MOLECULAR AND CELLULAR BIOLOGY, Jan. 1996, p. 236–246 Vol. 16, No. 1
0270-7306/96/$04.00+0

Intronic and Flanking Sequences Are Required To Silence Enhancement of an Embryonic β-Type Globin Gene

NANCY J. WANDERSEE,[1,2,3] RICHARD C. FERRIS,[1] AND GORDON D. GINDER[1,2]*

Department of Medicine[1] and Institute of Human Genetics,[2] University of Minnesota, Minneapolis, Minnesota 55455, and Genetics Ph.D. Program,[3] University of Iowa, Iowa City, Iowa 52242

Received 27 June 1995/Returned for modification 8 August 1995/Accepted 24 October 1995

In the course of studying regulatory elements that affect avian embryonic ρ-globin gene expression, the multipotential hematopoietic cell line K562 was transiently transfected with various ρ-globin gene constructs containing or lacking an avian erythroid enhancer element. Enhanced levels of ρ gene expression were seen from those constructs containing an enhancer element and minimal 5′ or 3′ flanking ρ sequences but were not seen from enhancer-containing constructs that included extensive 5′ and 3′ flanking sequences. Deletion analysis localized 5′ and 3′ "enhancer-silencing elements" to −2140 to −2000 and +1865 to +2180 relative to the mRNA cap site. A third element required for enhancer silencing was identified within the second intron of the ρ gene. The treatment of K562 cells with hemin, which induces erythroid differentiation, partially alleviated the enhancer-silencing effect. The silencer elements were able to block enhancement from a murine erythroid enhancer, but not from a nonerythroid enhancer. Electrophoretic mobility shift assays demonstrated that the transcription factor YY1 is able to bind both the 5′ and 3′ enhancer silencer elements; a point mutation of the single overlapping YY1/NF-Y binding site in the 3′ element completely abolished the enhancer-silencing effect. These results demonstrate a complex enhancer silencer that requires 5′ flanking, intronic, and 3′ flanking sequences for a single regulatory effect on a eukaryotic gene.

The control of transcription is a primary mechanism through which the regulation of vertebrate β-type globin genes is achieved. In the chicken, the embryonic ρ and ε genes are expressed in primitive erythroid cells through day 5 of development, after which expression of the β^H and β genes begins in definitive erythroid cells (3). The chicken β-like globin gene cluster is arranged such that the embryonic genes flank the adult genes, 5′-ρ-β^H-β-ε-3′. In contrast, the genes of the human β-like globin cluster are arranged 5′ to 3′ in the order in which the genes are expressed in development. Human globin genes are expressed in the following developmental stages: the ε gene is expressed in the embryonic stage, with expression of the $^G\gamma$ and $^A\gamma$ genes in the fetal stage and expression of the δ and β genes in the adult stage (reviewed in reference 50). These differences, coupled with the evolutionary divergence of birds and mammals before the duplication of the primordial β-type globin gene (45), might suggest that the mechanisms of developmental regulation between the species would be variant. However, studies using murine erythroleukemia cells and transgenic mice have shown that the genes of both the human (reviewed in reference 50) and chicken (20, 33) β-globin clusters are correctly expressed in murine development, suggesting that at least a majority of the factors responsible for stage- and tissue-specific globin gene expression have been conserved through the divergent evolution of birds and mammals.

The human β-globin cluster contains a series of erythroid cell-specific DNase I-hypersensitive sites (HS) upstream of the globin genes; these sites, collectively known as the locus control region (LCR) are necessary for high-level, position-independent gene expression in transgenic mice, as well as for chromatin opening in early erythroid cells (16–18, 22, 51). DNase I-HS with analogous functions have also been identified in the upstream region of the mouse β-globin locus (references 29, 30, and 36 and references therein). The chicken β-globin locus contains a similarly positioned set of upstream DNase I-HS, as well as a downstream HS between the β and ε genes that acts as a strong erythroid enhancer (9, 27, 33). It has been shown that both the downstream and upstream HS are critical to the expression of the chicken β-like globin genes in transgenic mice (33). Upstream HS2 and -3 have previously been shown individually to have moderate enhancer activity (1); in this paper, we show that upstream HS1 to -3 act together as a strong enhancer element.

The complex regulation of globin gene expression involves the positive influence of several erythroid cell-specific transcription factors. Binding sites for the erythroid cell-specific factors GATA-1 and NF-E2 (and related factors) have been identified in the promoters of all globin genes (and some nonglobin, erythroid cell-specific genes), as well as within the HS of the human LCR (50). The binding of these factors has been shown to be important for the enhancer capabilities of 5′ HS2 of the human LCR and, in some cases, for promoter function (reviewed in reference 50). In addition, potential binding sites for erythroid Krüppel-like factor, another erythroid cell-specific transcription factor identified by Miller and Bieker (35), have been found in the promoters of a number of globin genes, as well as in 5′ HS2 of the human LCR. To date, erythroid Krüppel-like factor seems to exert its influence primarily on transcription from the adult human β-globin gene promoter (12, 15, 35). However, the expression of some of these erythroid cell-specific factors has been shown to begin prior to the commitment of multilineage hematopoietic progenitor cells to the erythroid lineage (26, 34, 43). In addition, Jiménez et al. [30] have shown that 5′ HS1 and 5′ HS2 of the murine LCR are open and hypersensitive in multilineage hematopoietic progenitor cells. Therefore, it is reasonable to speculate that negative regulatory systems might exist to prevent the expression of

* Corresponding author. Mailing address: Box 286 UMHC, University of Minnesota, M506 Masonic Cancer Center, 424 Harvard St., SE, Minneapolis, MN 55455. Phone: (612) 624-7915. Fax: (612) 625-8966. Electronic mail address: ginde001@maroon.tc.umn.edu.

globin genes in hematopoietic progenitors prior to full commitment to the erythroid lineage.

The regulation of globin gene expression also has been shown to involve the action of negative elements, such as the promoter silencer element located in the upstream flanking sequences of the human ε-globin gene (6). Further studies have suggested that this region binds GATA-1 and the ubiquitous transcription factor YY1; the binding of YY1 displaces GATA-1 and represses transcription (25, 42). YY1, a protein that has been shown in different contexts to activate, repress, or initiate transcription (reviewed in reference 48), also binds within a putative repressor region located upstream of the human γ-globin gene (23). Recently, along with GATA-1 YY1 was shown to contribute to developmental stage-specific repression of the human ε-globin gene in transgenic mice (44).

In studies of the transient expression of the avian embryonic ρ-globin gene in the human erythroleukemic cell line K562, a multipotential cell line possessing erythroid as well as myeloid and megakaryocytic progenitor potential, a series of elements that act together to silence the effect of strong erythroid cell-specific enhancers on the promoter were identified. These "enhancer silencer elements" did not, however, block enhancement from an equally strong nonerythroid enhancer. This enhancer-silencing effect was partially abrogated by the treatment of K562 cells with hemin, which induces increased, but not complete, erythroid differentiation of these cells. To our knowledge, this is the first description of a eukaryotic gene regulatory effect requiring the cooperative interaction of 5′, intronic, and 3′ sequence elements.

MATERIALS AND METHODS

Cell culture. K562 cells (ATCC CCL243) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine, penicillin, and streptomycin. In some experiments, cells were induced by the addition of 20 μM hemin (Sigma). Primary avian erythroid cells were cultured after transfection in Leibovitz's L15 medium supplemented with 3.6% fetal calf serum, 22% chicken serum, glutamine, penicillin, and streptomycin (32).

Globin gene constructs. Construct pR contains a 4.6-kb ρ gene *Hin*dIII fragment (14) extending from approximately 2,400 bp upstream of the cap site to 1,200 bp downstream of the poly(A) site. All other constructs have the coordinates (relative to the cap site) listed in the corresponding figures. Construct pR3a was generated by partial digestion to truncate the 3′ end of pR.

Construct pR3 was generated by partial digestion of construct pR to truncate the 3′ end of the ρ fragment. Constructs pR5a and pR5b were generated by PCR amplification of pR with appropriate primers to generate constructs truncated from the 5′ end of the ρ fragment. Construct pR5c was generated by partial digestion of pR to truncate the 5′ end of the ρ fragment. Constructs pRΔI, pRΔI3′, and pRΔI5′ were generated by PCR amplification of pR with internal primers designed to delete either the entirety 3′ or 5′ portions of intron 2, respectively.

Constructs pR3a-1, pR3a-1β/ε, pR3a-2β/ε, and pR3a-3β/ε were constructed by combined PCR amplification and restriction enzyme digestion to contain various fragments of the 3′ enhancer silencer element (see Fig. 2A). These fragments were then inserted downstream of the ρ fragment in either construct pR3a or construct pR3β/εa. Construct pR3a-2mβ/ε was generated by overlapping PCR mutagenesis (references 31 and 46 and references therein) of the 3′ enhancer silencer element (+1865 to +2180) with appropriate external primers and internal primers spanning and mutating the putative YY1 site in this element (see Fig. 6), with subsequent insertion of this PCR product downstream of the ρ fragment in construct pR3β/εa. The final construct was sequenced to confirm mutation of only the appropriate sequence.

Constructs with the designation β/ε include a 480-bp fragment of the chick β-globin locus which contains the enhancer situated between the β and ε genes (Fig. 1). Constructs with the designation 5HS include a 4.0-kb fragment of the chick β-globin gene locus which contains upstream HS1, -2, and -3 (Fig. 1). Constructs with the designation RSV include a 250-bp fragment containing the Rous sarcoma virus (RSV) enhancer. Constructs with the designation mH2 include a 1.1-kb fragment of the murine β-globin LCR (kindly provided by Tim Ley) that contains 5′ HS2.

Transfection. K562 transient transfectants were generated by electroporation with a BTX transfector ECM 600 (Biotechnologies and Experimental Research, Inc.) and a 1.9-mm-gap electrode. Transfections were performed essentially as described previously (20) with the following modifications. Transfections were performed at room temperature, and each transfection contained supercoiled RSV-*neo* or SV40-CAT as internal transfection controls. Cells were incubated after electroporation for 40 to 48 h before being harvested. Each construct was transfected in quadruplicate in each experiment.

Primary avian erythroid cell transfections were performed by the protocol of Lieber et al. (32), except that osmotic shock was in 300 mM NH_4Cl (pH 7.4) for 60 min at room temperature. Cells were cultured for 40 to 48 h in 10 ml of Leibovitz's culture medium, as described above. Each construct was transfected in triplicate.

Cell harvesting. To obtain RNA, transfections of the same construct were pooled and RNA was harvested as previously described (8, 20). To obtain cytoplasmic extracts for chloramphenicol acetyltransferase (CAT) assay, cells from individual transfections (K562 cells or primary erythroid cells) were harvested according to the method of Gorman et al. (21).

CAT assay. Cytoplasmic extracts were assayed for CAT activities by using a liquid scintillation assay kit (catalog no. E1000; Promega, Madison, Wis.). After extraction, reactions were counted in a Beckman scintillation counter (model LS1801). As the variation between the three independent assays for each construct was ≤10%, the results expressed are mean values.

RNase protection assay. The generation of single-stranded, labeled cRNA probes and subsequent analysis of mRNA levels by RNase protection assay were done as previously described (4, 20). RNA from K562 transfectants was analyzed for expression of the transfected ρ construct, the cotransfected RSV-*neo* construct (as a control for transfection efficiency), and the endogenous human triose-phosphate isomerase (hTPI) gene (as a control for RNA quantitation). The hTPI probe was a kind gift from Lynne Maquat (Roswell Park Cancer Institute). Expression levels were quantitated on a Molecular Dynamics densitometer; the results cited have been corrected for transfection efficiency and RNA quantitation.

Electrophoretic mobility shift assays. DNA probes were end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Pharmacia). K562 nuclear protein extracts were prepared according to the method of Dignam et al. (13). Probe (54 to 56 fmol) was incubated with K562 nuclear extract (20 to 40 μg) in 20 mM HEPES (pH 7.9) (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–60 mM KCl–1 mM $MgCl_2$–0.1 mM EDTA–12% glycerol–1 mM dithiothreitol (24). Two micrograms of poly(dI-dC) was added per reaction as a nonspecific competitor; specific competitors were added at 25- to 500-fold molar excesses, as indicated. Reaction mixtures were incubated for 40 min at 4°C. Supershift reaction mixtures contained 2 μg of either polyclonal YY1 or NF-Y-A antibody or control preimmune serum and were incubated for 60 min at 4°C. Reaction products were electrophoresed on a 4% acrylamide gel in 0.5× TBE (45 mM Tris, 44 mM boric acid, 1 mM EDTA); gels were then dried and autoradiographed.

RESULTS

Enhancement of ρ gene expression is silenced by 5′ and 3′ flanking sequences. Previous studies using K562 cells have identified positive and/or negative elements involved in the developmental regulation of both the human embryonic ε-globin (6, 23, 42) and fetal γ-globin (23, 28) genes. In addition, studies by Glauber et al. (20) and Mason et al. (33) have indicated that intact chicken β-like globin genes are efficiently expressed and developmentally regulated in murine erythroleukemia cells and transgenic mice, respectively, suggesting an evolutionary conservation between birds and mammals of factors necessary for correct regulation of globin gene expression. Therefore, we transiently transfected constructs containing the avian embryonic ρ-globin gene and various lengths of 5′ and/or 3′ flanking sequences into K562 cells in order to identify potential regulatory elements. These constructs either contained or lacked the strong avian erythroid enhancer from the region between the β and ε genes (Fig. 1A; henceforth referred to as the 3′ β/ε enhancer). Subsequent to the initial design of these constructs, the results of Mason et al. (33) showed that the 5′ DNase I-HS located upstream of the ρ gene were critical for the expression of the ρ gene in transgenic mice. Therefore, additional constructs were made to test if 5′ HS1, -2, and -3 could together act as an enhancer of expression from the ρ promoter (Fig. 1A [5HS]). 5′ HS4 was not included in these constructs, as it is not erythroid cell specific (10).

The expression of the enhancerless pR3a construct (Fig. 1B, lane 4), which lacks 3′ flanking sequences, was consistently 1.5- to 2-fold higher than that of the enhancerless pR construct (lane 1), which contains both 5′ and 3′ flanking sequences.

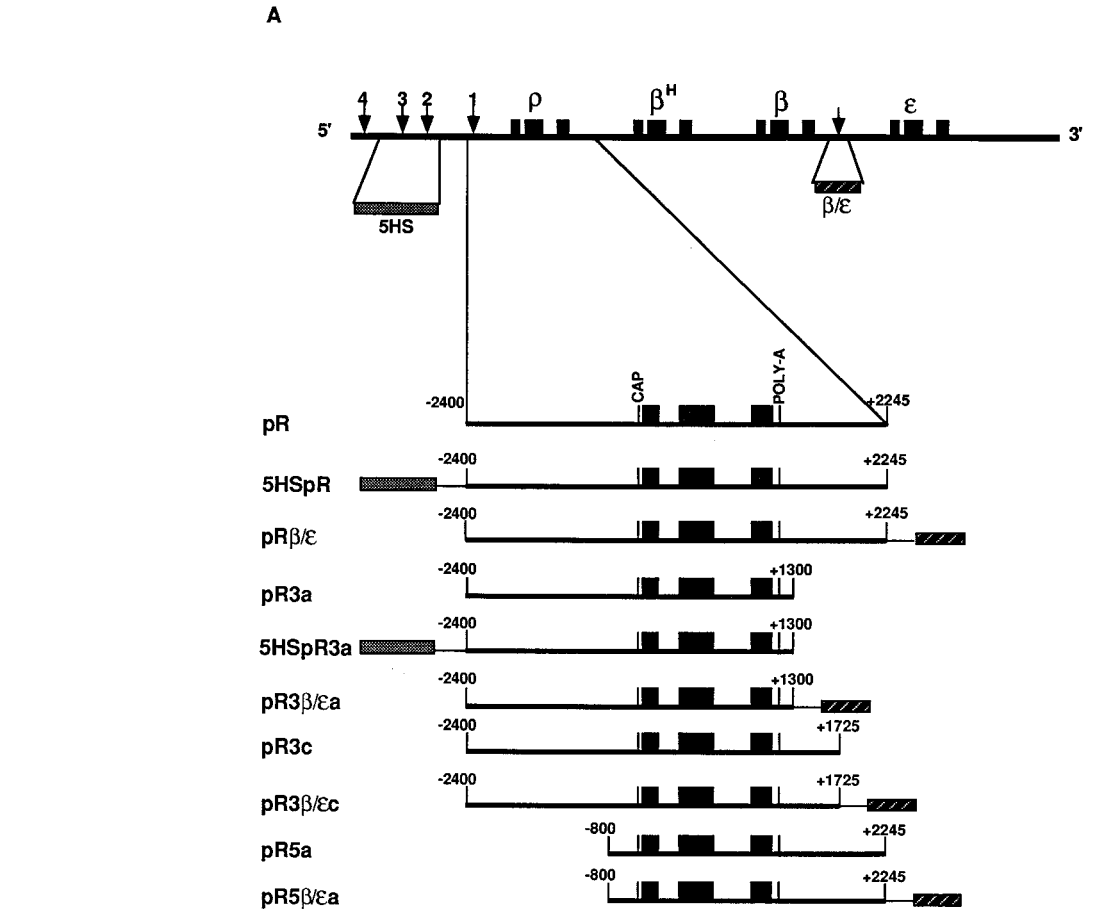

FIG. 1. (A) Schematic illustration of the chicken β-globin gene cluster, shown in the 5′-to-3′ orientation. Shown below are the constructs used in panel B; the name of each construct is indicated on the left. Numbers indicate positions relative to the mRNA cap site of the ρ gene. Solid boxes represent exons; each striped box represents the 3′ β/ε enhancer element; and stippled boxes represent 5′ HS2 and -3 from upstream of the ρ gene. A thick line represents ρ sequence; a thin line represents intervening vector sequence. (B) RNase protection assay of RNAs from K562 cells transiently cotransfected with RSV-neo and the constructs shown in panel A. The 140- and 110-nucleotide (nt) protected fragments for the ρ probe (Rho) correspond to correctly initiated and processed ρ-globin mRNA. The 360-nt protected fragment corresponds to expression of the cotransfected *neo* gene transcript (Neo), a control for transfection efficiency. RNA was also probed for expression of the endogenous hTPI gene as a control for RNA quantitation (data not shown). Expression levels were quantitated with a Molecular Dynamics densitometer; the ρ expression results presented in the text have been normalized for transfection efficiency and RNA quantitation.

However, the expression of additional constructs lacking 5′ or 3′ flanking sequences, including pR3c and pR5a, was equivalent to that of the pR construct (Fig. 1A and B [lanes 1, 7, and 9]). The small differences in expression between the pR3a construct and other constructs were not considered significant and were not pursued further.

In contrast, a striking difference in the expression from these constructs was observed when an avian erythroid enhancer was added. When the 3′ β/ε enhancer was included in a construct containing both 5′ and 3′ distal flanking sequences, construct pRβ/ε (Fig. 1A), no additional expression was seen (Fig. 1B, lane 3). However, when the same enhancer was included in constructs with less 3′ flanking sequence or less 5′ flanking sequence (constructs pR3β/εa, pR3β/εc, and pR5β/εa [Fig. 1A]), the expression levels increased by 6- to 10-fold (Fig. 1B, lanes 6, 8, and 10, respectively). When 5HS was included in enhancerless constructs, a similar effect was seen. The construct lacking 3′ flanking sequences exhibited an approximately 6- to 10-fold increase in expression when these HS were added (Fig. 1B, lane 5), demonstrating that together HS1 to -3 of the avian β-globin locus are capable of enhancing ρ gene expression as much as the 3′ β/ε enhancer in K562 cells (Fig. 1B; compare lanes 5 and 6). However, when both 5′ and 3′ flanking sequences were present, these HS had no effect on expression from the ρ promoter, a result identical to that observed with the 3′ β/ε enhancer (Fig. 1B, lane 2). These data demonstrate that elements in the 5′ and 3′ distal flanking sequences of the ρ gene are capable of silencing enhancement from both the 3′ β/ε enhancer and 5′ HS. These experiments indicate that the 5′ element involved in silencing the enhancer is located between

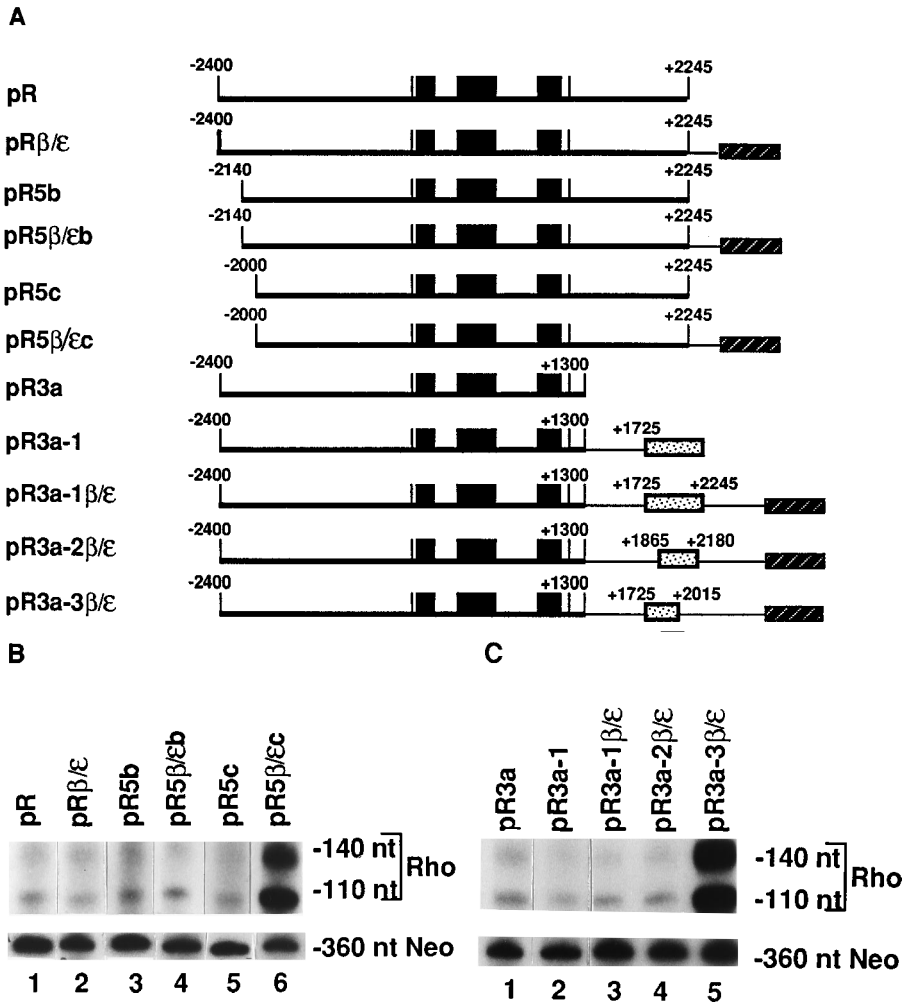

FIG. 2. (A) Schematic illustrations of the constructs used in this RNase protection assay. The name of each construct is indicated on the left. Numbers indicate positions relative to the mRNA cap site. Solid boxes represent exons, striped boxes represent the 3′ β/ε enhancer, and dotted boxes represent various portions of the 3′ ESE. Thick and thin lines represent ρ and vector sequences, respectively. (B and C) RNase protection assay of RNAs from K562 cells transiently cotransfected with RSV-*neo* and the constructs depicted in panel A. The probe fragments and expression quantitation are the same as those indicated in the legend to Fig. 1.

−2400 and −800 and that the 3′ silencer element is located between +1725 and +2245 (relative to the mRNA cap site). The increase in basal promoter activity seen with construct pR3a was not present for any of the other enhancerless constructs, indicating that sequences between +1300 and +1725 are responsible for the suppression of this activity. Thus, the sequences between −2400 and −800 and between +1725 and +2245 appear to be involved only in silencing the effects of the 3′ β/ε and 5′ HS enhancers. For this reason we have chosen to refer to these elements as enhancer silencer elements (ESE).

The 5′ ESE is located between −2140 and −2000 (relative to the mRNA cap site). To further define the 5′ ESE, deletions were made (see Materials and Methods) from the 5′ end of the ρ fragment in constructs pR and pRβ/ε, generating constructs pR5b, pR5c, pR5β/εb, and pR5β/εc (Fig. 2A). Transient transfections of these constructs into K562 cells showed that the levels of expression of constructs pR5b (Fig. 2B, lane 3) and pR5c (lane 5) were the same as that for construct pR (Fig. 2B, lane 1), suggesting that no negative regulatory elements affecting basal promoter expression reside in the sequences between −2400 and −2000. The expression from construct pR5β/εb was the same as that from construct pRβ/ε; no enhancement of expression was detected (Fig. 2B, lane 4). In contrast, the level of expression from construct pR5β/εc (Fig. 2B, lane 6) was six- to eightfold higher than that from pRβ/ε, indicating that the enhancement of expression had been restored through deletion of the region between −2140 and −2000. These experiments localize the 5′ ESE to between −2140 and −2000 and indicate that deletion of this element in the absence of an enhancer does not alter expression from the ρ promoter.

The 3′ ESE is contained within the region +1865 to +2180. The 3′ ESE (+1725 to +2245) was inserted downstream of the ρ fragment in construct pR3a, creating construct pR3a-1 (Fig. 2A). In addition, subfragments of the 3′ ESE were generated (see Materials and Methods) and inserted downstream of the ρ fragment in construct pR3a, with subsequent insertion of the 3′ β/ε enhancer, resulting in constructs pR3a-1β/ε, pR3a-2β/ε, and pR3a-3β/ε (Fig. 2A). When transiently transfected into K562 cells and analyzed for mRNA expression by RNase protection assay, construct pR3a-1 gave the same level of expression as that of construct pR3a (Fig. 2C), confirming the fact that the 3′ ESE in the absence of an enhancer had no effect on expression from the ρ promoter. In contrast, construct pR3a-1β/ε showed no enhancement of expression (Fig. 2C, lane 3),

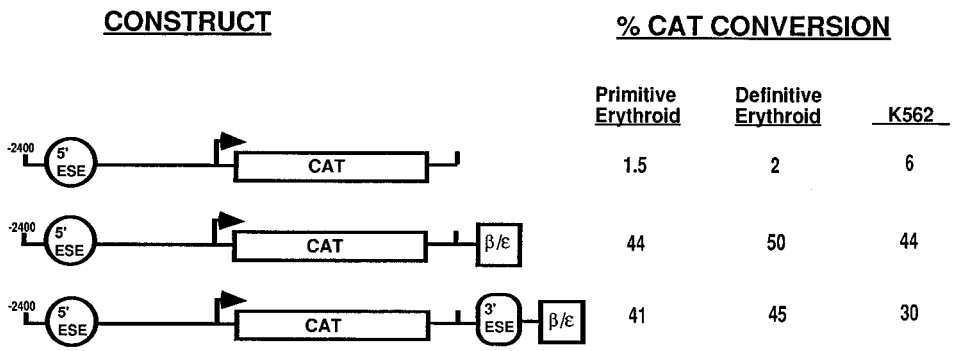

FIG. 3. Relative CAT expression in primitive primary erythroid, definitive primary erythroid, and K562 cells transiently transfected with the indicated constructs. The ρ promoter and 5′ flanking sequences (−2400 to +1) were inserted upstream of the CAT gene in pCATBasic (Promega); the 3′ β/ε enhancer and/or the 3′ ESE (+1725 to +2245) was inserted downstream of the CAT gene. Arrow, transcription start site; rectangle, CAT gene; square, 3′ β/ε enhancer; circle, 5′ ESE; oval, 3′ ESE. The relative expression values presented are CAT expression levels normalized to cotransfected RSV-βgal expression (data not shown). Each construct was transfected in triplicate (primary erythroid cells) or quadruplicate (K562 cells) in at least three separate experiments.

thus confirming the enhancer silencer activity of the 3′ ESE. Construct pR3a-2β/ε also demonstrated no enhancement of expression (Fig. 2C, lane 4), thus localizing the 3′ ESE to between +1865 and +2180. Construct pR3a-3β/ε, however, exhibited an expression level six- to ninefold greater than that seen for construct pRβ/ε (Fig. 2C, lane 5), indicating that the region between +1725 and +2015 did not completely contain the 3′ ESE. Taken together, these results show that the 3′ ESE is contained within sequences between +1865 and +2180.

An intragenic region of the ρ gene is necessary for the enhancer-silencing effect. In order to determine if the ESE could exert an effect on nontransformed erythroid cells, the activities of the 5′ and 3′ ESE were tested in both primitive and definitive primary avian erythroid cells. Constructs were made by linking the ρ promoter and 5′ flanking sequences (−2400 to +1, therefore including the 5′ ESE) (Fig. 3) to the CAT reporter gene; they either contained or lacked the 3′ β/ε enhancer and the 3′ ESE (Fig. 3). These constructs were transiently transfected into 5 (primitive) and 11-day-old (definitive) primary avian erythroid cells, and promoter activity was measured by CAT assay. In primitive erythroid cells, a 20- to 30-fold enhancement in expression was seen upon inclusion of the enhancer; this enhancement was not affected by the addition of the 3′ ESE (Fig. 3). Identical results were obtained when these same constructs were transiently transfected into definitive erythroid cells (Fig. 3). There are two likely explanations for these results. The first is that the enhancer silencer effect is peculiar to K562 cells and therefore not observed in primary avian erythroid cells. The second is that the enhancer silencer effect requires intragenic sequences (i.e., within the transcribed region) of the ρ gene. In order to distinguish between these two possibilities, the same CAT reporter constructs were transiently transfected into K562 cells. In these experiments, inclusion of the enhancer resulted in an 8- to 10-fold enhancement of expression which was not significantly decreased by the inclusion of the 3′ ESE (Fig. 3). This result suggests that the enhancer-silencing effect does require an intragenic region of the ρ gene.

Intron 2 of the ρ gene contains a third ESE. Upon inspection of the primary sequence of the ρ gene, several potential binding sites were noted in intron 2 for *trans*-acting factors previously shown to be involved in globin gene regulation (see Fig. 6A). Primers were synthesized to allow the deletion of intron 2 between +545 and +880 by PCR. The coordinates of this deletion were chosen so as to remove the maximum number of potential binding sites in intron 2 without disrupting splicing signals, which are critical to high-level globin gene expression (2, 11). In the absence of an enhancer, basal expression of the intron 2-deleted construct (Fig. 4B, lane 3) was the same as that of the wild-type construct (lane 1), thus excluding the possibility that the removal of intron 2 results in altered RNA processing or reduced gene expression. The 3′ β/ε enhancer was then added, resulting in construct pRβ/εΔI (Fig. 4A). When this construct was transiently transfected into K562 cells, no enhancer silencing was observed (Fig. 4B, lane 4). These results suggest that the second intron of the ρ gene

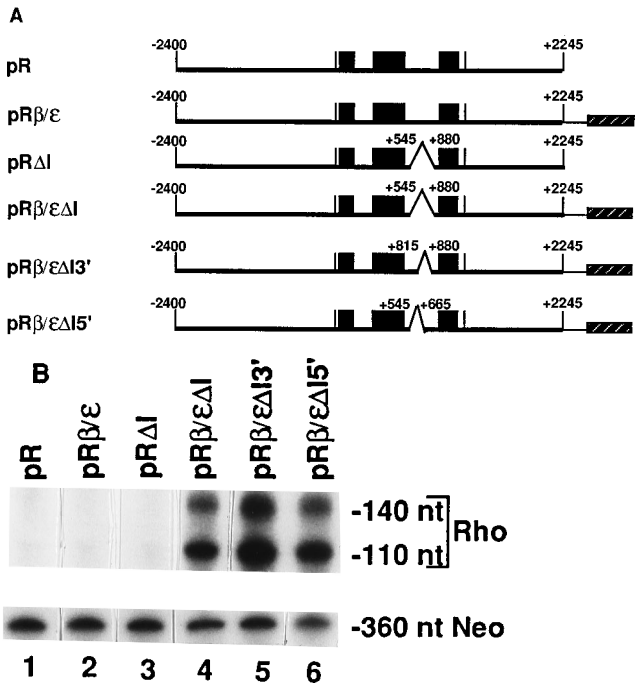

FIG. 4. (A) Schematic illustrations of the constructs used in this RNase protection assay. Numbers represent positions relative to the mRNA cap site. Solid and striped boxes represent exons and the 3′ β/ε enhancer, respectively. Thick and thin lines represent ρ and vector sequences, respectively. An upward notch represents a deletion of all or part of intron 2 of the ρ gene. (B) RNase protection assay of RNAs from K562 cells transiently cotransfected with RSV-*neo* and the constructs shown in panel A. The probe fragments and expression quantitation are the same as those indicated in the legend to Fig. 1.

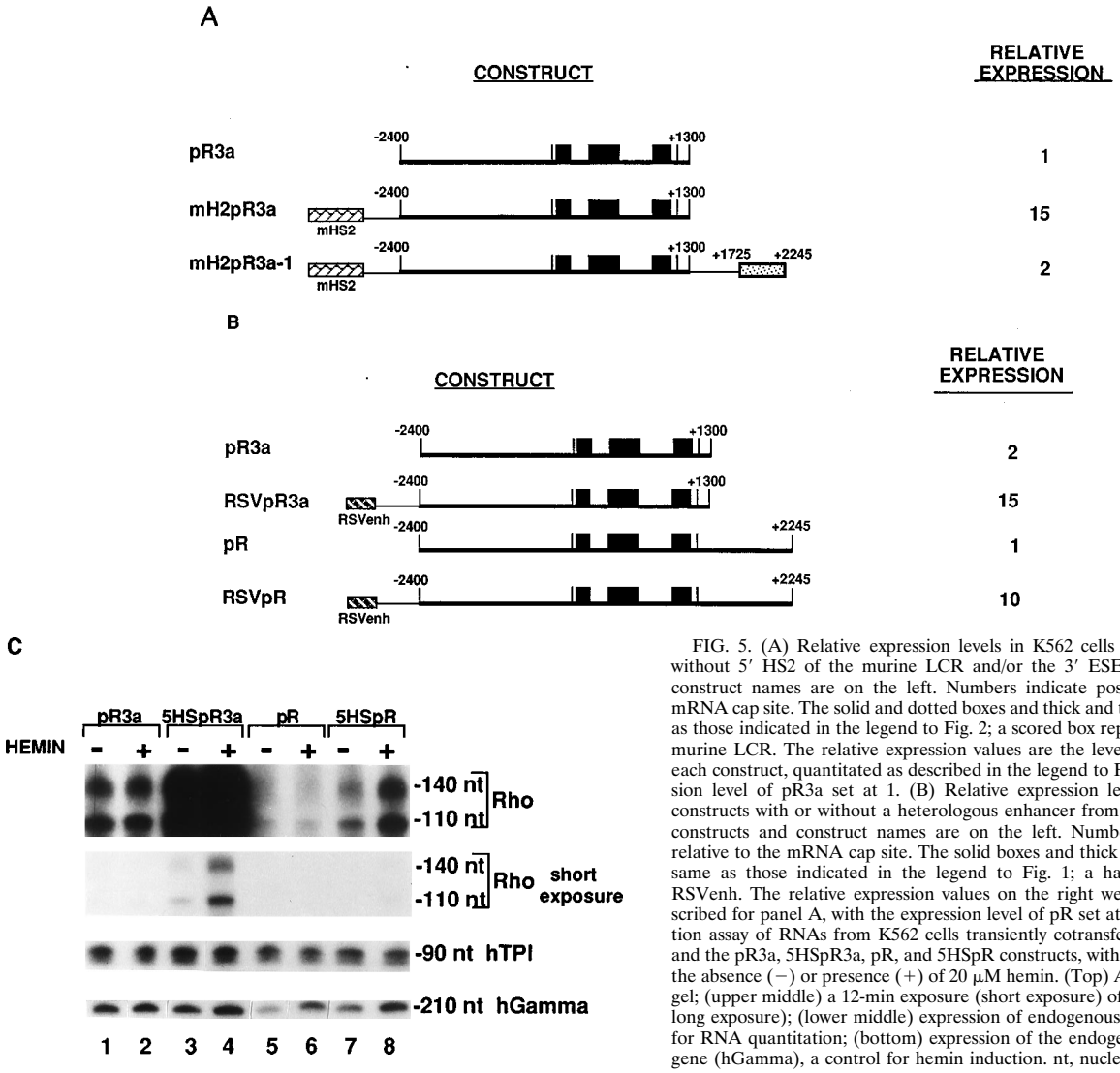

FIG. 5. (A) Relative expression levels in K562 cells of constructs with or without 5′ HS2 of the murine LCR and/or the 3′ ESE. The constructs and construct names are on the left. Numbers indicate positions relative to the mRNA cap site. The solid and dotted boxes and thick and thin lines are the same as those indicated in the legend to Fig. 2; a scored box represents 5′ HS2 of the murine LCR. The relative expression values are the levels of ρ expression for each construct, quantitated as described in the legend to Fig. 1, with the expression level of pR3a set at 1. (B) Relative expression levels in K562 cells of constructs with or without a heterologous enhancer from RSV (RSVenh). The constructs and construct names are on the left. Numbers indicate positions relative to the mRNA cap site. The solid boxes and thick and thin lines are the same as those indicated in the legend to Fig. 1; a hatched box represents RSVenh. The relative expression values on the right were determined as described for panel A, with the expression level of pR set at 1. (C) RNase protection assay of RNAs from K562 cells transiently cotransfected with SV40-CAT and the pR3a, 5HSpR3a, pR, and 5HSpR constructs, with subsequent culture in the absence (−) or presence (+) of 20 μM hemin. (Top) A 16-h exposure of the gel; (upper middle) a 12-min exposure (short exposure) of the same gel (1/80 of long exposure); (lower middle) expression of endogenous hTP1 gene, a control for RNA quantitation; (bottom) expression of the endogenous human γ-globin gene (hGamma), a control for hemin induction. nt, nucleotides.

contains one or more elements required for the enhancer-silencing effect.

In an attempt to define further the region of intron 2 required for enhancer silencing, additional primers were made to include the 5′ and 3′ portions of intron 2, resulting in constructs pRβ/εΔI3′ and pRβ/εΔI5′, respectively (Fig. 4A). As shown in Fig. 4B, neither of these constructs was able to silence the effect of the enhancer on expression from the ρ promoter (lanes 5 and 6, respectively). These results indicate that both the 5′ and 3′ portions of intron 2 are required for the enhancer-silencing effect, thus localizing the intron 2 ESE to sequences between +545 and +880.

The ρ gene enhancer-silencing effect is enhancer specific. To determine if the ρ gene enhancer-silencing effect is limited to avian erythroid enhancers, constructs containing 5′ HS2 from the murine β-globin LCR and containing or lacking the 3′ ESE were made. The constructs and the results of these experiments are shown in Fig. 5A. As can be seen, the addition of the mouse 5′ HS2 region to the pR3a construct lacking the 3′ ESE resulted in an approximately 15-fold increase in expression. The addition of the 3′ ESE to this construct resulted in a six- to eightfold reduction in expression levels (Fig. 5A). Thus, the presence of all three ESEs results in the same fold reduction in enhancement from mouse 5′ HS2 as that from the avian erythroid enhancer elements, thus demonstrating that the enhancer-silencing effect is not specific to avian erythroid enhancers.

In order to determine if the ρ gene ESE can affect enhancement from a nonerythroid enhancer, constructs linking the RSV enhancer to the pR and pR3a constructs were made. These constructs and their expression levels are illustrated in Fig. 5B. The addition of the RSV enhancer to construct pR3a, which lacks the 3′ ESE, increased expression from the ρ promoter by about 7.5-fold, which demonstrates approximately equal strengths for this enhancer and the erythroid cell-specific enhancers. The addition of this nonerythroid enhancer to construct pR, which contains all three ESE, increased expression by about 10-fold. Thus, the presence of all three ESE does not decrease the activity of the RSV enhancer, in contrast to the six- to eightfold reduction in enhancement from either avian or murine erythroid cell-specific enhancers. These results suggest that the enhancer-silencing effect is mediated, at least in part, through an interaction between the silencer elements and erythroid cell-specific enhancers that cannot be duplicated by the nonerythroid RSV enhancer.

Treatment of K562 cells with hemin partially alleviates the enhancer-silencing effect. In order to determine if the enhancer-silencing effect on embryonic ρ-globin gene expression in multipotential K562 cells would function in a more differentiated erythroid cell environment, transient transfectants were treated immediately posttransfection with 20 μM hemin for 40 to 44 h. Hemin induces the multipotential K562 cells to differentiate down the erythroid pathway, as evidenced by three- to fivefold increases in the expression of endogenous ε-, ζ-, and α-globin genes (7). Constructs containing or lacking the upstream HS of the avian β-globin locus (Fig. 1A) were chosen for testing because these sites had been shown by Mason et al. (33) to be critical for high-level ρ gene expression in transgenic mice. The results of these experiments are shown in Fig. 5C. Hemin-induced erythroid differentiation of K562 cells was monitored by analyzing the increase in expression of the endogenous human γ-globin gene (7). In these experiments, a consistent two- to threefold increase in γ-globin gene expression was observed (Fig. 5C [bottom panel]). The effect of hemin on enhancer silencing can best be appreciated by comparing the relative expressions of constructs with the same ρ gene sequences with or without an enhancer. In the case of constructs lacking the 3′ ESE (pR3a and 5HSpR3a), a significantly higher level of expression is seen upon the addition of an enhancer in both untreated and hemin-treated cells. In 3′ ESE-containing constructs (pR and 5HSpR), only a slight (<1.5-fold) increase in expression is observed in the presence of the enhancer in untreated cells (Fig. 5C; compare lanes 7 and 5 in the top panel), while a fivefold increase is seen after hemin induction (compare lanes 8 and lane 6 in the top panel). These data indicate that the treatment of K562 cells with hemin partially alleviates the enhancer-silencing effect. The partial alleviation of enhancer silencing through hemin-induced erythroid differentiation of K562 cells could be the result of the previously described increased activity of erythroid enhancers in hemin-induced K562 cells (37, 41), which could then partially overcome the silencing capabilities of the ESE. Alternatively, this result could reflect the involvement of a critical silencing factor, whose activity is decreased in hemin-induced cells. The partial nature of the alleviation of enhancer silencing through hemin induction may reflect the fact that hemin treatment does not result in terminal (complete) differentiation down the erythroid pathway.

The transcription factor YY1 binds to both the 5′ and 3′ ESE. An inspection of the sequences of the 5′, intron 2, and 3′ ESE revealed that each of them has one or more potential binding sites for the transcription factor YY1 (Fig. 6A; potential YYI sites are indicated by arrows). YY1 is a ubiquitous transcription factor that in different contexts has been shown to act as a repressor, activator, or initiator of transcription. Since YY1 has been shown to bind within repressor regions of both the human ε- (25, 42) and γ (24)-globin gene promoters, we examined whether YY1 binding could be detected in the ρ ESE. The 140-bp 5′ ESE (−2140 to −2000) was end labeled with T4 polynucleotide kinase (see Materials and Methods) and used as a probe in an electrophoretic mobility shift assay. As shown in Fig. 6B, lane 2, only one binding complex (the lower arrow) was observed when this probe was incubated with crude K562 nuclear extract. This complex was efficiently inhibited by both unlabeled 5′ ESE (Fig. 6B, lane 3) and an unlabeled, double-stranded oligonucleotide containing a consensus YY1 binding site (lane 4). The sequence of the consensus YY1 oligonucleotide is shown in Fig. 6C; the fact that YY1 binds to this consensus site was verified by gel shift and supershift assays (data not shown). In addition, the 5′ ESE complex was supershifted by the addition of 2 μg of polyclonal YY1 antibody (Santa Cruz Biotechnologies) to the binding reaction mixture (Fig. 6B, lane 5 [the upper arrow]). Negative control experiments with preimmune sera excluded a nonspecific antibody interaction (data not shown). This indicates that at least one of the potential YY1 binding sites in the 5′ ESE is capable of binding the transcription factor YY1.

In order to determine if YY1 also binds to the single potential site within the 3′ ESE, a 185-bp fragment (+1995 to +2180) of the 3′ ESE was end labeled and tested in a similar binding assay. The pattern of binding to this fragment with both crude and partially purified (over a nickel-nitrilotriacetic acid column [47]) K562 extracts was so complex that YY1 binding could not be clearly distinguished (data not shown). Therefore, a double-stranded oligonucleotide containing the putative YY1 site in the 3′ ESE was synthesized (Fig. 6C; wild-type 3′ enhancer silencer) and tested in a binding assay with crude K562 nuclear extract. Two complexes were observed (Fig. 6D, lane 2); both complexes were inhibited by 25- to 200-fold molar excesses of unlabeled wild-type 3′ enhancer silencer (YY1) oligonucleotide (lanes 3 to 6). The addition of 25- to 200-fold molar excesses of unlabeled consensus YY1 oligonucleotide resulted in competition of only the faster-migrating complex (Fig. 6D, lanes 7 to 10). This suggests that the formation of the faster-migrating complex involves YY1 binding and that the formation of the more slowly migrating complex does not involve YY1 binding. This conclusion is supported by the fact that the addition of YY1 antibody to the binding reaction mixture supershifts only the faster-migrating complex (Fig. 6D, lane 11 [middle arrow]). The faster-migrating complex was completely inhibited by a 25-fold molar excess of unlabeled YY1 consensus oligonucleotide (Fig. 6D, lane 7), whereas complete inhibition of this complex by unlabeled wild-type 3′ enhancer silencer oligonucleotide required a 50-fold molar excess (lane 4). This suggests that the binding of YY1 to the 3′ ESE is slightly weaker than its binding to the consensus YY1 site.

In an effort to confirm YY1 binding to the site in the 3′ ESE, an additional pair of complementary oligonucleotides containing a two-base mutation that abolishes the core of the YY1 binding site [mutant 3′ enhancer silencer (YY1) (Fig. 6C)] were annealed and used as an unlabeled competitor for binding to the wild-type probe at 50- to 500-fold molar excesses. As shown in Fig. 6D, lanes 12 to 15, this sequence was unable to compete for the formation of the faster-migrating complex, even at a 500-fold molar excess (lane 15), confirming that YY1 does not bind efficiently to the mutant sequence. Somewhat surprisingly, this same mutant oligonucleotide was also unable to compete for the formation of the more slowly migrating complex, indicating that the mutated bases are also critical to the binding of the factor (or factors) involved in the formation of the more slowly migrating complex.

Further inspection of the sequence of the 3′ ESE identified a putative binding site for the CCAAT box binding factor NF-Y (Fig. 6A [dashed line]) overlapping the YY1 binding site (solid arrow) in this element. To determine if the formation of either of the binding complexes in Fig. 6D involved the binding of NF-Y, an oligonucleotide containing a consensus NF-Y binding site (a gift of Linda Burns, University of Minnesota) was used to compete for binding to the wild-type 3′ ESE probe. As shown in Fig. 6E, when this oligonucleotide was added at a 100- to 500-fold molar excess, it competed for the formation of the more slowly migrating complex (middle arrow), not the faster-migrating YY1-containing complex (lower arrow). Furthermore, the addition of 2 μg of polyclonal antibody directed against the A subunit of NF-Y to the binding reaction mixture resulted in a supershift of the more slowly migrating complex,

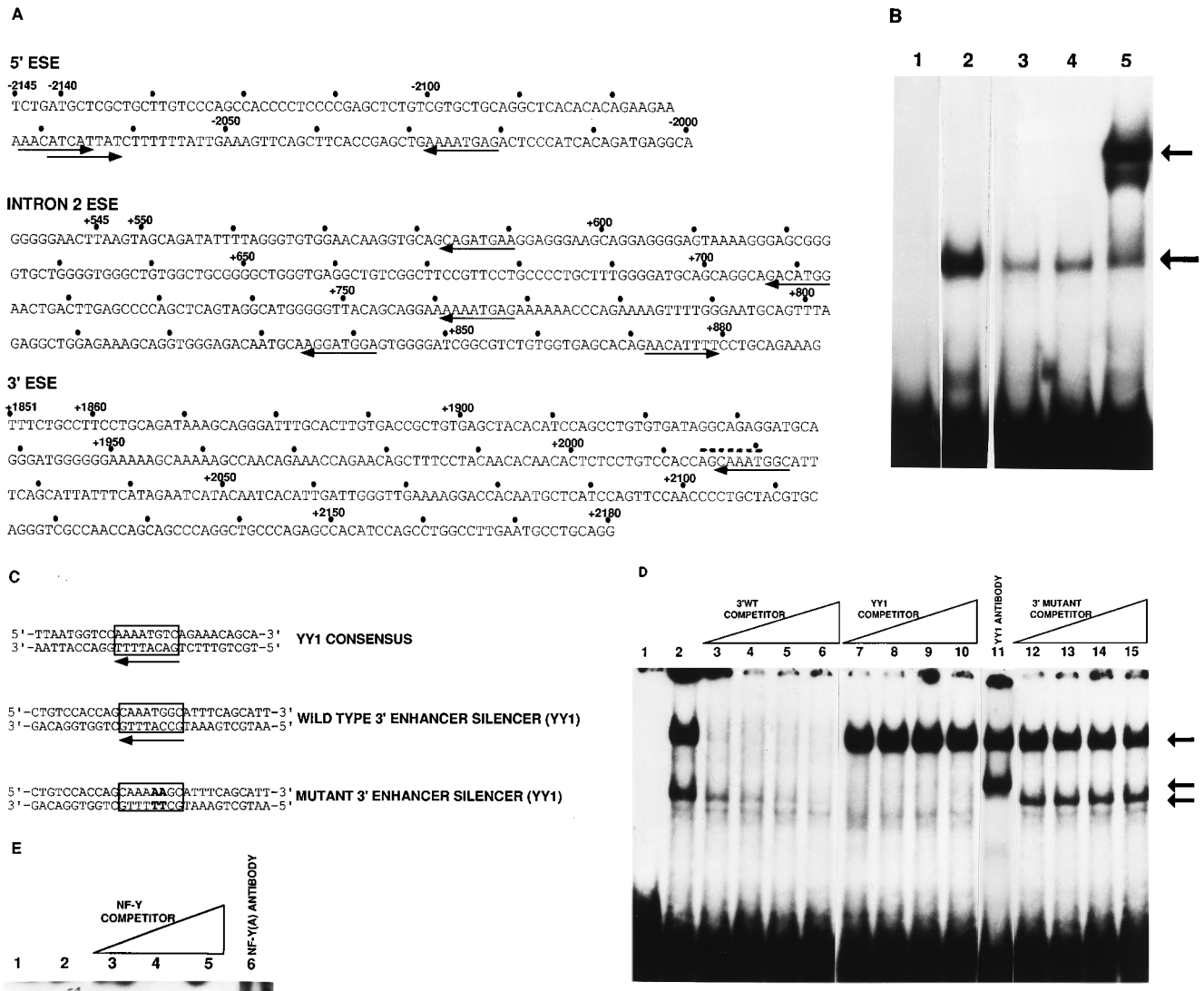

FIG. 6. (A) Sequences of the 5′, intron 2, and 3′ ESE; numbers indicate positions relative to the ρ mRNA cap site. Potential YY1 sites are indicated by arrows; the potential NF-Y binding site in the 3′ ESE is indicated by a dashed line. (B) Electrophoretic mobility shift assay with the 5′ ESE as probe. Lane 1, probe alone; lanes 2 to 5, 25 μg of K562 nuclear extract and competition with 100-fold molar excess of unlabeled 5′ ESE (lane 3) or unlabeled YY1 consensus oligonucleotide (shown in panel C; lane 4) or supershift with 2 μg of polyclonal YY1 antibody (Santa Cruz Biotechnologies) (lane 5). The lower arrow indicates YY1 binding; the upper arrow indicates the supershifted complex. (C) Sequences of the YY1 consensus oligonucleotides used in the competitions of panels B and D, as well as the wild-type and mutant 3′ enhancer silencer (YY1) oligonucleotides used in panels D and E. Each YY1 site is boxed; mutated bases are shown in bold. (D) Electrophoretic mobility shift assay with the 3′ wild-type (WT) enhancer silencer (YY1) oligonucleotide as probe. Lane 1, probe alone; lanes 2 to 15, 25 μg of K562 nuclear extract. Lanes 3 to 6, competition with unlabeled WT 3′ enhancer silencer (YY1) at a 25-, 50-, 100-, and 200-fold molar excess, respectively. Lanes 7 to 10, competition with unlabeled YY1 consensus site oligonucleotide at a 25-, 50-, 100-, and 200-fold molar excess, respectively. Lane 11, supershift with 2 μg of polyclonal YY1 antibody. Lanes 12 to 15, competition with unlabeled mutant 3′ enhancer silencer (YY1) oligonucleotide at a 50-, 100-, 200-, and 500-fold molar excess, respectively. The bottom arrow indicates YY1 binding; the middle arrow represents a supershift of the YY1-containing complex. (E) Electrophoretic mobility shift assay with the 3′ wild-type enhancer silencer (YY1) oligonucleotide as probe. Lane 1, probe alone, lanes 2 to 6, 25 μg of K562 nuclear extract. Lanes 3 to 5, competition with unlabeled NF-Y consensus site oligonucleotide at a 100-, 200-, and 500-fold molar excess, respectively. Lane 6, supershift with 2 μg of polyclonal antibody to the A subunit of NF-Y [NF-Y(A) antibody]. Bottom arrow, YY1-containing complex; middle arrow, NF-Y-containing complex; top arrow, supershift of NF-Y complex.

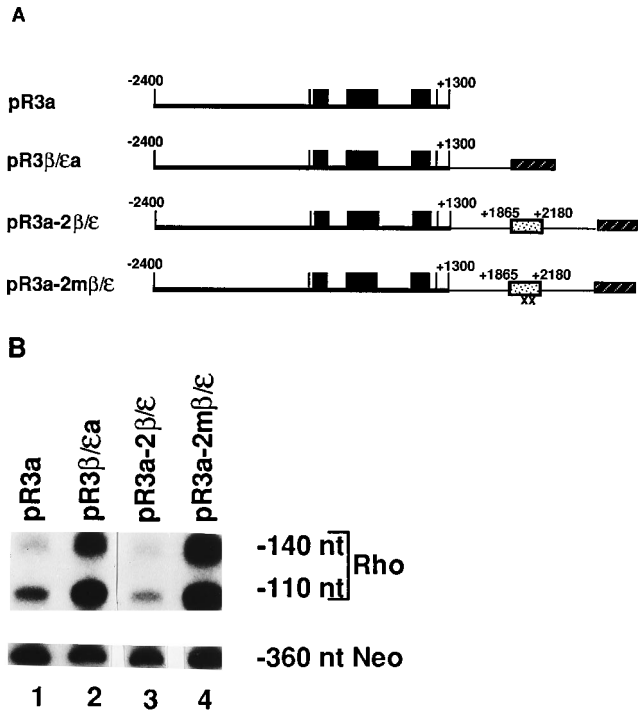

FIG. 7. (A) Schematic illustrations of the constructs used in this RNase protection assay. The name of each construct is indicated on the left. Numbers indicate positions relative to the mRNA cap site. The solid, striped, and dotted boxes represent exons, 3′ β/ε enhancer, and 3′ ESE, respectively. Thick and thin lines represent ρ and vector sequences, respectively; XX, the two mutated bases of the YY1/NF-Y binding sites (Fig. 6A). (B) RNase protection assay of RNAs from K562 cells transiently cotransfected with RSV-*neo* and the constructs shown in panel A. The probe fragments and expression quantitation are the same as those indicated in the legend to Fig. 1.

but not the faster-migrating complex (Fig. 6E, lane 6). The addition of antibody to the B subunit of NF-Y abolished the formation of the more slowly migrating complex (data not shown). These data indicate that the formation of the more slowly migrating complex involves the binding of the transcription factor NF-Y.

Mutation of the overlapping YY1/NF-Y binding site in the 3′ ESE abolishes enhancer silencing. The mutant 3′ enhancer silencer oligonucleotides were used in the generation of a 315-bp (+1865 to +2180) 3′ ESE containing the two-base mutation depicted in Fig. 6C. This mutant 3′ ESE was then inserted into construct pR3β/εa, generating construct pR3a-2mβ/ε (Fig. 7A). The constructs shown in Fig. 7A were transiently transfected into K562 cells, and expression was analyzed by RNase protection assay. In these experiments, the enhancer conferred a 6- to 10-fold increase in expression from the ρ promoter in the pR3β/εa construct (which lacks the 3′ ESE) (Fig. 7B, lane 2). The addition of the wild-type 3′ ESE completely silenced this enhancer effect, as previously demonstrated (Fig. 7B, lane 3). However, the addition of the mutant 3′ ESE to the pR3β/εa construct had no effect on the enhanced level of expression from the ρ promoter (Fig. 7B, lane 4). These data show that the two mutated bases in the 3′ ESE are critical to the function of the 3′ ESE in particular and thus to the enhancer-silencing effect in general. The fact that the mutation introduced was in the overlapping YY1/NF-Y binding site indicates that interactions at this site are important to the enhancer-silencing effect; however, because the mutation abolished the binding of both YY1 and NF-Y, it is not yet known whether YY1, NF-Y, or both are required for the enhancer-silencing effect.

DISCUSSION

In this paper, we have delineated three ESE within the avian embryonic ρ-globin gene and its flanking sequences that act together to silence the enhancement of ρ gene expression by erythroid enhancers in human K562 cells, We have also shown that 5′ HS1 to -3 from the avian β-globin locus act together as an enhancer of ρ gene expression that is equal in potency to the 3′ β/ε enhancer of the avian β-globin locus. In addition, we have demonstrated that the enhancer-silencing effect in K562 cells can be partially abrogated by the induction of erythroid differentiation through treatment with hemin. Finally, we have demonstrated that the transcription factor YY1 can bind to both the 5′ and 3′ ESE and that a region of the 3′ ESE containing YY1 and NF-Y binding sites is critical to enhancer silencer function.

While no striking positive or negative regulatory effects were identified in the examination of expression from enhancerless ρ constructs, a striking negative regulatory effect was identified when an avian erythroid enhancer was added to these constructs. The presence of these silencing elements in the absence of an enhancer element did not alter basal expression from the ρ promoter; therefore, we have chosen to refer to these sequences as ESE. When an enhancer was included in constructs lacking 5′ or 3′ flanking sequences, 6- to 10-fold increases in expression levels were observed. These increases were observed with either the 3′ enhancer from the avian β-globin locus (the 3′ β/ε enhancer) or 5′ HS1 to -3 from the upstream portion of the avian β-globin locus. When both 5′ and 3′ flanking sequences were included in the construct, however, the effect of the enhancer was silenced; no increase in expression was observed in the presence of either of the avian erythroid enhancer elements used. Deletional analysis localized the 5′ flanking sequence necessary for the silencing effect to −2140 to −2000 and the necessary 3′ flanking sequence to +1865 to +2180. Additional experiments indicated that the region of intron 2 between +545 and +880 (which is most of intron 2) is also necessary for the enhancer-silencing effect. Both the 5′ and 3′ portions of the +545 to +880 region appear to be critical for the enhancer-silencing effect. It is unclear whether the middle portion of the intron also is required for enhancer silencing; it remains possible that the intron 2 ESE described in this report is composed of two separate, smaller elements. This possibility is suggested by the presence of YY1 binding sites clustered at the two ends of the intronic silencing element (Fig. 6A), although direct evidence is lacking.

The binding of the ubiquitously expressed transcription factor YY1 to the 5′ and 3′ ESE and the presence of potential binding sites for this factor within the intron 2 ESE add an additional level of complexity to any postulated mechanism of ESE function. Although the results of mutation analysis indicate that the YY1 binding site in the 3′ ESE is critical for the ρ gene enhancer-silencing effect, the presence of an NF-Y binding site that is also abolished by the YY1 binding site mutation makes it impossible to state with certainty that YY1 is required for the enhancer-silencing effect. However, several lines of evidence suggest that YY1 is involved in the ρ gene enhancer-silencing effect. In an analysis of the genes that are regulated by YY1, Shrivastava and Calame (48) found that the majority of those genes for which YY1 acts as a repressor of expression are highly regulated and expressed in a tissue-specific manner. In analyzing the surrounding sequences of these YY1 sites, they identified separate consensus sequences for the

sites in genes at which YY1 acts as an activator, as opposed to the sites in genes at which YY1 acts as a repressor. Without exception, the potential YY1 sites identified in the ESE of the ρ gene match the repressor consensus sequence more closely than they do the activator consensus sequence. In addition, Raich et al. recently reported the involvement of a YY1 site in the development-specific silencing of the embryonic ε-globin gene, which may be mediated by interference with the action of the β-globin LCR on the ε promoter (44). Finally, YY1 has been shown to repress the expression of the c-*fos* promoter by bending the DNA by approximately 80°, which blocks the ability of the cyclic AMP response element to interact with and stimulate expression from the promoter (40). It seems likely that if such DNA bending is critical to the enhancer-silencing effect seen here, which involves three widely separated regulatory elements, the effect of bending would be on local factor binding at each individual element. In this regard, elucidation of the role of NF-Y and other potential factors binding to the ESE will be critical to a full understanding of the mechanism of silencing.

The observation that the ρ ESE can act on three different erythroid cell-specific enhancers but not on the equally strong, non-tissue-specific RSV enhancer suggests that the enhancer-silencing effect is somehow tissue specific. While this result does not prove that no nonerythroid enhancer would be affected by the ESE, it does show that this is not an effect mediated by nonspecific blocking of the promoter. The partial alleviation of silencing by treatment with hemin could also be interpreted to suggest that enhancer silencing is a tissue-specific effect. The silencing of the enhancement of expression of an embryonic globin gene in the embryonic and fetal erythroid environment of K562 cells could be due to the influence of some nonerythroid characteristics of this transformed cell line. The fact that hemin-induced erythroid differentiation partially abrogates the enhancer-silencing effect supports the hypothesis that the effect in untreated cells is the result of the active influence of some nonerythroid factor in K562 cells or, alternatively, of the lack of the fully erythroid environment needed to overcome silencing. In support of the possibility of a tissue-specific effect, Jiménez et al. (30) have shown that 5′ HS1 and 5′ HS2 of the murine LCR are already hypersensitive in multilineage hematopoietic progenitor cells yet do not stimulate globin gene transcription. These HS subsequently disappear if the progenitor cells are induced to differentiate down a nonerythroid pathway. Other studies have shown that erythroid cell-specific factors critical to globin gene promoter and erythroid LCR enhancer effects, such as GATA-1 and NF-E2, begin to be expressed prior to full commitment to the erythroid lineage (26, 34, 43). Therefore, while speculative, it is reasonable that a negative regulatory system might operate to prevent the high-level expression of globin genes at a point prior to full erythroid commitment, when both erythroid cell-specific LCR HS and erythroid cell-specific factors requisite for enhancer and promoter function are already present. The proof of such a role for the enhancer silencer mechanism described here will require testing in the context of normal hematopoietic progenitor cell differentiation; these studies are under way.

Although the classical definition of a silencer includes a requirement for position- and orientation-independent function, as well as the ability to affect a heterologous promoter or enhancer, several silencers that do not fit all of these criteria have been described. Most of these nonclassical silencers have been shown to be involved in tissue-specific silencing of gene expression. Examples include upstream silencers that control tissue-specific expression of the rat SCG10 gene (38), human α-fetoprotein gene (39), and human urokinase plasminogen activator gene (5); a tissue-specific intronic silencer in the human platelet-derived growth factor A-chain gene (52); and a tissue-specific bipartite silencer in exon 1 and the border between exon 1 and intron 1 of the rat osteocalcin gene (19). Since they did not silence the enhancement from a nonerythroid viral enhancer, the ESE described in this report may fit more appropriately into this latter category of nonclassical silencers. Finally, while previous reports have described the involvement of intronic or intronic and flanking (5′ or 3′) sequences in the negative regulation of gene expression (19, 49, 52), this report emphasizes the potential complexity of negative regulation by showing the simultaneous requirement for 5′ flanking, intronic, and 3′ flanking sequences in the execution of a single negative regulatory effect on a eukaryotic gene.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant DK29902 from NIDDKD (G.D.G.) and by the Masonic Cancer Center Fund (G.D.G.).

We thank Linda Burns, Kathleen Conklin, and Jane Little for helpful advice and critical review of the manuscript and Judy Goetzke for assistance in preparation of the manuscript.

REFERENCES

1. **Abruzzo, L. V., and M. Reitman.** 1994. Enhancer activity of upstream hypersensitive site 2 of the chicken β-globin cluster is mediated by GATA sites. J. Biol. Chem. **269:**32565–32571.
2. **Bordonaro, M., and J. L. Nordstrom.** 1994. Different mechanisms are responsible for the low accumulation of transcripts from intronless and 3′ splice site deleted genes. Biochem. Biophys. Res. Commun. **203:**128–132.
3. **Brown, J. L., and V. M. Ingram.** 1974. Structural studies on chick embryonic hemoglobins. J. Biol. Chem. **249:**3960–3972.
4. **Burns, L. J., J. G. Glauber, and G. D. Ginder.** 1988. Butyrate induces selective transcriptional activation of a hypomethylated embryonic globin gene in adult erythroid cells. Blood **72:**1536–1542.
5. **Cannio, R., P. S. Rennie, and F. Blasi.** 1991. A cell-type specific and enhancer-dependent silencer in the regulation of the expression of the human urokinase plasminogen activator gene. Nucleic Acids Res. **19:**2303–2308.
6. **Cao, S. X., P. D. Gutman, H. P. G. Dave, and A. N. Schechter.** 1989. Identification of a transcriptional silencer in the 5′-flanking region of the human ε-globin gene. Proc. Natl. Acad. Sci. USA **86:**5306–5309.
7. **Charnay, P., and T. Maniatis.** 1983. Transcriptional regulation of globin gene expression in the human erythroid cell line K562. Science **220:**1281–1283.
8. **Chen, E., R. W. Karr, J. P. Frost, T. A. Gonwa, and G. D. Ginder.** 1986. Gamma interferon and 5-azacytidine cause transcriptional elevation of class I major histocompatibility complex gene expression in K562 leukemia cells in the absence of differentiation. Mol. Cell. Biol. **6:**1698–1705.
9. **Choi, O.-R., and J. D. Engel.** 1986. A 3′ enhancer is required for temporal and tissue-specific transcriptional activation of the chicken adult beta-globin gene. Nature (London) **323:**731–734.
10. **Chung, J. H., M. Whiteley, and G. Felsenfeld.** 1993. A 5′ element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. Cell **74:**505–514.
11. **Collis, P., M. Antoniou, and F. Grosveld.** 1990. Definition of the minimal requirements within the human β-globin gene and the dominant control region for high level expression. EMBO J. **9:**233–240.
12. **Crossley, M., A. P. Tsang, J. J. Bieker, and S. H. Orkin.** 1994. Regulation of the erythroid Kruppel-like factor (EKLF) gene promoter by the erythroid transcription factor GATA-1. J. Biol. Chem. **269:**15440–15444.
13. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
14. **Dodgson, J. B., S. J. Stadt, O.-R. Choi, M. Dolan, H. D. Fischer, and J. D. Engel.** 1983. The nucleotide sequence of the embryonic chicken β-type globin genes. J. Biol. Chem. **258:**12685–12692.
15. **Feng, W. C., C. M. Southwood, and J. J. Bieker.** 1994. Analysis of β-thalassemia mutant DNA interactions with erythroid Krüppel-like factor (EKLF), and erythroid cell-specific transcription factor. J. Biol. Chem. **269:**1493–1500.
16. **Forrester, W. C., E. Epner, M. C. Driscoll, T. Enver, M. Brice, T. Papayannopoulou, and M. Groudine.** 1990. A deletion of the human β-globin locus activation region causes a major alteration in chromatin structure and replication across the entire β-globin locus. Genes Dev. **4:**1637–1649.
17. **Forrester, W. C., S. Takegawa, T. Papayannopoulou, G. Stamatoyannopoulos, and M. Groudine.** 1987. Evidence for a locus activation region: the

formation of developmentally stable hypersensitive sites in globin-expressing hybrids. Nucleic Acids Res. **15:**10159–10177.
18. **Forrester, W. C., C. Thompson, J. T. Elder, and M. Groudine.** 1986. A developmentally stable chromatin structure in the human β-globin gene cluster. Proc. Natl. Acad. Sci. USA **83:**1359–1363.
19. **Frenkel, B., J. Mijnes, M. A. Aronow, G. Zambetti, C. Banerjee, J. L. Stein, J. B. Lian, and G. S. Stein.** 1993. Position and orientation-selective silencer in protein-coding sequences of the rat osteocalcin gene. Biochemistry **32:** 13636–13643.
20. **Glauber, J. G., N. J. Wandersee, J. A. Little, and G. D. Ginder.** 1991. 5′-flanking sequences mediate butyrate stimulation of embryonic globin gene expression in adult erythroid cells. Mol. Cell. Biol. **11:**4690–4697.
21. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
22. **Grosveld, F., G. B. van Assendelft, D. R. Greaves, and G. Kollias.** 1987. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell **51:**975–985.
23. **Gumucio, D. L., H. Heilstedt-Williamson, T. A. Gray, S. A. Tarlé, D. A. Shelton, D. A. Tagle, J. L. Slightom, M. Goodman, and F. S. Collins.** 1992. Phylogenetic footprinting reveals a nuclear protein which binds to silencer sequences in the human γ and ε globin genes. Mol. Cell. Biol. **12:**4919–4929.
24. **Gumucio, D. L., K. L. Rood, T. A. Gray, M. F. Riordan, C. I. Sartor, and F. S. Collins.** 1988. Nuclear proteins that bind the human γ-globin gene promoter: alterations in binding produced by point mutations associated with hereditary persistence of fetal hemoglobin. Mol. Cell. Biol. **8:**5310–5322.
25. **Gumucio, D. L., D. A. Shelton, W. J. Bailey, J. L. Slightom, and M. Goodman.** 1993. Phylogenetic footprinting reveals unexpected complexity in trans factor binding upstream from the ε-globin gene. Proc. Natl. Acad. Sci. USA **90:**6018–6022.
26. **Hannon, R., T. Evans, G. Felsenfeld, and H. Gould.** 1991. Structure and promoter activity of the gene for the erythroid transcription factor GATA-1. Proc. Natl. Acad. Sci. USA **88:**3004–3008.
27. **Hesse, J. E., J. M. Nickol, M. R. Lieber, and G. Felsenfeld.** 1986. Regulated gene expression in transfected primary chicken erythrocytes. Proc. Natl. Acad. Sci. USA **83:**4312–4316.
28. **Jane, S. M., D. L. Gumucio, P. A. Ney, J. M. Cunningham, and A. W. Nienhuis.** 1993. Methylation-enhanced binding of Sp1 to the stage selector element of the human γ-globin gene promoter may regulate developmental specificity of expression. Mol. Cell. Biol. **13:**3272–3281.
29. **Jiménez, G., K. B. Gale, and T. Enver.** 1992. The mouse β-globin locus control region: hypersensitive sites 3 and 4. Nucleic Acids Res. **20:**5797–5803.
30. **Jiménez, G., S. D. Griffiths, A. M. Ford, M. F. Greaves, and T. Enver.** 1992. Activation of the β-globin locus control region precedes commitment to the erythroid lineage. Proc. Natl. Acad. Sci. USA **89:**10618–10622.
31. **Kuipers, O. P., H. J. Boot, and W. M. de Vos.** 1991. Improved site-directed mutagenesis method using PCR. Nucleic Acids Res. **19:**4558.
32. **Lieber, M. R., J. E. Hesse, J. M. Nickol, and G. Felsenfeld.** 1987. The mechanism of osmotic transfection of avian embryonic erythrocytes: analysis of a system for studying developmental gene expression. J. Cell Biol. **105:** 1055–1065.
33. **Mason, M. M., E. Lee, H. Westphal, and M. Reitman.** 1995. Expression of the chicken β-globin gene cluster in mice: correct developmental expression and distributed control. Mol. Cell. Biol. **15:**407–414.
34. **Mignotte, V., L. Wall, E. deBoer, F. Grosveld, and P.-H. Romeo.** 1989. Two tissue-specific factors bind the erythroid promoter of the human porphobilinogen deaminase gene. Nucleic Acids Res. **17:**37–54.
35. **Miller, I. J., and J. J. Bieker.** 1993. A novel, erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the *Krüppel* family of nuclear proteins. Mol. Cell. Biol. **13:**2776–2786.
36. **Moon, A. M., and T. J. Ley.** 1990. Conservation of the primary structure, organization, and function of the human and mouse β-globin locus-activating regions. Proc. Natl. Acad. Sci. USA **87:**7693–7697.
37. **Moon, A. M., and T. J. Ley.** 1991. Functional properties of the β-globin locus control region in K562 cells. Blood **77:**2272–2284.
38. **Mori, N., R. Stein, O. Sigmund, and D. J. Anderson.** 1990. A cell type-preferred silencer element that controls the neural-specific expression of the SCG10 gene. Neuron **4:**583–594.
39. **Nakabayashi, H., T. Hashimoto, Y. Miyao, K.-K. Tjong, J. Chan, and T. Tamaoki.** 1991. A position-dependent silencer plays a major role in repressing α-fetoprotein expression in human hepatoma. Mol. Cell. Biol. **11:**5885–5893.
40. **Natesan, S., and M. Z. Gilman.** 1993. DNA bending and orientation-dependent function of YY1 in the c-*fos* promoter. Genes Dev. **7:**2497–2509.
41. **Ney, P. A., B. P. Sorrentino, C. H. Lowrey, and A. W. Nienhuis.** 1990. Inducibility of the HS II enhancer depends on binding of an erythroid specific nuclear protein. Nucleic Acids Res. **18:**6011–6017.
42. **Peters, B., N. Merezhinskaya, J. F. X. Diffley, and C. T. Noguchi.** 1993. Protein-DNA interactions in the ε-globin gene silencer. J. Biol. Chem. **268:** 3430–3437.
43. **Pevny, L., M. C. Simon, E. Robertson, W. H. Klein, S.-F. Tsai, V. D'Agati, S. Orkin, and F. Constantini.** 1991. Erythroid differentiation in chimeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature (London) **349:**257–260.
44. **Raich, N., C. H. Clegg, J. Grofti, P.-H. Roméo, and G. Stamatoyannopoulos.** 1995. GATA1 and YY1 are developmental repressors of the human ε-globin gene. EMBO J. **14:**801–809.
45. **Reitman, M., J. A. Grasso, R. Blumenthal, and P. Lewit.** 1993. Primary sequence, evolution and repetitive elements of the *Gallus gallus* (chicken) β-globin cluster. Genomics **18:**616–626.
46. **Sharrocks, A. D., and P. E. Shaw.** 1992. Improved primer design for PCR-based, site-directed mutagenesis. Nucleic Acids Res. **20:**1147.
47. **Shi, Y., E. Seta, L.-S. Chang, and T. Shenk.** 1991. Transcriptional repression by YY1, a human GLI-Krüppel-related protein, and relief of repression by adenovirus E1A protein. Cell **67:**377–388.
48. **Shrivastava, A., and K. Calame.** 1994. An analysis of genes regulated by the multi-functional transcriptional regulator Yin Yang-1. Nucleic Acids Res. **22:**5551–5155.
49. **Siu, G., A. L. Wurster, D. D. Duncan, T. M. Soliman, and S. M. Hedrick.** 1994. A transcriptional silencer controls the developmental expression of the CD4 gene. EMBO J. **13:**3570–3579.
50. **Stamatoyannopoulos, G., and A. W. Nienhuis.** 1994. Hemoglobin switching, p. 107–155. *In* G. Stamatoyannopoulos et al. (ed.), The molecular basis of blood diseases. The W. B. Saunders Co., Philadelphia.
51. **Tuan, D., W. Solomon, Q. Li, and I. M. London.** 1985. The "β-like-globin" gene domain in human erythroid cells. Proc. Natl. Acad. Sci. USA **82:** 6384–6388.
52. **Wang, Z.-Y., N. Masaharu, Q.-Q. Qiu, Y. Takimoto, and T. F. Deuel.** 1994. An S1 nuclease-sensitive region in the first intron of human platelet-derived growth factor A-chain gene contains a negatively acting cell type-specific regulatory element. Nucleic Acids Res. **22:**457–464.