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trans-Activation of a Globin Promoter in Nonerythroid Cells

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We show that expression in fibroblasts of a single cDNA, encoding the erythroid DNA-binding protein Eryf1 (GF-1, NF-E1), very efficiently activates transcription of a chicken α-globin promoter. *trans*-Activation in these cells occurred when Eryf1 bound to a single site within a minimal globin promoter. In contrast, efficient activation in erythroid cells required multiple Eryf1 binding sites. Our results indicate that mechanisms exist that are capable of modulating the *trans*-acting capabilities of Eryf1 in a cell-specific manner, without affecting DNA binding. The response of the minimal globin promoter to Eryf1 in fibroblasts was at least as great as for optimal constructions in erythroid cells. Therefore, the assay provides a very simple and sensitive system with which to study gene activation by a tissue-specific factor.

The regulation of globin genes involves restriction of expression to cells of the erythroid lineage; other (perhaps distinct) mechanisms determine which members of the gene family are expressed during a particular stage of development. A number of DNA-binding proteins have been identified which interact with sequences that are important for transcription of the various chicken globin genes (reviewed in reference 7); at least some of these factors are present in many cell types and therefore may also regulate the expression of nonerythroid genes. Other binding activities are abundant only at particular stages of erythroid development and therefore might be involved in regulating stage-specific expression of particular globin genes (5, 10, 13).

We identified an erythroid DNA-binding protein named Eryf1 (mammalian homologs are also referred to as GF-1 [21] and NF-E1 [31]) that binds to regulatory regions of every chicken globin gene (8) and have cloned the Eryf1 cDNA (6). Because the factor is expressed in all erythroid lineages, we proposed that Eryf1 may be a key regulatory molecule in the early events determining erythroid-specific expression (8). As further evidence, Eryf1 interacts with regulatory elements of erythroid-specific genes in addition to globin genes (2, 24, 29).

We have previously shown that the Eryf1 cDNA encodes the sequence-specific DNA-binding activity identified in extracts of chicken erythroid cells (6) and that mutation of Eryf1 binding sites results in a decrease in expression from a linked promoter in the same cells (8, 27). However, it is possible that for activation of globin gene expression, additional cell-specific factors are required. To address this question, we have asked whether a globin promoter can be activated in a nonerythroid environment by Eryf1.

MATERIALS AND METHODS

Cell culture and DNA transfection. Chicken fibroblast cells (CEFs) were prepared by trypsin digestion of 11-day embryos. Following expansion of the adherent cell population, cells were divided into aliquots and frozen in 10% dimethyl sulfoxide. Cells were then thawed for use in transfection experiments; an aliquot could be passaged up to 10 times without significant changes in growth rates, at which time a fresh aliquot was expanded. Cells were maintained in Dulbecco modified Eagle medium containing 8% fetal bovine serum, 2% chick serum, and antibiotics, including amphotericin B (Fungizone) (all from GIBCO). Similar results were obtained from different passages and aliquots of CEF cells or from cells prepared instead from breast muscle tissue. Chicken erythroid cells were prepared by bleeding 10-day embryos.

Transfection into embryonic erythroid cells was performed as described previously (12, 25). For each assay, a cell concentration corresponding to an A_{412} value of 20 (about 1.7×10^8 cells; 18) was osmotically shocked in 0.25 M NH_4Cl for 35 min at 25°C. For transfection, swelled cells were incubated in a 0.5-ml solution of L-15 medium (Flow Laboratories) containing DNA (2 μg/ml) and DEAE-dextran (200 μg/ml); (Pharmacia) for 10 min at 37°C. After washing, cells were incubated for 40 to 48 h in L-15 medium containing 5% fetal bovine serum, 2% chick serum, and antibiotics. Cells were transfected and incubated in sterile 1.5-ml microcentrifuge tubes (Sardstedt). Transfection of nonerythroid cells was performed by using a cationic lipid (DOTMA or lipofectin; 9) according to the instructions of the manufacturer (Bethesda Research Laboratories). In a standard assay, 0.75 ml of medium (without serum) containing DNA (3 μg of reporter plasmid, 1 μg of Eryf1 expression vector, and 1 μg of β-galactosidase (β-gal) expression vector [pCH110; Pharmacia]) was added to 0.75 ml of medium containing lipofectin (40 μg). After 15 min, the solution was placed onto 5×10^5 cells (seeded 18 to 24 h previously) in a 35-mm culture dish. After 5 h at 37°C, the transfection mixture was aspirated and cells were incubated in medium with serum for 40 to 48 h prior to harvesting. For some experiments (see Fig. 5), we used 0.25 μg of Eryf1 expression vector supplemented with 0.75 μg of pUC18; we found that additional expression vector can lead to slight inhibition of activation, apparently as a result of competition from overexpressed Eryf1 which is proteolyzed but still binds specifically to DNA. The plasmids used for all transfections were purified by banding twice in CsCl gradients (20).

Analysis of *trans*-activation. Nonerythroid cells were washed with phosphate-buffered saline (PBS), harvested into TEN buffer (40 mM Tris [pH 7.5], 10 mM EDTA, 150 mM NaCl) by scraping, and lysed in 150 μl of 0.25 M Tris (pH 8.0) by three cycles of freeze-thawing as described

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previously (28). For each transfection, a 25-μl sample was analyzed for β-gal activity at 37°C for 20 min. A volume (usually about 5% of the sample) containing a defined amount of β-gal activity was then used to assay for CAT activity. Erythroid cells were spun down, washed in PBS, and similarly lysed. One half of each sample was then assayed for chloramphenicol acetyltransferase (CAT) activity.

All CAT assays were performed for 30 min at 37°C and analyzed by thin-layer chromatography as described previously (28). Following autoradiography, the regions of the thin-layer chromatography plate containing labeled chloramphenicol or its acetylated forms were individually cut out and counted by liquid scintillation to determine the conversion percentage. In the case of transfections including $p\alpha^D3$ and pRSV20-2, this was typically about 40%. Assays in which the conversion was greater than 60% were repeated with less lysate. After subtraction of the percent conversion using lysate from a mock transfection (less than 0.1%), all results from a single set of transfections were expressed relative to the basal activity obtained from $p\alpha^D3$ in the same set. The results from multiple independent experiments were then combined to determine the mean activities and standard sample deviations shown in the figures.

RNA for primer extension experiments was prepared from transfections performed under standard conditions except scaled up threefold, using 100-mm culture dishes. Cells were harvested, and a sample from each was used to measure β-gal activity. The remaining cells were lysed in 4.0 ml of RNAzol (Cinna Biotecx); RNA was prepared as instructed by the manufacturer. DNA was removed by treatment with RNase-free DNase I (Boehringer Mannheim). For each sample, 0.2 pmol of 5'-end-labeled primer complementary to CAT message was hybridized to 50 μg of total RNA in 20 μl of a solution consisting of 0.2 M NaCl and 0.01 M piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES; pH 6.4). The solution was heated at 75°C for 10 min and allowed to cool slowly to 37°C to facilitate annealing. The primer was extended by avian reverse transcriptase (Bethesda Research Laboratories). RNA was then hydrolyzed by incubation of the purified sample in 0.1 N NaOH for 10 min at 37°C prior to analysis of the extension products by gel electrophoresis. Initiation that occurs at the proper start site of the α^D-globin promoter results in an extended product of 161 nucleotides.

Plasmid constructions and oligomer probes. The cDNA expression vectors were derived by using DNA fragments from pRSVCAT (11). The vectors were constructed by inserting a *Hin*dIII fragment containing the enhancer and promoter of the Rous sarcoma virus long terminal repeat upstream of the full-length Eryf1 cDNA (clone 20; 6) in the vector pBSIISK- (Stratagene). Downstream of the cDNA sequences, the simian virus 40 splicing and polyadenylation signals from pRSVCAT were inserted. Vector pRSV20-2 contains the cDNA sequences in the correct orientation for proper expression from the long terminal repeat, while in pRSV20-1 the cDNA is inverted so that the noncoding strand is transcribed.

The reporter plasmids $p\alpha^D1$ and $p\alpha^D/J$ were provided by J. Knezetic and have been described elsewhere (referred to as D': and D':J, respectively; 17). To construct the other reporter plasmids, the sequences from a *Pst*I site at −10 in the α^D-globin promoter to a *Pst*I site downstream of the CAT gene were isolated from $p\alpha^D1$ and inserted into the *Pst*I site in the polylinker of pBSIISK- (Stratagene). The resulting plasmid is designated $p\alpha^D2$. The natural ($p\alpha^D3$) or mutated ($p\alpha^D4$, $p\alpha^D9$, $p\alpha^D10$, $p\alpha^D11$, and $p\alpha^D12$) promoter sequences were then synthesized from the −10 *Pst*I site to −65 (where an *Spe*I site was created) and inserted in the proper orientation of *Pst*I-*Spe*I-digested $p\alpha^D2$. Synthetic (20-mer) Eryf1 binding sites (or mutated derivatives) were concatamerized and inserted into the *Spe*I site of $p\alpha^D3$ or $p\alpha^D4$ to construct $p\alpha^D5$, $p\alpha^D6$, $p\alpha^D7$, and $p\alpha^D8$. The sequence of the oligonucleotides used for concatamerization is GATCCCT GCAGATAAACATG (the consensus binding sequence is underlined). The promoter region of each reporter construct was confirmed by sequencing. Each Eryf1 binding site mutation involves an alteration in the core recognition motif of the nucleotides GAT to CTG.

The reporter plasmids illustrated in Fig. 5b were constructed by ligating the purified 60- or 100-bp *Spe*I insert from $p\alpha^D5$ or $p\alpha^D6$, respectively (containing 3 or 5, respectively, synthetic Eryf1 binding sites) in either orientation into the *Spe*I site of $p\alpha^D4$. Again, each construct was confirmed by sequencing.

The sequences of the oligomers used in the gel mobility shift assays of Fig. 6 are as follows (top strand only; the consensus binding site or GAT motif is underlined):

Eryf1:	AGCTTCGGTTGCAGATAAACATTGAATTCA
Sp1:	AATTGCAGAGCTGGGAATCCTAACTGGGCGGAGTTATGCTGGTGGTGTGG
probe 2:	CAGGTTGCAGATAAACATTTTGCTATCAAGACTTGCACAGACCTTGTTTCTAGA
probe 3:	AGCTTGCGGATAAGATAAGGCCGGAATTCA
probe 4:	AGCTTGCGCTGAAGATAAGGCCGGAATTCA
probe 5:	AGCTTGCGGATAACTGAAGGCCGGAATTCA
probe 6:	AGCTTGCGCTGAACTGAAGGCCGGAATTCA

DNA-protein binding assays. Nuclear extracts were prepared from 10-day embryonic erythroid cells as described previously (8). To prepare extracts from CEF cells, the cells were first transfected under standard conditions except scaled up threefold, using 100-mm culture dishes. After 40 to 48 h, cells were harvested into TEN buffer by scraping and washed in PBS. Nuclear extracts were prepared exactly as described previously (4); all buffers included 87.5 μg of phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin, 0.7 μg of pepstatin, and 50 μg of antipain per ml. The nonerythroid extracts were made by using fivefold fewer cells (per volume of extract) relative to those made from erythroid cells (erythroid cell extracts were more concentrated).

The gel mobility shift assays were performed as described previously (6); gels were 8% polyacrylamide and were run in 10 mM Tris base–10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES)–1 mM EDTA. When used, unlabeled specific competitors were added to the reaction mix prior to addition of protein. DNase I footprinting experiments were performed as described previously (13); the amount of DNase I used in each digestion was determined

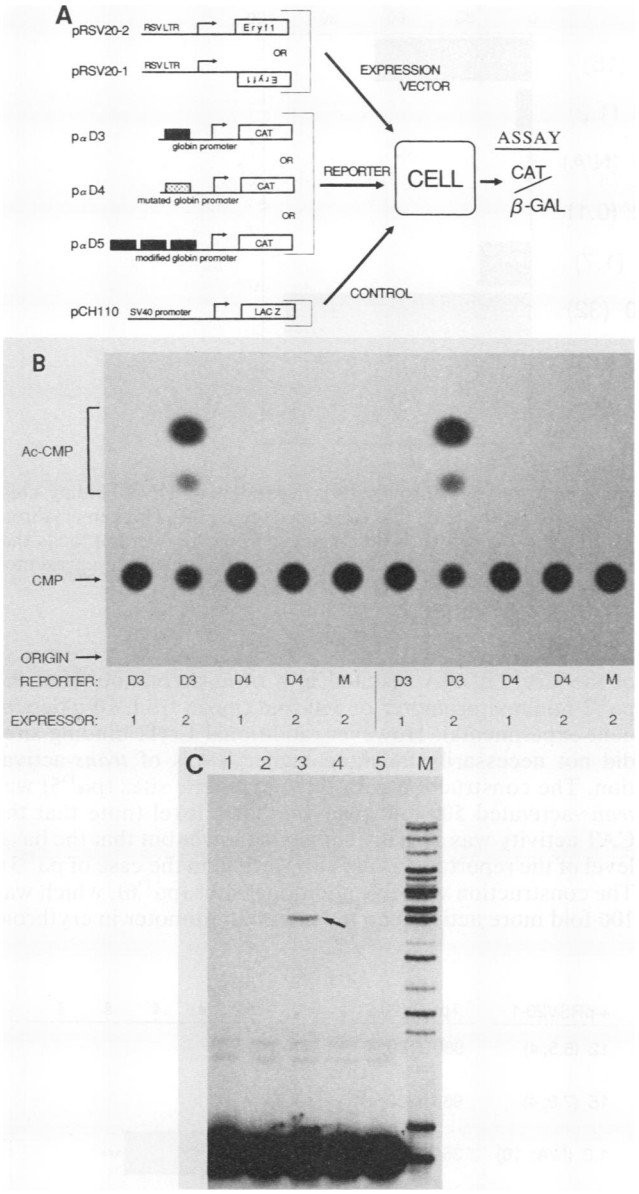


FIG. 1. *trans*-activation of the chicken α^D-globin promoter in nonerythroid cells. (A) Strategy. Three plasmids were cotransfected into cells: a vector containing the Eryf1 cDNA in the proper sense orientation (pRSV20-2) for expression of Eryf1 protein or in the opposite orientation (pRSV20-1) as a control for nonspecific effects of the expression vector, a reporter plasmid containing the minimal α^D-globin promoter ($p\alpha^D3$) or a related construct, and an internal control plasmid that expresses β-gal (pCH110). Following transfection, cells were harvested and CAT activity was measured after correction for differences (usually no more than twofold) in β-gal activity (see Materials and Methods). (B) Example of CAT assays from two independent experiments, performed by using lysates from CEF cells transfected with expression vector pRSV20-2 (2) or the control pRSV20-1 (1) and the minimal α^D-globin reporter $p\alpha^D3$ (D3), the minimal promoter in which the Eryf1 binding site is mutated ($p\alpha^D4$; D4), or a mock reporter with no CAT gene (M). Shown is an autoradiograph of a thin-layer chromatography plate in which the acetylated forms (Ac-CMP) have been separated from [^{14}C]chloramphenicol (CMP) after incubation in cell lysates containing equal amounts of β-gal activity. (C) Hybridization assay. A labeled primer specific for CAT mRNA was hybridized to total RNA prepared from transfected cells and extended by using reverse transcriptase, and the products were electrophoresed through an 8% polyacrylamide-urea gel. Lanes contained transfections as follows: 1, pRSV20-2 plus a mock reporter; 2, pRSV20-1 plus $p\alpha^D3$; 3, pRSV20-2 plus $p\alpha^D3$; 4, pRSV20-2 plus $p\alpha^D4$; 5, pRSV20-2 plus the TATA box mutant $p\alpha^D9$ (see Fig. 5). Lane M contains labeled DNA size standards; the arrow indicates an extended product that initiates at the mRNA cap site of the α^D-globin promoter. A portion of each transfection was also tested for β-gal; all samples contained approximately equal activities.

empirically in preliminary experiments. Following digestion, each DNA sample was purified by phenol extraction and ethanol precipitation. The samples were then denatured and analyzed on 6% polyacrylamide-urea gels in 1× TBE buffer. The probes were prepared by first digesting $p\alpha^D3$ with *Bss*HII, which cuts 63 bp upstream of the *Spe*I site in the pBSIISK-vector. The DNA was either 5′ or 3′ end labeled (20) and secondarily digested with *Eco*RI (which cuts within the CAT gene of $p\alpha^D3$). The 430-bp fragments labeled at the *Bss*HII site upstream of the α^D-globin promoter region were then purified from a preparative nondenaturing polyacrylamide gel (23).

RESULTS

The Eryf1 cDNA encodes a positive transcription factor. To investigate the ability of Eryf1 to affect globin expression in a nonerythroid cell, we used reporter plasmids containing the chicken α^D-globin promoter upstream of the coding sequence for the bacterial CAT gene. This promoter was chosen as a target for Eryf1 *trans*-activation because it contains an Eryf1 binding site occupied by protein specifically in erythroid cells that express the α^D-globin gene (16). Furthermore, the adult α-globin genes (α^D and α^A) are expressed throughout development (unlike the other members of the chicken globin gene family) and are therefore less likely to be regulated by stage-specific factors.

The *trans*-activation assay is outlined in Fig. 1A. The $p\alpha^D3$ reporter contains sequences extending from 65 bp upstream of the α^D-globin mRNA cap site to position +38, inserted adjacent to the CAT gene coding sequence. This plasmid was cotransfected into CEF cells with a plasmid containing the Eryf1 cDNA expressed from the long terminal repeat of Rous sarcoma virus (pRSV20-2). In control experiments, the expression construct contained the Eryf1 cDNA in a reverse orientation (pRSV20-1); in addition, all transfections included a plasmid expressing β-gal as an internal control for transfection efficiency and recovery of protein activity. To determine whether binding to the Eryf1 site was indeed responsible for the effect on CAT expression, we used the control reporter plasmid $p\alpha^D4$, containing a mutation in the site.

An example of CAT assays performed following cotransfection into CEF cells and normalization for β-gal activity is shown in Fig. 1B. Abundant expression from the $p\alpha^D3$ reporter (quantitated below) was obtained only when the Eryf1 cDNA was expressed in the proper orientation. Mutation of the Eryf1 binding site ($p\alpha^D4$) largely abolished the ability of the expressed cDNA to activate the α^D-globin promoter. Results of primer extension experiments using RNA prepared from cotransfected CEF cells (Fig. 1C) demonstrate that activated transcription began at the proper mRNA start site of the α^D-globin gene.

Expression from the α^D-globin promoter in erythroid cells is mediated by Eryf1 binding sites. The efficient *trans*-activation by Eryf1 of the $p\alpha^D3$ promoter was perhaps surprising;

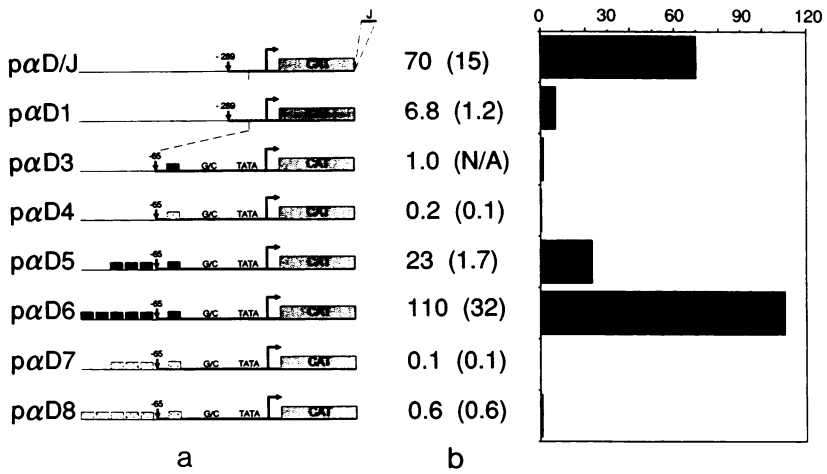


FIG. 2. Demonstration that expression of the α^D-globin promoter in primary embryonic erythroid cells is dependent on Eryf1 binding sites. (a) Structures of CAT plasmids based on the minimal α^D-globin promoter (described in the text) that were transfected into chicken erythroid cells. Symbols: ■, Eryf1 binding site; ▩, mutated site that cannot bind Eryf1. The J fragment is an enhancer from the α-globin locus that contains three Eryf1 binding sites. (b) CAT activities from the various reporters relative to that obtained by using $p\alpha^D3$. Results are presented as the means of three independent experiments, with standard sample deviations in parentheses. N/A, Not applicable.

previous results had shown a more extensive α^D-globin promoter region, containing 289 bp upstream of the mRNA cap site ($p\alpha^D1$), to be only weakly active in embryonic erythroid cells (17). In that study, the 289-bp promoter was stimulated by an enhancer sequence from the α-globin locus containing three Eryf1 binding sites ($p\alpha^D/J$). Therefore, we analyzed the effect of additional Eryf1 binding sites on the activity of the α^D-globin promoter in either primary erythroid cells or the nonerythroid CEF cells.

The reporter constructs were first transfected into primary embryonic erythroid cells (12, 18) to determine whether expression from the CAT gene is dependent on the presence of binding sites for the endogenous Eryf1 (Fig. 2). Deletion of the α^D-globin promoter containing 289 bp upstream of the mRNA cap site ($p\alpha^D1$) to position −65 ($p\alpha^D3$) resulted in a sevenfold drop in CAT activity, indicating that positive regulatory elements are present in the upstream promoter region (−289 to −65). Mutation of $p\alpha^D3$ at the Eryf1 binding site (as in $p\alpha^D4$) reduced expression a further fivefold, essentially to a background level. Therefore, the $p\alpha^D3$ reporter contains a minimal promoter that in erythroid cells is dependent on a single Eryf1 binding site.

Consistent with previous results (17), we found that addition of the α-globin enhancer downstream of the CAT gene stimulated expression from $p\alpha^D1$ about 10-fold in erythroid cells. Addition of synthetic Eryf1 binding sites (derived from one of the sites present in the β-globin enhancer) upstream of $p\alpha^D3$ also increased expression; a reporter containing three ($p\alpha^D5$) or five ($p\alpha^D6$) additional upstream Eryf1 binding sites expressed CAT at levels 23- or 110-fold, respectively, over $p\alpha^D3$. In the latter case, this is even higher than activity from the $p\alpha^D/J$ construct containing the enhancer, demonstrating that the synthetic sites function as positive elements in the erythroid cells. The effect on expression was entirely dependent on binding by Eryf1, as mutation of the binding sites abolished the effect ($p\alpha^D4$, $p\alpha^D7$, and $p\alpha^D8$).

Efficient *trans*-activation in CEF cells requires only a single Eryf1 binding site. We next assayed the activity of these reporters in CEF cells (Fig. 3), either in control experiments (cotransfecting pRSV20-1) or in the presence of the expressed Eryf1 cDNA (cotransfecting pRSV20-2). Expression of the Eryf1 cDNA resulted in a *trans*-activation from the $p\alpha^D3$ minimal promoter of 360-fold (mean from 10 independent experiments). However, additional Eryf1 binding sites did not necessarily facilitate higher levels of *trans*-activation. The construction with three synthetic sites ($p\alpha^D5$) was *trans*-activated 500-fold over the basal level (note that the CAT activity was actually somewhat lower but that the basal level of the reporter was also lower than in the case of $p\alpha^D3$). The construction with five additional sites ($p\alpha^D6$), which was 100-fold more active than the minimal promoter in erythroid

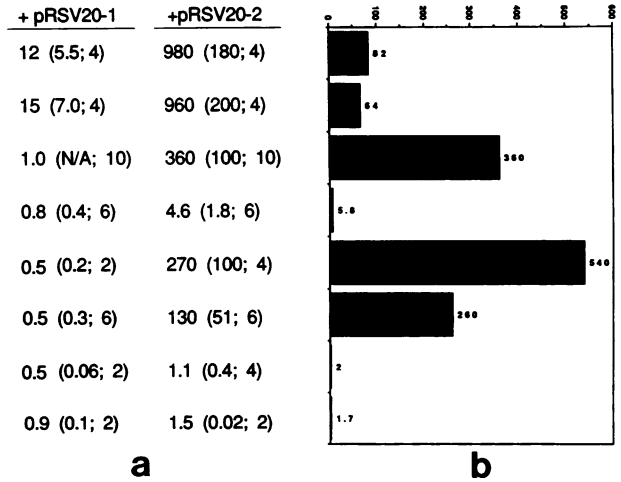


FIG. 3. Demonstration that a single binding site promotes efficient *trans*-activation of the α^D-globin promoter by Eryf1 expressed in CEF cells. The reporters shown in Fig. 2a were transfected into CEF cells with either the Eryf1 expression vector pRSV20-2 or the control vector pRSV20-1. (a) Results from β-gal-normalized CAT assays relative to the basal activity of the $p\alpha^D3$ reporter ($p\alpha^D3$ plus pRSV20-1); given are the means of the CAT activities from multiple experiments. Values in parentheses represent standard sample deviation and number of experiments used to derive the data. (b) Level of *trans*-activation by Eryf1 (+pRSV20-2/+pRSV20-1) for each reporter.

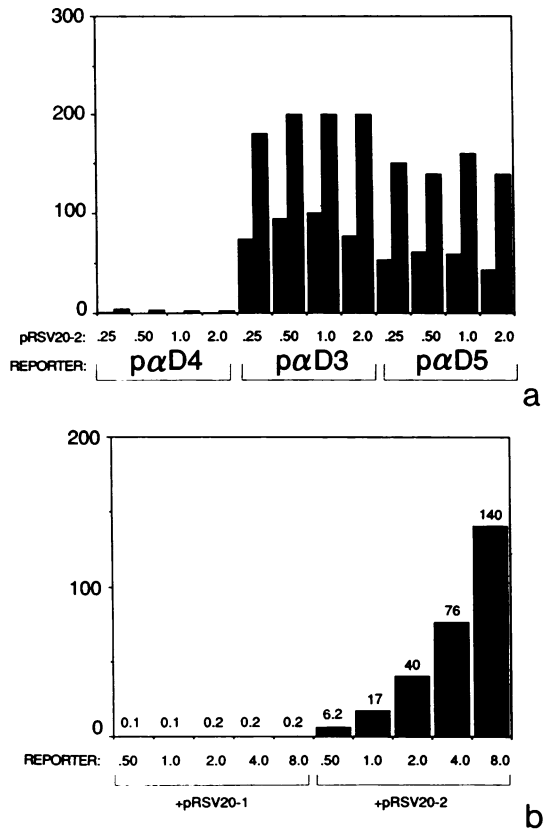


FIG. 4. Demonstration that the response of the α^D-globin promoter is not limited by expressed Eryf1 or other cellular factors. (a) The reporter plasmids $p\alpha^D4$, $p\alpha^D3$, and $p\alpha^D5$ (■, 2 μg; ▨, 6 μg) were transfected into CEF cells with increasing amounts of the Eryf1 expression vector pRSV20-2. (b) Increasing amounts of the $p\alpha^D3$ reporter were cotransfected into CEF cells with 1 μg of either the Eryf1 expression vector (pRSV20-2) or the control expression vector (pRSV20-1).

cells, was activated by expressed Eryf1 somewhat less than $p\alpha^D3$ in CEF cells. In all cases, *trans*-activation was entirely dependent on expression of the Eryf1 cDNA (pRSV20-1 did not activate).

As expected, mutation of the Eryf1 binding site in $p\alpha^D3$ did not affect the basal level of the α^D-globin promoter ($p\alpha^D4$) because CEF cells do not contain endogenous Eryf1 (shown below). However, *trans*-activation of the mutated minimal promoter by expression of Eryf1 was less efficient by 2 orders of magnitude. Reporters in which all of the multiple Eryf1 binding sites were mutated ($p\alpha^D7$ and $p\alpha^D8$) also failed to be *trans*-activated.

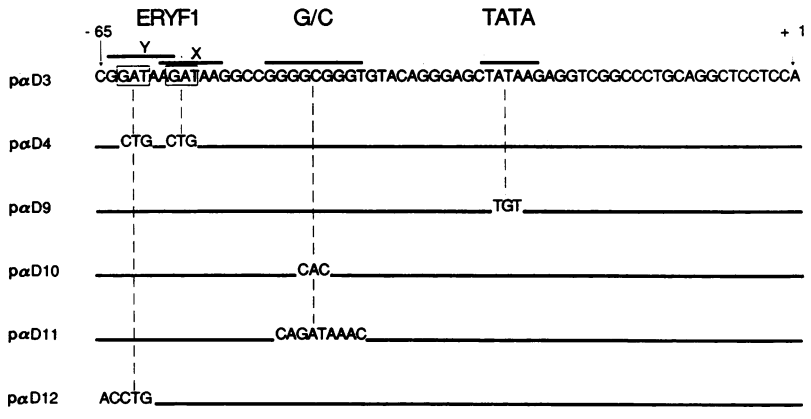
trans-activation of the $p\alpha^D1$ construct yielded CAT activity threefold above that obtained by *trans*-activation of the minimal (−65) $p\alpha^D3$ promoter (Fig. 3). However, because basal expression of the $p\alpha^D1$ reporter in CEF cells (in control experiments cotransfecting pRSV20-1) was 10-fold greater than that of $p\alpha^D3$, the activation of $p\alpha^D1$ relative to its basal expression was actually somewhat less than observed with $p\alpha^D3$. This result indicates that the contribution of positive elements upstream of the minimal promoter, detected also by transfection into erythroid cells (Fig. 2), is likely to be due to interaction with factors shared between erythroid and CEF cells rather than with erythroid-specific factors. We conclude that the Eryf1 cDNA encodes the erythroid activity necessary and sufficient (in combination with any required factors that are shared between erythroid cells and fibroblasts) for activation of the minimal globin promoter. In CEF cells, addition of the enhancer 3′ to the $p\alpha^D1$ promoter ($p\alpha^D$/J) did not result in further stimulation. In summary, in erythroid cells multiple Eryf1 binding sites are required for efficient activation, whereas in nonerythroid CEF cells only a single proximal promoter binding site is required for near-maximal activation.

Does our quantitation of CAT activity truly reflect promoter potential? In preliminary experiments, we determined that the various DNA constructions introduced into either erythroid or CEF cells (reporter plasmid, expression construct, and internal control plasmid) are not in competition for cellular factors (Fig. 4 and data not shown). CAT activity obtained from CEF cells was the same regardless of whether 0.25, 0.5, 1.0, or 2.0 μg of pRSV20-2 was included in the transfection (Fig. 4a). (In some experiments, we have found that the use of excess expression vector results in slightly reduced activation [compare, for example, the experiments using 1.0 and 2.0 μg of pRSV20-2 in Fig. 4]. This inhibition is apparently due to an accumulation of proteolysed Eryf1 molecules that compete with the full-length protein [see below]). In all cases, a single Eryf1 binding site was necessary (compare $p\alpha^D4$ and $p\alpha^D3$) and sufficient (compare $p\alpha^D3$ and $p\alpha^D5$) for maximal *trans*-activation; furthermore, the level of CAT activity was directly proportional to the amount of reporter (2 or 6 μg). This result indicates that under the conditions used in the experiments of Fig. 3 (1 μg of expression construct and 3 μg of reporter), the level of expressed Eryf1 is not limiting. The level of CAT expression observed was directly proportional to the amount of reporter included in the transfection (Fig. 4b); in other words, the assay is linear with respect to promoter elements.

We considered whether the efficient *trans*-activation of $p\alpha^D3$ in CEF cells might be a consequence of overexpression of the Eryf1 cDNA relative to the endogenous level of Eryf1 in erythroid cells. However, cotransfection of the α^D-globin reporters with the cDNA expression construct into 10-day embryonic erythroid cells led to only about a twofold enhancement of all reporters containing nonmutated binding sites (data not shown); multiple binding sites were still required for efficient expression.

Because these results indicate a fundamental difference in the mechanism by which *trans*-activation occurs in CEF cells, we have analyzed the activity of the reporter plasmids in other nonerythroid cells (data not shown). *trans*-activation of the α^D-globin promoter by Eryf1 is less efficient in monkey COS cells than in CEF cells. We believe that this is due, at least in part, to a lower activity of the expression vector in mammalian cells, so that Eryf1 becomes limiting (30a). The difference in activity may also reflect a cell-specific modulation of Eryf1 activity in CEF versus COS cells, given the results obtained for erythroid cells. Nevertheless, data from activation of reporters containing multiple binding sites are analogous to results obtained in CEF cells: a single site is sufficient for activation. *trans*-activation in a quail embryo fibroblast cell line (QT6) is similar to that observed in CEF cells with respect to both the magnitude of the activation and the lack of a requirement of multiple sites for efficient activation.

Promoter elements cooperate with Eryf1 for efficient *trans*-activation. In a previous analysis of the minimal α^D-globin promoter region (16), the Eryf1 binding site, located between positions −63 and −55, was shown to be occupied by protein



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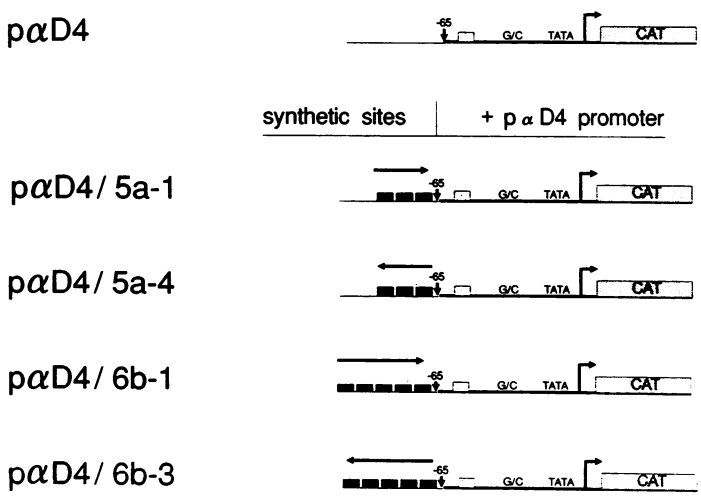

b

REPORTER	ACTIVITY IN RBC	ACTIVITY IN CEF +pRSV20-1	ACTIVITY IN CEF +pRSV20-2
MOCK	0.64 (0.20)	(ND)	0.12 (0.02)
pαD3	1.0 (N/A)	0.13 (0.02)	18 (0.47)
pαD4	0.70 (0.18)	0.12 (0.02)	0.39 (0.04)
pαD5	12 (5.6)	0.12 (0.02)	16 (3.2)
pαD6	48 (27)	0.12 (0.02)	3.7 (0.70)
pαD9	0.76 (0.20)	0.14 (0.01)	1.4 (0.25)
pαD10	0.76 (0.29)	0.14 (0.02)	4.2 (1.2)
pαD11	0.91 (0.28)	0.13 (0.01)	13.3 (2.7)
pαD12	0.86 (0.24)	0.12 (0.01)	5.2 (0.30)
pαD4/5a-1	1.9 (0.54)	0.12 (0.02)	4.7 (0.31)
pαD4/5a-4	2.0 (0.84)	0.12 (0.02)	5.8 (0.55)
pαD4/6b-1	11 (3.3)	0.12 (0.03)	3.0 (0.08)
pαD4/6b-3	8.1 (2.2)	0.12 (0.03)	2.8 (0.37)

ACTIVATION BY ERYF1

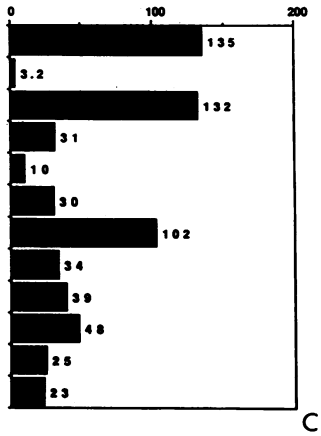

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FIG. 5. Demonstration that mutation of promoter elements distinct from the Eryf1 binding site affects the efficiency by which Eryf1 *trans*-activates the α^D-globin promoter. (a) Sequence in the promoter region of each reporter. These plasmids are all based on pα^D3 but contain mutations as indicated by the nucleotides on the lines below the pα^D3 sequence. The solid lines indicate nucleotide identities. (b) Additional reporters based on the pα^D4 reporter. Synthetic Eryf1 binding sites from pα^D5 (three sites) or pα^D6 (five sites) have been inserted just upstream. Arrows indicate the relative orientation of the oligomerized sites; these reporters are similar to pα^D5 or pα^D6 but with the native Eryf1 binding site mutated. (c) Mean results of CAT assays from four independent experiments, expressed as in Fig. 2 and 3. For experiments in CEF cells, transfections included only one-fourth the amount of expression vector compared with the experiments of Fig. 3 (see Materials and Methods). Also, CAT activity from mock transfections was not subtracted from each result (as the mock activity was not significantly lower than the basal level of the reporters). Therefore, the levels of *trans*-activation illustrated on the graph relative to basal levels (+pRSV20-2/+pRSV20-1) may represent minimum estimates. RBC, Chicken erythroid cells; ND, not determined; N/A, not applicable.

in the nuclei of erythroid cells. Other protein binding sites were mapped in vitro by using concentrated nuclear extracts; it seemed possible that these sites might be important for facilitating the Eryf1 effect. These sites include a sequence just upstream of and adjacent to the consensus Eryf1 binding site and a G+C-rich element centered at position −48, very similar to an Sp1 binding site (14). In addition, the TATA box at −30 presumably binds the general RNA polymerase factor TFIID (reference 3 and references therein). We introduced mutations into each of these putative elements within the $p\alpha^D3$ promoter region (Fig. 5a) and assayed for the effect of these mutations on expression in erythroid cells and *trans*-activation by Eryf1 in CEF cells.

The results of the promoter mutations are shown in Fig. 5c; in all cases, the mutations had deleterious effects on the activity of the promoter in erythroid cells, although the significance is unclear, as the normal activity of the minimal promoter is quite low in these cells. In CEF cells, mutation of the TATA box ($p\alpha^D9$) resulted in a 15-fold drop in CAT activity relative to activation from the wild-type minimal promoter ($p\alpha^D3$). *trans*-activation still occurred, at a level about 10-fold above the basal level of the reporter. However, we were unable to detect correctly initiated message from *trans*-activated $p\alpha^D9$ (Fig. 1c, lane 5).

Mutation of the G+C region ($p\alpha^D10$) resulted in a smaller but significant (fourfold) drop in *trans*-activation by Eryf1. However, replacing the putative Sp1 site with a single synthetic Eryf1 binding site ($p\alpha^D11$) restored the induction by expressed Eryf1, indicating that a specific interaction with a distinct factor (such as Sp1) is not required for Eryf1 activity, at least in the context of this reporter.

The Eryf1 binding site in $p\alpha^D3$ consists of two adjacent GAT core consensus binding sequences, both of which are mutated in the non-*trans*-activated minimal promoter construct $p\alpha^D4$. Although only the downstream GAT element was protected from DNase I digestion in nuclei, the upstream GAT was also protected by concentrated protein extracts in vitro (16). We found that mutation of the upstream GAT motif ($p\alpha^D12$) decreased *trans*-activation about fourfold, similar to mutation of the G+C element, demonstrating that primary activation by Eryf1 occurs through the downstream GAT motif. As shown below, the α^D promoter bound only one molecule of Eryf1, at the downstream motif; mutation of the upstream GAT motif in $p\alpha^D12$ resulted in a weaker binding site.

Finally, we considered whether the Eryf1 binding site in the minimal promoter is functionally distinct from the synthetic binding sites that we have appended. The synthetic sites were derived from a strong binding site present in the chicken β-globin enhancer and functioned in erythroid cells (Fig. 2). Nevertheless, it was in principle possible that they function in erythroid cells in conjunction with a factor missing from CEF cells, while only the native α^D promoter site (which has distinct adjacent sequences) can function in CEF cells. This activity would presumably be mediated by a cooperative interaction between expressed Eryf1 and a factor present in CEF cells but absent from erythroid cells. To test this, we inserted the synthetic sites upstream of the mutated ($p\alpha^D4$) promoter (Fig. 5b) and assayed activity in erythroid or CEF cells. In either cell type, the synthetic sites (in either orientation) functioned for activation of the minimal promoter (Fig. 5c). In all cases, the mutation of the native site caused a significant decrease in the activity of the promoter that was not entirely compensated for by the upstream synthetic sites. The data indicate either that the function of Eryf1 under these condition is dependent on its relative distance from the cap site (consistent with the high level of activity observed with the $p\alpha^D11$ construct) or that the native binding site functions as a particularly strong positive regulatory element (consistent with a higher affinity for Eryf1, as shown below).

These results demonstrate that activation is enhanced by, but not dependent on, promoter elements distinct from a single Eryf1 binding site and demonstrate that Eryf1 is necessary and sufficient (presumably in cooperation with TFIID and perhaps factors that do not bind DNA) for efficient *trans*-activation of the globin promoter. The results obtained by using the $p\alpha^D1$ reporter indicate that other promoter elements may be located upstream of the minimal promoter and that these also contribute to the basal expression of the α^D-globin promoter.

Eryf1 activates through binding to the α^D-globin promoter. We sought to show directly that *trans*-activation of the minimal α^D-globin promoter occurs through binding of the expressed Eryf1. Nuclear extract prepared from embryonic chicken erythroid cells protected the Eryf1 binding site of $p\alpha^D3$ from digestion by DNase I (Fig. 6a). No protection of the Eryf1 binding site was detected in extract prepared from CEF cells transfected with the control expression vector (pRSV20-1); this result confirms that CEF cells do not contain endogenous Eryf1 binding activity. However, extract from CEF cells transfected with pRSV20-2 protected the Eryf1 binding site, on both DNA strands, yielding a DNase I protection pattern essentially the same as observed when erythroid extract was used.

Results of gel mobility shift experiments (Fig. 6b) demonstrate that the Eryf1 expressed in CEF cells had the same specificity and affinity for the $p\alpha^D3$ Eryf1 binding site as did Eryf1 isolated from erythroid cells. In control experiments, the Sp1 from CEF cells and erythroid cells displayed similar specificities and affinities to a strong Sp1 binding site. If we assume that Sp1 is present in similar abundance in erythroid cells and CEF cells, then the amount of full-length Eryf1 binding activity present in CEF cells transfected by pRSV20-2 is similar (at least of the same order of magnitude) to the amount of endogenous Eryf1 binding activity present in embryonic erythroid cells. This is a particularly rough estimate, as we have no evidence for the percentage of transfected cells that are expressing the Eryf1 cDNA. Therefore, we may be underestimating the local concentration of Eryf1 in a transfected CEF cell. We note that the promoter region of $p\alpha^D3$, containing the G+C element, is unable to compete for the Sp1 binding activity. This result indicates that the activity previously identified in concentrated extract (16) as binding in vitro to this site (a site that does contribute to transcriptional activity; Fig. 5) either is not Sp1 or else binds to this site with a relatively low affinity.

Additional, faster-mobility complexes were also formed with the Eryf1 probe; these resulted from binding of proteolytic products of the expressed cDNA that retained DNA-binding activity and appeared to accumulate as a consequence of overexpression in the nonerythroid cells (data not shown). Because this is the only obvious difference that we find between endogenous Eryf1 and the protein expressed in CEF cells, it is important to determine that the full-length and not the proteolytic products are responsible for the efficient *trans*-activation of the α^D-globin reporters in CEF cells. It is conceivable that proteolysis of Eryf1 in fibroblasts removes a repressor domain to release the protein from normal suppressed regulation. For example, removal of such a domain in the N-terminal region of c-*jun* results in significantly greater transcriptional activity (1). Preliminary mu-

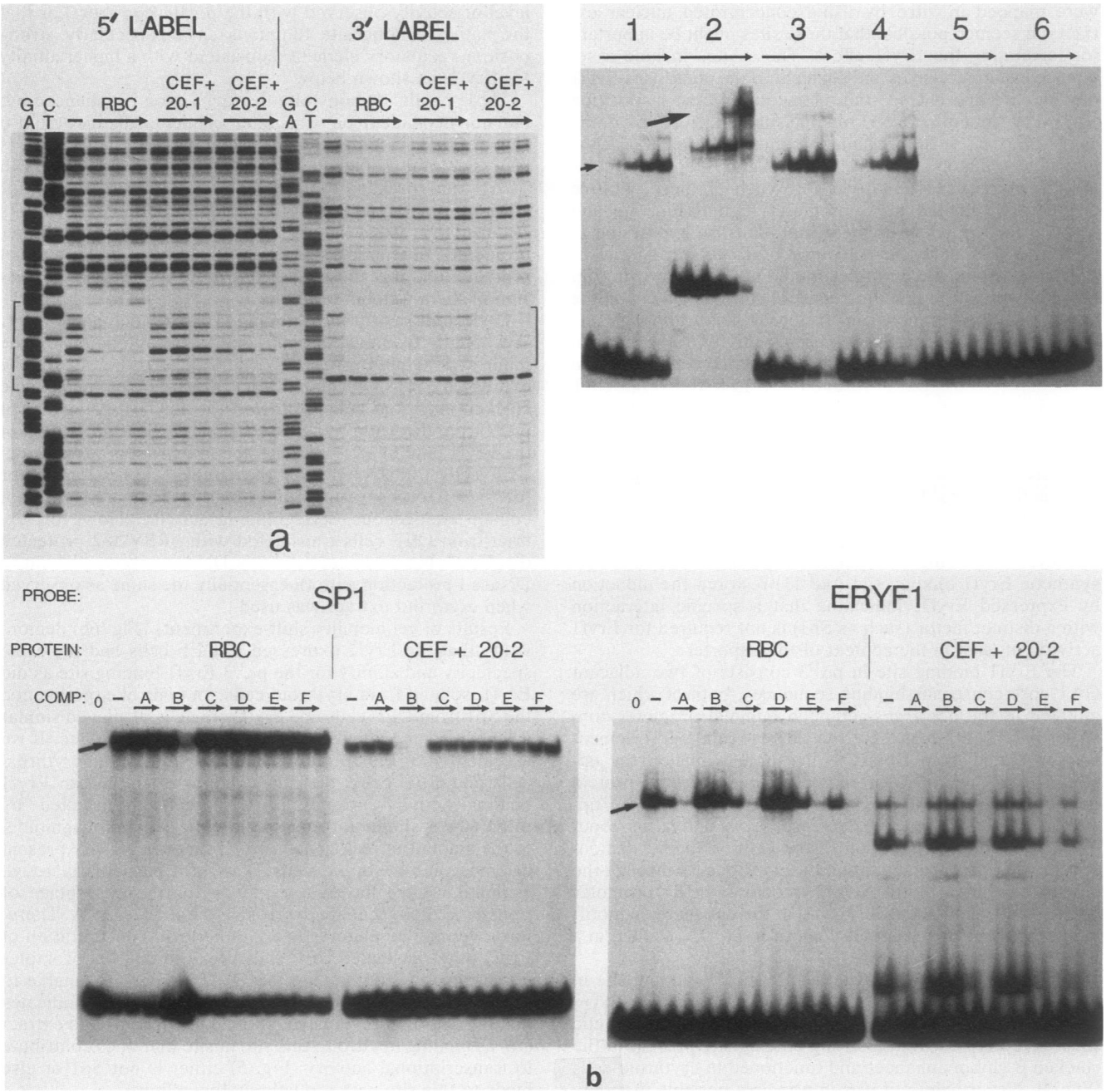

FIG. 6. Demonstration that Eryf1 *trans*-activates by binding to the $p\alpha^D3$ promoter. (a) The probe was a DNA fragment from $p\alpha^D3$, end labeled upstream of the α^D-globin promoter sequence (see Materials and Methods), on either the coding (5′-labeled) or noncoding (3′-labeled) strand. It was incubated with nuclear extract (arrow; 2.5, 5.0, or 10.0 μl, left to right) from 10-day embryonic chicken erythroid cells (RBC) or CEF cells transfected with either the vector expressing the Eryf1 cDNA (CEF + 20-2) or the control vector (CEF + 20-1). Following digestion with DNase I, the DNA was purified and electrophoresed on a 6% polyacrylamide-urea gel. Lanes marked GA and CT contain marker ladders generated by chemical degradation of the probe (23); in lanes marked −, the probe was incubated in buffer alone prior to DNase I digestion. The bracket indicates the position of the Eryf1 binding site. (b) Gel mobility shift assays were performed by using either 2.5 μl of erythroid nuclear extract or 5 μl of nuclear extract from transfected CEF cells (lanes marked −). In addition to the labeled probe (lanes marked 0), each assay contained a 20- or 80-fold molar excess (left to right) of unlabeled oligonucleotide competitor (see Materials and Methods and Fig. 5): A, Eryf1 binding site; B, Sp1 binding site; C, the $p\alpha^D3$ promoter region; D, the $p\alpha^D4$ promoter region; E, the $p\alpha^D9$ promoter region; F, the $p\alpha^D10$ promoter region. (c) Oligonucleotides were incubated with increasing amounts (0, 1, 2, 4, or 8 μl, left to right) of nuclear extract prepared from 10-day embryonic chicken erythroid cells. Lanes contained probes (see Materials and Methods and Fig. 5) as follows: 1, a single Eryf1 binding site; 2, two adjacent binding sites (en4; 8); 3, the $p\alpha^D3$ Eryf1 binding site; 4, the $p\alpha^D12$ Eryf1 binding site; 5, the $p\alpha^D3$ binding site mutated in the downstream GAT motif; 6, the double GAT mutant ($p\alpha^D4$) binding site. The small and large arrows indicate complexes formed by binding of one and two molecules, respectively, of Eryf1.

tagenesis experiments indicate that truncated Eryf1 is not a superactivator and that regions (at both the N and C termini) not required for efficient binding of Eryf1 to DNA are required for transcriptional activity.

To determine whether the Eryf1 binding site in $p\alpha^D3$, containing two adjacent GATA consensus core motifs, binds one or two molecules of Eryf1 (or other proteins), we used mutated oligonucleotide duplexes as probes in the gel mobility shift assay (Fig. 6c). As controls, we used DNA probes that are known to contain either a single binding site (probe 1) or two adjacent sites from the β-globin enhancer that bind independently (8) two molecules of Eryf1 (probe 2).

When probe 3 (containing the nonmutated sequence) was used, a single complex was formed that was identical to the complex formed by using control probe 1, containing only a single binding site. Even under conditions of increased protein, in which both sites of probe 2 were bound to form a dimer complex (large arrow, Fig. 6c), probe 3 did not form a dimer complex. Mutation of the upstream GAT motif (probe 4) resulted in about a twofold reduction in affinity relative to the wild-type sequence, whereas mutation of the downstream GAT (probe 5) or mutation of both sites (probe 6) resulted in a complete loss of complex formation. Identical results were obtained by using extracts from CEF cells transfected with pRSV20-2 (not shown). We conclude that the α^D-globin minimal promoter binds a single molecule of Eryf1 that recognizes primarily the downstream GAT motif, but that in the presence of both GAT motifs, Eryf1 binds with a twofold-higher affinity, facilitating a fourfold-higher level of *trans*-activation (compare $p\alpha^D3$ with $p\alpha^D12$ in Fig. 5). Mutation of a complex Eryf1 binding site that affects transcription but does not strictly correlate with a change in binding affinity has been noted in other systems (21).

DISCUSSION

We have shown that the Eryf1 cDNA, previously found to encode a tissue-specific DNA-binding activity (6), also encodes the transcriptional activation function implied by mutation studies of DNA regulatory elements (8, 21, 27).

In drawing conclusions from these experiments, it must be kept in mind that we detect CAT activity from the reporter as an indirect measure of transcriptional enhancement. We note that reporters containing mutated Eryf1 binding sites (e.g., $p\alpha^D4$) show a small (about fivefold) but consistent level of *trans*-activation. This could be due to any of several causes: binding of the expressed Eryf1 at fortuitous sites present in the reporter vector, true *trans*-activation in the absence of DNA-binding, or activation of transcription elsewhere on the reporter plasmid that reads through the CAT gene. We are further investigating this phenomenon.

Nevertheless, in CEF cells that do not contain endogenous Eryf1, with a reporter containing only a single Eryf1 binding site, we find that Eryf1 expression stimulates CAT activity 2 orders of magnitude over the basal level of the promoter. This *trans*-activaton is dependent both on expression of the cDNA in the correct orientation and on a functional DNA-binding site. In the case of the $p\alpha^D3$ reporter, we have shown that Eryf1 directs accurate initiation of RNA transcripts.

The mechanism by which Eryf1 stimulates transcription appears to be different or modified in erythroid compared with nonerythroid cells. A single high-affinity binding site suffices to direct efficient *trans*-activation in the nonerythroid cells, while high levels of activation in erythroid cells requires either multiple Eryf1 binding sites or the addition of an enhancer. Our data indicate that Eryf1 is not limiting in either case. Furthermore, we find no obvious difference in the DNA-binding affinity or specificity between the endogenous and expressed proteins, nor do we find other cell-specific DNA-binding activities in the region of the minimal promoter.

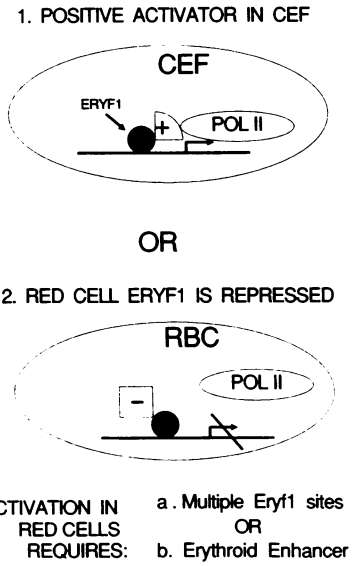

FIG. 7. Two distinct models that might explain cell-specific differences in activation by Eryf1. (1) In nonerythroid CEF cells, a positive factor (or modification), not present in erythroid cells, may fortuitously cooperate with Eryf1 to activate efficient transcription from a single proximal binding site. Alternatively, (2) Eryf1 may be repressed by a factor or modification that acts specifically in erythroid cells (RBC); this suppression could be overcome by multiple bound Eryf1 molecules or by the presence of an enhancer containing multiple bound factors. Pol, Polymerase.

At this point, we are unable to distinguish between two models, either of which could explain these observations (Fig. 7). One possibility is that Eryf1 activity is suppressed in erythroid cells, directly or indirectly, to make it a less active transcription factor, without altering its DNA-binding function. In this case, full activity could be obtained only when multiple molecules are bound in a regulatory region. According to this view, the role of an enhancer would be to serve as a site for interactions that overcome the repression placed upon Eryf1 by the erythroid environment. Such a regulatory mechanism might be important to prevent a powerful transcriptional activator from wreaking havoc in the cell in which it is expressed. The repression mechanism need not be present in nonerythroid cells (such as CEF cells), because Eryf1 is normally never expressed in these cells.

An alternative possibility is that Eryf1 is modified or complemented to make it a superactivator in CEF cells. Binding of additional Eryf1 molecules at the upstream synthetic sites may have little effect if the minimal promoter is already operating at the maximal rate. Similar cell-specific activities of a transiently expressed transcription factor have recently been noted in other systems (30). One possibility is that Eryf1 requires a coactivator, as has recently been suggested for activation by Sp1 and several viral *trans*-activators (15, 19, 26); cell-specific coactivators might result in very different mechanisms of *trans*-activation, all still dependent on Eryf1. For example, a CEF factor might fortuitously interact with Eryf1 to allow efficient activation

from a proximal site, while the erythroid coactivator may be less potent but allow activation from a distance. Recent experiments (not shown) suggest that enhancer sequences containing Eryf1 sites can readily activate a promoter from a distance in erythroid cells but function efficiently in CEF cells only when located proximally. Resolution of this question will require a more complete understanding of the species- and cell-type-specific effects on transiently expressed Eryf1. For example, the matter may be complicated by differential message stability, translational efficiency, and interactions with cell-specific factors.

We find, using the α^D-globin promoter region as a model, that other promoter elements facilitate the maximal enhancement of expression by Eryf1. These include upstream promoter sequences as well as proximal elements. Although expression from the minimal promoter ($p\alpha^D3$) is entirely dependent on the presence of Eryf1, mutations in other promoter elements decrease the level by which Eryf1 *trans*-activates. In addition, a mutation that decreases the binding affinity at the native Eryf1 binding site only twofold results in lower levels of expression that cannot be entirely compensated for by additional upstream binding sites. We infer from these results that the affinity for a particular site or its position relative to other promoter elements may be critical for determining the efficiency of Eryf1 activation. The data indicate that Eryf1 is the only erythroid protein necessary for activation of the α^D-globin promoter and that maximal activity is obtained in combination with other factors that are not tissue specific.

In erythroid cells, in contrast to CEF cells, multiple Eryf1 interactions are required for full promoter activity. In this case, protein-protein interaction between Eryf1 molecules or between Eryf1 and other DNA-binding factors may be critical for Eryf1 to function as a transcription factor (27). In some respects, the α^D-globin promoter and assay system that we use may constitute too simple a model to explain certain aspects of globin gene regulation: the transient assay probably does not address the formation of the active chromatin domain required for regulated expression of the endogenous globin genes. In addition, the α^D-globin gene is expressed in all erythroid cells, whereas transcription of many globin genes is limited to a particular developmental period. Because Eryf1 is present throughout erythroid development, regulation of these genes likely involves stage-specific factors as well.

A requirement for additional erythroid-specific factors under certain circumstances may be relevant in light of recent results indicating that in mammalian cells, Eryf1 (GF-1) is also expressed in developmentally related megakaryocytes (22). Expression of genes that are restricted to the two cell types could be regulated by Eryf1. Proper expression of other, entirely cell-specific genes may be regulated by cooperative interactions between Eryf1 and other factors that are unique to the erythroid or megakaryocytic lineage. Our results indicate that the way in which Eryf1 activates a particular promoter is dependent on cell type.

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