globin promoter *in vitro* was achieved by the combined use of DNase I digestion and LMPCR. Plasmid DNA probes (1–2 ng) containing the human α-globin promoter were digested with DNase I (Worthington, grade DPFF) in the presence of appropriate nuclear extracts (31) as described (21). The DNase I cleavage sites on the human α-globin promoter were then mapped by LMPCR as described above.

Plasmid Construction and Site-Directed Mutagenesis. All recombinant DNA work was done by using standard techniques (32). All transient expression studies used the human growth hormone (hGH) gene derived from pOGH as the reporter gene (33). To construct pB-α590GH, a 590-bp *Hin*dIII–*Dde* I fragment encompassing the α1-globin promoter region from −574 to +21 was first blunt-end ligated into the *Bam*HI site immediately upstream of the hGH gene in pOGH. The α1-globin promoter and the adjacent hGH gene were then excised as a single *Hin*dIII–*Eco*RI fragment, which was then subcloned into the *Hin*dIII–*Eco*RI sites within the polylinker of pBluescript II KS(−) to produce pB-α590GH.

Plasmid pB-HS40-α590GH was constructed by cloning a 350-bp HS-40-enhancer-containing fragment (13) as an *Eco*RI-*Hin*dIII fragment into the corresponding sites of the polylinker of pBluescript. The HS-40 enhancer element was then excised as an *Xba* I fragment and blunt-end ligated in the genomic orientation into the *Hin*dIII site of pB-α590GH. Site-directed mutagenesis of the α-globin promoter was done by using a commercially available kit (Stratagene). Specific oligonucleotide primers 5′-GGCTCCGCGCCAtatgAT-GAGCGCCGCC-3′, 5′-CCCGCGCAGGCCgaattCGG-GACTCCCCT-3′, and 5′-GTCCAGGCCGCGgtaCGGGCT CCGCGC-3′ were used to mutagenize the CCAAT box-binding factor (CBF), transcription factor Sp1, and α-globin factor 1 (AF1) sites in the α-globin promoter of pB-α590GH, thus producing pB-α590(CBF)GH, pB-α590(Sp1)GH, and pB-α590(AF1)GH, respectively. After mutagenesis, a wild-type HS-40 enhancer was cloned into each of these mutant promoter-carrying plasmids as described above for pB-HS40-α590GH, to generate the constructs pB-HS40-α590(CBF)GH, pB-HS40-α590(Sp1)GH, and pB-HS40-α590(AF1)GH.

Site-directed mutagenesis of the HS-40 enhancer was done as described in detail elsewhere (34). Each mutated HS-40 element was subsequently cloned into pB-α590GH, as described for the construction of pB-HS40-α590GH.

Transient Expression Analysis. Human α-globin promoter activity and HS-40 enhancer effect were analyzed by transient expression in transfected K-562 cell cultures. Procedures for DNA transfection, hGH assay, RNA quantitation by primer extension, and the use of cotransfected pSV2CAT as a control plasmid follow those described (19, 35). Experimental data are derived from multiple sets of DNA transfection experiments.

RESULTS

Genomic Footprinting of Human α-Globin Promoter(s). Within the α-globin promoter region from −200 to +40 (Fig. 1) there are four potential Sp1-binding sites (37), two other ubiquitous factor-binding sites, CBF and nuclear factor I (NFI) (38, 39), and an α-inverted repeat protein (α-IRP) motif first identified within the murine and rabbit α-globin promoters (23, 25). The rabbit α-IRP motif is identical to the human sequence and has been shown to bind a ubiquitous factor in addition to Sp1 (25). There are no putative binding sites for the erythroid-enriched factors GATA-1 (40) or NF-E2 (41).

An example of the genomic footprinting analyses is presented in Fig. 2, and the data are summarized in Fig. 1. Interestingly, only one of the four Sp1 motifs, located at −122 to −116, appears to bind nuclear factor(s), presumably Sp1, *in vivo*. This binding is evidenced by specific protection of guanidine residues at −120, −118, −117, −116, and hyperreactivity to DMS at G^{-121} in erythroid HEL cells, K-562 cells, and adult erythroblasts (lanes 3, 4, 7, 8, 11, and 12).

Similar to the Sp1 motif at −122 to −116, the two adjacent NFI and CBF motifs are also protected mainly in erythroid cells, although protection of guanidine residues −75 and −74 in K-562 cells (Fig. 2 *Left*, lane 3) is not as extensive as in the adult erythroblasts (Fig. 2 *Left*, lane 4). Protection of the α-IRP motif is weak but is, nevertheless, detectable only in the erythroid cells (Fig. 2) (30). In addition, a G+C-rich sequence, 5′-GCCGCGCCCC-3′, immediately upstream of the NFI motif, which we termed AF1, is well protected in erythroid cells (lanes 3, 4, 7, 8, 11, and 12; Fig. 2).

***In vitro* DNase I Footprinting by LMPCR.** We combined the use of DNase I digestion and LMPCR to analyze protein binding to the human α-globin promoters in nuclear extracts derived from erythroid K-562 cells and nonerythroid HeLa cells. As exemplified by the footprinting pattern in Fig. 3 for the noncoding strand and summarized in Fig. 1, similar regions

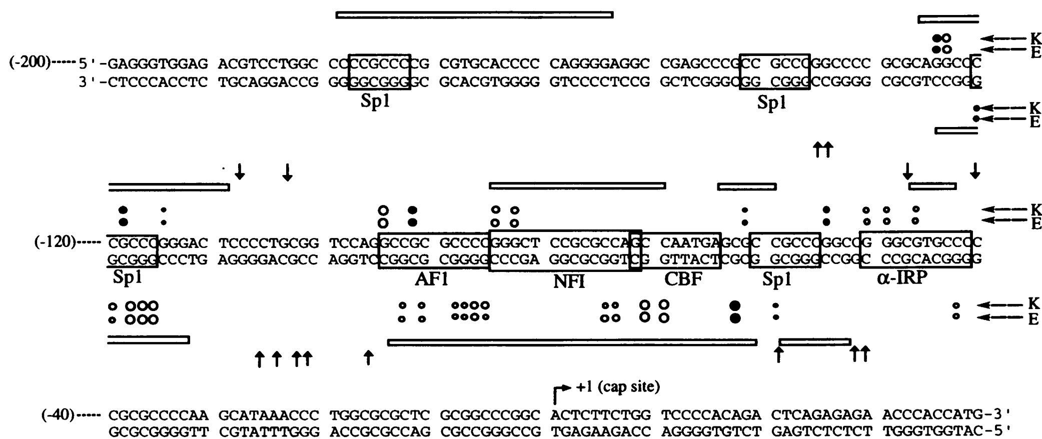


FIG. 1. Summary of binding sites of nuclear factors within the human α-globin promoter. Nucleotides are numbered relative to the cap (+1) site. Nuclear factor-binding motifs predicted on the basis of known consensus sequences are indicated by boxes. The *in vivo* DMS footprints observed in adult erythroblasts (E) and K-562 cells (K), derived from the experiments of Fig. 2, are shown for each strand. Protected and hyperreactive guanidine residues are represented by open and closed circles, respectively; circle size indicates relative extents of protection or enhancement toward the DMS reaction. Open bars represent regions of protection during *in vitro* DNase I footprinting experiments, as shown in Fig. 3, and vertical arrows indicate DNase I HSs. The noncoding strand upstream of −145 was not assayed in the *in vitro* DNase I protection experiments.

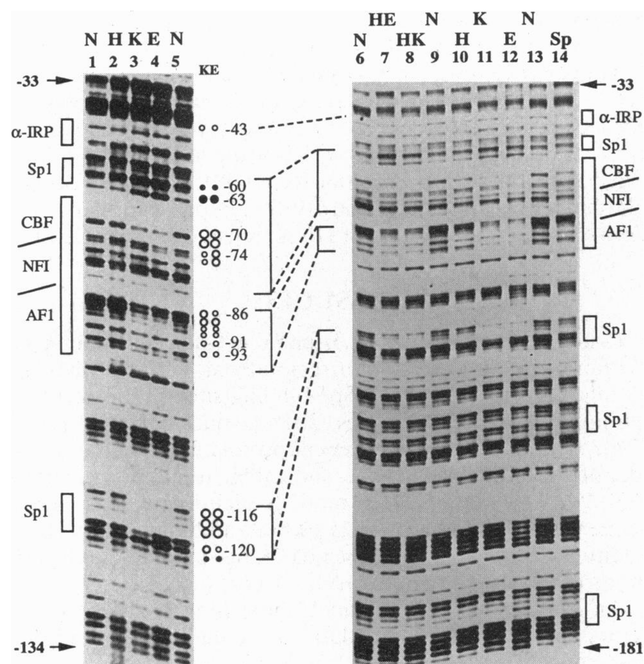


FIG. 2. *In vivo* DMS footprinting pattern of noncoding (lower) strand of human α-globin promoter. Vertical open bars indicate locations of different binding motifs. Nucleotide positions of top- and bottom-most guanidine residues are shown at left. Protected and hyperreactive guanidine residues are represented as in Fig. 1. Lanes: 1, 5, 6, 9, and 13, naked DNA controls (N); 2 and 10, HeLa cells (H); 3 and 11, K-562 cells (K); 4 and 12, adult erythroblasts (E); 7, HEL cells (HE); 8, hemin-induced K-562 cells (HK); 14, adult spleen cells (Sp).

are protected from DNase I digestion in both K-562 and HeLa nuclear extracts. Furthermore, the *in vitro*-protected DNA sequences include those exhibiting genomic footprints—e.g., the Sp1-binding site at −122 to −116, as well as those that did not—e.g., the Sp1-binding site at −61 to −56 (blank boxes, Fig. 1).

Site-Directed Mutagenesis of Human α-Globin Promoter(s). The functional roles of the protein-binding motifs that are occupied *in vivo* have been characterized by site-directed mutagenesis and transient expression in K-562 cells (Fig. 4). Comparison of the mutant and wild-type promoter activities, as measured by hGH assay, showed that mutagenesis of either the AF1 motif or the Sp1 motif at −122 to −116 reduced α-globin promoter activity by ≈30%. As a corollary, a 2-bp mutation of the Sp1 site, in which the 5′-CC-3′ residues at −122 and −121 were changed to 5′-TA-3′, also caused a 30% decrease in the α-globin promoter activity in K-562 cells (data not shown). By contrast, mutation of the CBF motif greatly decreased (by 85%) the α-globin promoter activity in transfected K-562 cells (Fig. 4*B*, left column).

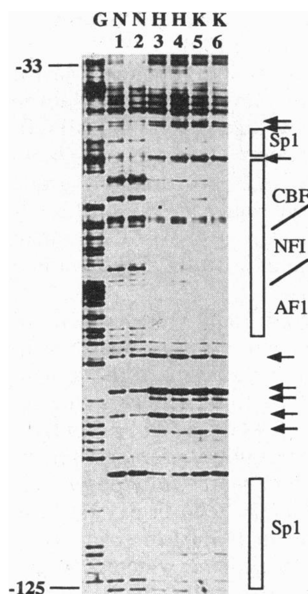


FIG. 3. *In vitro* DNase I footprinting of the lower (noncoding) strand. Data from duplicate experiments are shown. Vertical open bars represent regions of protection, whereas horizontal arrows indicate positions of DNase I HSs. Lanes: 1 and 2, naked DNA controls (N); 3 and 4, HeLa nuclear extracts (H); 5; and 6, K-562 nuclear extracts (K); G, products of a guanidine-specific chemical sequencing reaction.

Enhancer Response of Mutant α-Globin Promoters. The functional contributions of different nuclear factor sites within the human α-globin promoter to its overall response to the HS-40 enhancer have also been analyzed by transient expression assay (Figs. 4 and 5). As summarized in the right column of Fig. 4*B*, mutation of CBF again gave rise to a significant decrease of ≈95% in the expression level. On the other hand, similar to the corresponding enhancerless plasmids, expression levels of pB-HS40-α590(Sp1)GH and pB-HS40-α590(AF1)GH are not significantly lower than wild-type pB-HS40-α590GH.

Function of Nuclear Factor-Binding Motifs of HS-40 in Human α-Globin Promoter Activation. There are at least seven nuclear factor-binding motifs in the HS-40 enhancer, as identified by protein–DNA binding studies *in vitro* (13), six of which are occupied by nuclear factors *in vivo* in an erythroid lineage- and developmental stage-specific manner (19, 20). To determine the functional contribution of these latter motifs to the transcriptional activation of the human α-globin gene, seven different site-directed mutants of HS-40 were cloned upstream of the human α-globin promoter–GH hybrid gene. These constructs were then separately transfected into K-562 cells, and their expression levels were quantitated by primer-extension analysis, as exemplified in Fig. 6*B* and summarized in Fig. 6*A*.

Relative to the wild-type plasmid, pB-HS40-α590GH (Fig. 6*B*, lanes 1 and 2), there were large decreases (75–85%) in

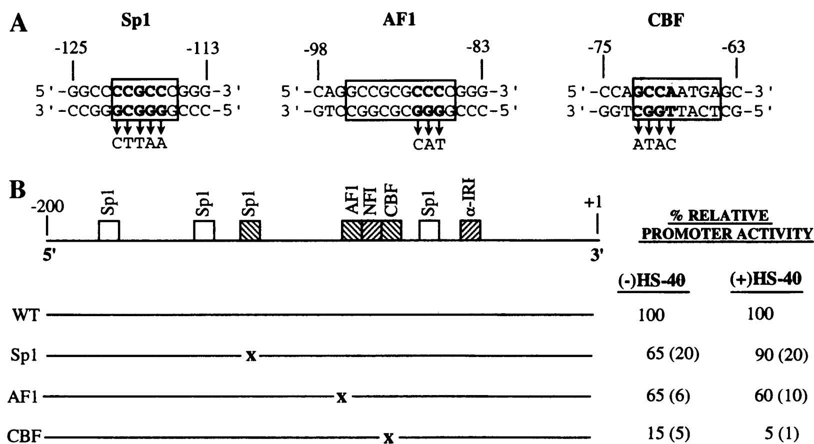


FIG. 4. Transient expression analysis of functional roles of nuclear factor-binding motifs within human α-globin promoter(s). (*A*) Wild-type and mutant motifs. Consensus binding sequences for nuclear factors CBF, AF1, and Sp1 are indicated by boxes. Residues altered by site-directed mutagenesis are indicated by boldface type, while newly substituted nucleotides are indicated by vertical arrows. (*B*) Human α-globin promoter activities in transfected K-562 cells. Motifs in the 200-bp region 5′ of α-globin promoter(s) are shown at top; those occupied *in vivo* are indicated by hatched boxes. Singly mutated sites are indicated below by x; α-globin promoter activities of the corresponding plasmid constructs in transfected K-562 cells are at right. Average expression level of each construct was calculated relative to that of the appropriate wild-type plasmid. SDs in parentheses.

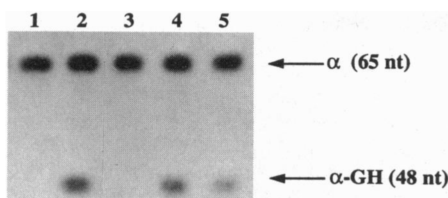


FIG. 5. Primer-extension analysis of cytoplasmic RNA from K-562 cells transfected with pB-HS40-α590GH (lane 2), pB-HS40-α590(CBF)GH (lane 3), pB-HS40-α590(Sp1)GH (lane 4), or pB-HS40-α590(AF1)GH (lane 5). As a negative control, K-562 cells were transfected with the enhancerless construct, pB-α590GH (lane 1). Primer-extension products of the endogenous α-globin and α-GH hybrid mRNAs are 65 and 48 nt, respectively, and their positions are indicated by arrows. The amount of α-GH hybrid product was measured and normalized relative to the product of the internal α-globin control gene (see Fig. 4*B*).

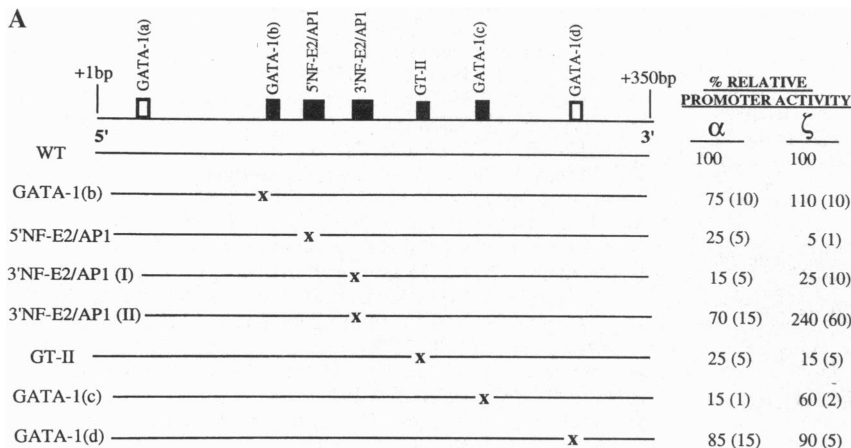
α-globin promoter activity resulting from mutagenesis of the 3′NF-E2/AP1 [the 3′NF-E2/AP1(I) mutation; Fig. 6*B*, lanes 3 and 4], 5′NF-E2/AP1 (Fig. 6*B*, lanes 7 and 8), GTII, and GATA-1(c) motifs in HS-40. These results indicate that protein–DNA interactions occurring at these motifs contribute significantly to the HS-40 enhancer effect on α-globin gene expression in K-562 cells. By contrast, only a modest decrease (from 15 to 30%) in expression was seen for plasmids containing GATA-1(b), GATA-1(d), or 3′NF-E2/AP1(II) mutation (Fig. 6*B*, lanes 5 and 6) in the HS-40 element. Transcriptional levels of the human embryonic ζ-globin promoter as mediated by these HS-40 mutants (34) are also listed in Fig. 6*A* for comparison.

DISCUSSION

The regulation of different human α-globin genes during erythroid development is likely modulated, in part, by the functioning of multiple nuclear factor–DNA complexes formed at the LCRs and different human globin promoters. However, unlike the other human globin promoters, all nuclear factors capable of binding to 200 bp of the human α-globin promoter DNA—namely, Sp1, AF1, CBF, and α-IRP—appear to be ubiquitous factors (Fig. 2; refs. 23, 25, 30, and 37–39). This observation may partially explain the relatively high activity of human α-globin promoter in transfected nonerythroid cell cultures (36, 42, 43). Interestingly, the nuclear factor-binding pattern of the human α-globin promoter bears a striking resemblance to the human phosphoglycerate kinase I promoter on the active X chromosome, which is a ubiquitous CpG island, in that both of them consist of mainly protected regions at Sp1, NFI, CBF, and α-IRP motifs (44). Our genomic footprinting data indicate that only a subset of these nuclear factor-binding sites on the human α-globin promoters are occupied *in vivo*, and the observed genomic footprints are highly restricted to erythroid, α-globin-expressing cells (Figs. 1 and 2; ref. 30). Consequently, there must exist a mechanism(s) preventing the binding of these ubiquitous transcription factors to the endogenous α-globin promoters in nonerythroid cells, thus rendering them transcriptionally repressed or inactivated.

We have analyzed the function of these erythroid cell-specific, genomic-footprinted motifs of the human α-globin promoter(s) by transient expression assay of site-directed promoter mutants in K-562 cells. This approach most likely measures the transcription process and thus reflects the roles of nuclear factor–DNA complexes, during the final steps of activation. The essential function of CBF is evidenced by the large decrease in α-globin promoter activity after mutation of the motif (Fig. 4). In contrast, mutagenesis experiments of the other two motifs, Sp1 and AF1, suggest that they only contribute modestly to the α-globin promoter activity in erythroid cells (Fig. 4), despite their erythroid lineage-specific occupancy by nuclear factors *in vivo* (Figs. 1 and 2). It is possible that certain critical regulatory events preceding the final stage of transcription, such as chromatin remodeling (45), may not occur on the episomal DNA in the transiently transfected cells. Also, factor-binding sites such as Sp1, AF1, or α-IRP may contribute significantly to α-globin promoter activity only under appropriate physiological conditions, as suggested by previous studies of Sp1 (38) and AP2 (46, 47), a most likely candidate for AF1.

We have also used the above approaches to analyze the functions of different nuclear factor-binding motifs in the HS-40 element (Fig. 6). Relative to the wild-type HS-40-containing plasmid, a marked decrease in α-globin promoter activity (≈65–85%) was observed for plasmids containing HS-40 sequences mutated in the motifs 5′NF-E2/AP1, 3′NF-



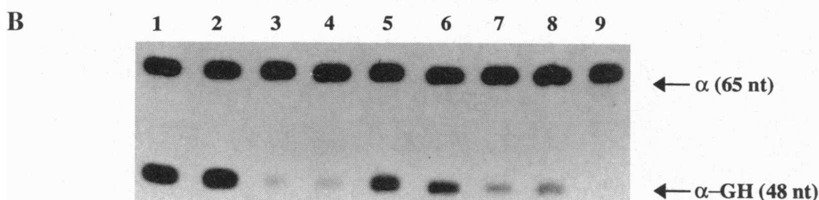


FIG. 6. (*A*) Function of nuclear factor-binding sites within α-globin HS-40 enhancer element. Motifs within the HS-40 enhancer bound *in vivo* with factors in K-562 cells are indicated by shaded boxes. The seven mutagenized HS-40 elements are shown below; a mutated site is indicated by X. The α-globin promoter activities of the corresponding plasmid constructs in transfected K-562 cells, as measured by primer-extension analysis (see Fig. 6*B* for typical result), are shown at right. For comparison, promoter activities of similar constructs containing the human ζ-globin promoter linked in cis with each of the mutant HS-40 enhancers (34) are also presented. WT, wild type. (*B*) Primer-extension analysis from a duplicate set of transfection experiments. K-562 cells were transfected with wild-type pB-HS40-α590GH (lanes 1 and 2) and plasmids containing the following HS-40 mutations: 3′NF-E2/AP1(I) (lanes 3 and 4), 3′NF-E2/AP1(II) (lanes 5 and 6), and 5′NF-E2/AP1 (lanes 7 and 8). Lane 9, enhancerless pB-α590GH.

E2/AP1 [the 3′NF-E2/AP1(I) mutation], GTII, or GATA-1(c) (Fig. 6*A*). This result, together with previous genomic footprinting analysis of HS-40 (19, 20), indicates that these four motifs very likely constitute the central functional core of the HS-40 region that intimately interacts with the α-globin promoters during the final steps of transcriptional activation in embryonic/fetal erythroid cells.

Mutations in the two GATA-1 motifs peripheral to the above functional core of HS-40, GATA-1(b) and GATA-1(d), and a single base mutation in the 3′NF-E2/AP1 motif, 3′NF-E2/AP1(II), all resulted in relatively modest decreases of the enhancer function (Fig. 6*A*). The absence of a detectable effect for the GATA-1(d) site in the transient expression assay in K-562 cells is consistent with the fact that it is bound by nuclear factor(s) only in adult erythroid cells but is not bound in K-562 cells (19, 20). The relatively small contribution of the GATA-1(b) motif to HS-40 function on promoter activity of both α-globin genes (this study) and the ζ-globin gene (34) is puzzling at this moment, in view of its erythroid-specific occupancy by nuclear factor(s) in K-562 cells, as well as in adult erythroblasts (19). Again, GATA-1(b) could be involved at an earlier step(s) of transcriptional regulation of the human α-like globin locus by HS-40.

The HS-40-mediated transcriptional activities of the α- and ζ-globin promoters in transiently transfected K-562 cells, as compared in Fig. 6*A*, are affected differently in response to mutation of each of the factor-binding motifs in HS-40—in particular, the 1-bp mutation of the 3′NF-E2/AP1 motif (designated II in Fig. 6*A*). As discussed elsewhere (34), the binding of NF-E2 at this motif likely plays a role in silencing ζ-globin promoter activity in fetal erythroid cells. Overall, these results suggest that the assembly and functioning of multiple nuclear factor–DNA complexes at HS-40 are promoter-specific.

In summary, we have used transient expression assay to analyze the functional roles of different nuclear factor-binding motifs in the transcriptional activation of the human α-globin promoters by the HS-40 enhancer. The apparent functioning of most of these motifs, either in the α-globin promoter or the HS-40 enhancer, in the final stage of the enhancer–promoter interaction correlates well with their erythroid cell-specific and developmental stage-specific occupancy by nuclear factors *in vivo*. Further functional analysis of these promoter and enhancer mutants in stably integrated chromosomes should reveal their roles at earlier steps of regulation of the human α-like globin genes.

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