Identification of Functional Elements of the Chicken ε-Globin Promoter Involved in Stage-specific Interaction with the β/ε Enhancer*

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Expression of the chicken globin genes is regulated in part by competition between the β^-globin and ε-globin promoters for the enhancer bound between the genes. To understand the determinants of the enhancer-promoter interaction in stage-specific regulation, the functional elements of the embryonic chicken ε-globin promoter were characterized. In vitro assays demonstrated that: (a) the TATA motif at −30 bound GATA-1, (b) Sp1 bound to an element centered at −54, and (c) both Sp1 and another factor, designated CACCC (which appears related to erythroid Krüppel-like factor, EKLF) bound in the −120 to −128 region. The functions of these motifs were tested using transient expression in embryonic erythroid cells. In the absence of the enhancer, promoter point mutants showed that the TATA, Sp1, and CCAAT motifs (but not the CACCC motif) contributed to promoter activity. In contrast, in the presence of the enhancer, both motifs contributed to transcription. Developmental regulation of the enhancer activity was observed, with enhancement decreasing sharply from 185-fold at 4 days (cells expressing ε-globin) to 16-fold at 10 days (when ε-globin is no longer expressed). Taken together, the data suggest that multiple transcription factors contribute to promoter-enhancer interaction and the developmental regulation of ε-globin expression, with EKLF-like factors having an especially important role. Regulation of stage specificity occurs at the level of enhancer/ε-promoter interaction, even in the absence of competition, and is not simply a property of the enhancer or promoter in isolation.

Vertebrates express different β-like globin genes at different stages of their development, a process termed gene switching (1, 2). As a result of gene switching, organisms express hemo-globins whose physicochemical properties are adapted to the physiologic requirements of each developmental stage. In the chicken, the embryonic ε- and ρ-globin genes are expressed in primitive lineage erythroid cells at days 2–5 of development, while the adult β^- and β^H^-globins are produced in definitive lineage cells, beginning on embryonic day 5 (3).

The mechanisms responsible for developmental stage-specific expression of globin genes are beginning to be understood.

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The abbreviations used are: LCR, locus control region; bp, base pair(s); DTT, dithiothreitol; UTR, untranslated region.

Interaction between the individual globin genes and distant regulatory elements is important for this process. Control by a series of upstream DNase I-hypersensitive sites, referred to as a locus control region (LCR), was first identified in the human β-globin cluster (4). These hypersensitive sites are important for opening the globin cluster chromatin (5–7). The LCR is also needed for developmental regulation of the individual globin genes, whose promoters are thought to compete for interaction with the LCR (8–11). The strength of a distant element’s interaction with a particular promoter is determined by the distance between the element and the promoter (12), the order of the promoters within the cluster (13, 14), and the developmental specificity intrinsic to the promoter and upstream site (15). One mechanistic model of LCR action is that the individual hypersensitive sites form simultaneous binary interactions with different globin promoters (16). Elegant evidence that the LCR functionally interacts with only one gene at a time is consistent with a model in which the LCR sites together form a single active complex (17).

Comparison of the chicken and human β-like globin clusters should yield important insights into gene cluster regulation. Unlike the human genes, the chicken β^-globin genes (5′^-β^-H^-^A^-β^-^E^-3′) are not arranged in order of developmental expression. Also unlike the human cluster, a strong enhancer (the β/ε enhancer), with LCR-like properties, is located inside the cluster, between the β^- and ε-globin genes (18–20). Both the upstream sites and the β/ε enhancer contribute to the expression of all the genes in the cluster (21). Finally, developmental regulation of the β-globin cluster probably evolved independently in the two species (22, 23).

Although the chicken ρ- and ε-globins are expressed simultaneously at similar levels and their proximal promoters are similar, the proposed mechanisms for their regulation are different. Transcription of ρ-globin is largely dependent on a promoter cis-element that binds the GATA-1 transcription factor (24). The ε promoter lacks this GATA site, and ε-globin expression is dependent on the β/ε enhancer located 2.4 kilobase pairs upstream. To understand more about stage-specific regulation, we have undertaken a detailed characterization of the ε-globin promoter and its interaction with the β/ε enhancer at different developmental stages.

EXPERIMENTAL PROCEDURES

Standard cloning methods were used (25). Sequence numbers refer to GenBank accession no. L17432 (23).

Preparation of Nuclear Extracts—Nuclear extracts (26) from embryonic chicken erythroid cells were prepared at 4 °C with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 0.5 μg/ml chymostatin) in all

1 The abbreviations used are: LCR, locus control region; bp, base pair(s); DTT, dithiothreitol; UTR, untranslated region.
Enhancer-Promoter Interaction

solutions. Washed cells were swollen (10 min, 0.67% phosphate-buff-}
ered, resuspended in the base nuclei, and homogen-}{
ed with 12 strokes in a Dounce homogenizer (type B pestle). After}
centrifugation (20,000 × g, 4 °C, 20 min), the pellet was resuspended}
(1.67 × 10⁹ cells/ml in 20 mM HEPES, pH 7.9, 25% glycerol, 420 mM}
NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibi-
tors). The suspension was homogenized as above, gently stirred for 30}
min, adjusted as above, and the supernatant was dialyzed (6, 4 × 4}
against 50 volumes of 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM}
KCl, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitors). Aliquots}
(200–500 μl) were frozen on dry ice and stored at –70 °C. Protein}
concentrations were determined using the Bio-Rad protein assay.

DNase I Footprinting—DNA probes were labeled with [α-32P]dATP} and [α-32P]dCTP (6000 Ci/mmole) using Klenow fragment and gel-puri-
fied. Binding reactions (50 μl) containing extract, probe (1–5 ng,}
50–5000 cpm), 15 μg of poly(dI·dC), 100 ng of salmon sperm DNA, 10}
mM HEPES, pH 7.9, 60 mM KCl, 1 mM MgSO₄, 5 mM DTT, 10% glycerol,
and protease inhibitors were incubated for 20 min at 23 °C. DNase I}
digestion (2 min, 23 °C) was performed with added CaCl₂ (3 mM) and}
stopped with 200 μl of 20 mM NaCl, 20 mM EDTA, 1% SDS, and
0.5 μg of poly(dI·dC) were incubated on ice for 15 min and electrophoresed in an
8% polyacrylamide gel. DNA was electrophoresed on an 8% denaturing acryla-

d acid gel using 10 mM Tris base, 10 mM HEPES, pH 7.9, 3 mM MgCl₂,0.5 mM DTT,
and protease inhibitors. The suspension was homogenized as above, gently stirred for 30
min, adjusted as above, and the supernatant was dialyzed (6, 4 × 4}
against 50 volumes of 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM
KCl, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitors). Aliquots}
(200–500 μl) were frozen on dry ice and stored at –70 °C. Protein}
concentrations were determined using the Bio-Rad protein assay.

Electrophoretic Mobility Shift Analysis—Gel-purified single-strand}
oligonucleotides were kinased with [γ-32P]ATP (6000 Ci/mmole) and
annealed with a molar excess of unlabeled complementary strand.
Binding reactions (20 μl) containing extract (1–5 μg of protein), probe (10–}
50 μg of DNA), 20 mM HEPES, pH 7.9, 100 mM KCl, 3 mM MgCl₂,1 mM DTT,
and 0.5 μg of poly(dI·dC) were incubated on ice for 15 min and electrophoresed in an
8% acrylamide gel using 10 mM Tris base, 10 mM HEPES, pH 7.9, and 1 mM DTT as
running buffer. Competitor oligonucleotides were added prior to
addition of extract; antiserum (0.1 μg/mlbovineserumalbumin,0.0125%TritonX-100,4%Ficoll
l) were frozen on dry ice and stored at –70 °C. Protein}
concentrations were determined using the Bio-Rad protein assay.

Results are expressed as the mean ± S.E.

RESULTS

Enhancerless e- and ρ-Globin Promoters Express at Similar Levels—In embryos, e- and ρ-globin are strongly expressed and at similar levels (see Ref. 21 and references therein). To}


c ompare the promoter strength of these genes independent of dis-
t elements, we transfected plasmids carrying a luciferase
reporter driven by the ρ or e promoter into 4-day erythroid cells.
We expected that e expression in the absence of the βe enhancer
would be much lower than ρ expression. However, the enhancerless constructs were expressed at similar levels (Fig. 1).
Inclusion of the βe enhancer in the e construct greatly
stimulated e expression (Fig. 1).

Construction of Reporter Plasmids—Construction of pY726-lu-
ferase, containing the chicken e-globin promoter driving lu-
ferase, has been described. 2 The 5’ UTR of the chicken e-globin gene was marked by
insertion of 40-bp (5’-CGAGTATAGCTGAGCTGCAGAGCCG-
GATGTTCTGAGT) between bases 20332 and 20333. The insert
did not alter luciferase expression (Fig. 1). Mutations were introduced into the e promoter by site-directed mutagenesis (29) (Transformer kit,
Clontech), and all mutated regions were sequenced. The e promoter DNA was from pc2cat (Ref. 30, derived from Ref. 31) and differed from
the L17432 sequence at the following positions: 19906, T → G; 20068, G → T; 20075, C → A; 20078, T → C; 20081, G → T; 20110, T → G; and
20181, C → T.
The wild type and mutated Ncol fragments (19795–20347; from 510 bp upstream of the e cap site to the initiator codon) were individually
inserted into the NcoI site of pGL2-Basic plasmid (Promega), thus
fusing the e and luciferase initiator codons (this involved a
conversion of the luciferase initiation codon to a Ncol site). The unmu-
tated version is designated e510 (p1291). The mutated plasmids are
named using the number of the 3’ most base in the mutation. Our
labo
atory designations for these plasmids are: e252, p1292; e35, p1293;
e35, p1304; m131Enh, p1384; m33Enh, p1385; m52Enh, p1386;
m74Enh, p1387; m93Enh, p1541; m111Enh, p1389; m120Enh, p1390; m123Enh, p1391; m124Enh, p1542;
m124Enh, p1392; m133Enh, p1393; e7Enh, p1405; and e142Enh, p1394. Plasmids e510Enh (p1306) and e85Enh (p1308) were generated by
HindIII digestion of p1353 (wild type with enhancer), Klenow fill-in,
EEI digestion and insertion of the 730-bp HpaI-EcoRI fragment of
p1295 (m52) or the 765-bp HpaI-EcoRI fragment of p1297 (m93).
Transient Expression—Transient transfections were performed with
4-, 5-, and 6-day chicken embryos was performed essentially as
described previously (19, 32). Typically, 125 ng of luciferase reporter
plasmid and 125 ng of RSV-cat control plasmid were transfected into 3
A₅₅₆ units of cells using osmotic shock. Transient expression in 10-day
erythroid cells was performed by electroporation. Cells (10⁹ in 0.5 ml of L-
15 medium) were mixed with 2.5 μg of RSV-cat and 10 μg of test
plasmid. After 5 min at room temperature, the cells were electroporated
(0.4-cm cuvette, 500 microfarads, 450 V, giving a τ of ~9 ms) using a
Bio-Rad Gene Pulser. The cells were cultured with 1.4 ml of complete
medium at 37 °C for 40–48 h, as described for the cells transfected by
osmotic shock. Luciferase activity was measured (Luciferase Assay
System, Promega) normalized to the activity from the RSV-cat (33) system, and stopped by
Hin digestion (2 min, 23°C) was performed with added CaCl₂ (3 mM) and
stopped with 200 μl of 20 mM NaCl, 20 mM EDTA, 1% SDS, and
0.5 μg of poly(dI·dC) were incubated on ice for 15 min and electrophoresed in an
8% acrylamide gel using 10 mM Tris base, 10 mM HEPES, pH 7.9, and 1 mM DTT as
running buffer. Competitor oligonucleotides were added prior to
addition of extract; antiserum (0.1 μg/mlbovineserumalbumin,0.0125%TritonX-100,4%Ficoll
l) were frozen on dry ice and stored at –70 °C. Protein}
concentrations were determined using the Bio-Rad protein assay.

2 O. Gavrilova, M. M. Mason, and M. Reitman, submitted for publication.
terized further using electrophoretic mobility shift assays. Like many other globins, the ε promoter contains a GATA motif in place of a canonical TATA box. When this region was used as a probe (oligonucleotide A), a specific complex with the mobility of cGATA-1 bound to DNA was formed (Fig. 4, lanes 1–6). Mutation of the TATA element (G → T at −33, oligonucleotide Am33) destroyed the GATA motif and prevented formation of the GATA-1-DNA complex (Fig. 4, lanes 7–10). These results indicate that the cGATA-1 protein binds to the GATA/TATA motif of the ε promoter.

Oligonucleotide B, spanning the GC-rich, Sp1-like region immediately upstream of the TATA box (Fig. 3), yielded a band with the mobility and binding specificity of an Sp1-DNA complex (Fig. 4, lanes 11–19). Antibody to Sp1 inhibited formation of this complex (Fig. 4, lanes 20–22). These data demonstrate that this region of the ε promoter can bind Sp1 (or an antigenically related protein).

Oligonucleotide E corresponds to the protected region of the promoter containing EKLF/CACCC-like (34, 35) and Sp1-like sequence motifs and yielded two distinct complexes in mobility shift assays (Fig. 5A). The slower migrating complex had the mobility of Sp1 bound to probe; its formation was competed by an authentic Sp1 oligonucleotide, by oligonucleotide B (shown above to contain an Sp1 site), and by antibodies to Sp1. In contrast, the faster migrating complex was not competed by Sp1 oligonucleotides or antibodies to Sp1. These results demonstrate that this region binds two distinct factors in the erythroid nuclear extracts; the slower complex is due to Sp1 (or a related factor that is indistinguishable by antibody reactivity and binding site specificity), while the faster migrating band is not due to Sp1 and is here designated CACCC.

Methylation interference analysis was used to analyze the Sp1- and CACCC-binding region further (Fig. 5B). Methylation of guanosine residues at positions −124, −125, −126, −128, −130, and −131 inhibited binding of the CACCC factor. Methylation of these same residues inhibited Sp1 binding, as did methylation of an additional guanosine at −120. Substitution of bases −123 to −126 (CCCT → GTAC; E’m124e) prevented binding of CACCC with little effect on Sp1 (Fig. 5C, lanes 6, 7, and 10–15). Mutation at −120 (C → A; E’m120) reduced Sp1 binding but had little effect on CACCC binding (Fig. 5C, lanes 8, 9, and 16–21). These data demonstrate that Sp1 binds to a motif that overlaps, but is distinct from, that bound by CACCC.

The CACCC motif centered at −128 of the chicken ε-globin promoter is a perfect match (CCACCCCT) to the EKLF-binding sequence of certain mammalian globin promoters (34, 35). In addition, the methylation interference pattern of CACCC is

![Graph](image-url)
identical to that reported for murine EKLF (36). Thus, we examined binding of recombinant murine EKLF to this region (Fig. 5D). EKLF formed a complex with oligonucleotide E (the wild type chicken sequence) that comigrated with EKLF bound to the classical murine b-globin promoter site. The point mutation, which inhibited Sp1 binding but not CACCC complex formation (E m120), did not significantly affect EKLF binding. Conversely, the mutation that abolished CACCC binding with little effect on Sp1 (E m124c) did abolish EKLF binding. These data suggest that the faster migrating complex (designated CACCC) in chicken erythroid extracts may be due to a chicken homologue of EKLF or to a closely related protein.

Despite the presence of footprints in at 286 to 2105 and 2155 to 2183, no mobility shifts were observed with oligonucleotides comprising these regions of the e-globin promoter (data not shown). Under the assay conditions used, no protein binding was observed to the CCAAT box at 275.

**Enhancer-Promoter Interaction**

**Effect of Deletions on e Promoter Activity**—To examine the
functionally important regions of the \( e \)-globin promoter, a series of deletion mutants were tested with and without the \( \beta e \) enhancer. In reporter plasmids lacking the enhancer (Fig. 6, solid bars), deletion upstream of –142 did not reduce expression. Deletion to –88, removing the CACCC/Sp1 element, reduced activity 2- to 5-fold in the primitive cells but to a lesser extent in definitive cells. Deletion to –73, destroying the CCAAT motif, and to –51, ablating the Sp1 site, each reduced expression further. These data suggest that full promoter activity resides in the proximal 142 bp of promoter. To examine the effect of the enhancer on \( e \)-globin expression, plasmids containing the enhancer (as a 2.7-kilobase pair fragment, from –3229 to –510) was cloned upstream of the various promoter deletions. Constructs containing at least 73 bp of promoter

Fig. 5. Protein-binding analysis of the distal \( e \)-globin promoter. A and C, electrophoretic mobility shift assays using oligonucleotides E and E' (see “Experimental Procedures” and Fig. 3 for sequences). Nuclear extracts were from 10-day (11.4 \( \mu g \), panel A, lanes 2–9 and 12–14; panel C, lanes 2–9, 11–15, and 17–21) embryonic chicken erythroid cells, or were omitted (panel A, lanes 1 and 11; panel C, lanes 1, 10, and 16). Competitor oligonucleotides were added in a 10- or 100-fold molar excess. The positions of free probe, a complex with the CACCC site, and authentic Sp1-DNA complexes are indicated. Lanes 12–14 of panel A contained no antiserum, preimmune serum, or anti-Sp1. B, methylation interference patterns of DNA from protein complexes with oligonucleotide E (with the upper strand labeled). The regions of a gel corresponding to the free probe and the protein-DNA complexes (labeled Sp1 and CACCC in C) were eluted, the DNA was cleaved with piperidine and electrophoresed on 8% denaturing acrylamide gel. Bases whose relative intensity was decreased maximally are designated by solid circles; open circles indicate bases that were reduced in intensity, but to a lesser extent. D, electrophoretic mobility shift assays using recombinant EKLF. Purified GST-EKLF (34) was provided by J. Bieker, and electrophoretic mobility shift was performed as described (36). Competitor oligonucleotides were added as indicated in a 100-fold molar excess.
showed strong enhancement (Fig. 6, open bars; note the different scales), suggesting that the proximal 73 bp of the $\epsilon$-globin promoter is sufficient for productive interaction with the enhancer.

Enhancement Decreases with Development—The decrease in $\epsilon$-globin transcription that occurs with development could be due to diminished enhancer activity or to diminished promoter activity. Promoter activity, in the absence of the enhancer, was comparable in erythroid cells from days 4–6 (Fig. 6). (Because different methods were used to transfect the 10-day cells, the data from that stage cannot be compared directly with the results from primitive cells.) To examine the role of the enhancer, enhancer activity (defined as the ratio of activity of constructs containing the promoter and enhancer to those with the promoter only) for each of the promoter deletions was plotted as a function of the age of the cells used for transfection (Fig. 7). In the four promoter constructs containing at least 73 bp of promoter, the average enhancement was 185-, 66-, 18-, and 16-fold for expression in 4-, 5-, 6-, and 10-day cells, respectively. These data suggest that the decrease in $\epsilon$-globin expression with development is due to a decrease in the effect of the enhancer on the $\epsilon$ promoter, and not to a decrease in intrinsic $\epsilon$ promoter activity.

Expression of $\epsilon$-Globin Promoter Point Mutants—We next studied the effect of clustered point mutations on transcription from the $\epsilon$-globin promoter in chicken erythroid cells (Fig. 8). Sequencing of the mutated constructs revealed a T at −123 in our parent construct, rather than the reported C (31). However, introduction of the C into several constructs showed no difference in expression in the absence of the enhancer (compare m123c versus $\epsilon$510, m111c versus m111, and m124c versus m124). When not otherwise noted, the sequence is a T at this position.

In the absence of the enhancer, mutation of the TATA site (m25, m31), of the Sp1 motif (m52), or the CCAAT box (m74) reduced promoter activity. Interestingly, mutation of sites farther upstream than the CCAAT box did not affect expression levels. In addition, conversion of the TATA site from a GATA to a canonical TATA (m33) had no significant effect on $\epsilon$ expression.

In plasmids containing the enhancer, like those without the enhancer, point mutations in the TATA (m25, m31), Sp1 (m52), and CCAAT (m74) motifs reduced promoter activity (Fig. 9, upper panels). However, in contrast to the enhancerless plasmids, mutations in the CACCC site (m111c, m123c, m124, m124c), in the presence of the enhancer, also decreased reporter expression. Consistent with the greater enhancement in earlier stage cells noted earlier with the deletion mutants, the reduced activity resulting from the point mutants was more striking in 4-day than in 10-day cells.

A measure of the relative contribution of the promoter sites to enhancer-promoter interaction was obtained by comparing the effects of mutated promoter elements in enhancerless and enhancer-containing constructs (Fig. 9, lower panels). Mutations of the CACCC element (m111c, m123c, m124, m124c) affected enhancement more than mutations of the CCAAT (m74), Sp1 (m52), and TATA (m25, m31, m33) regions. Since mutations affecting CACCC factor binding lowered expression more than the mutations affecting Sp1 binding (compare m124 and m124c with m120), promoter-enhancer interaction at this site is probably due to the EKLF-like proteins rather than Sp1.
DISCUSSION

**Functional Elements in the Chicken \(\epsilon\)-Globin Promoter**—Using a combination of protein binding and transient expression assays, we have characterized the cis-elements and trans-factors of the \(\epsilon\)-globin promoter. The proximal 142 bp were sufficient for full promoter activity, with four sequence motifs contributing to this activity: the TATA box at \(-30\), an Sp1-binding site (GGTGGG) at \(-54\), a CCAAT sequence at \(-75\), and a CACCC motif at \(-127\). These elements are similar to those observed in other globin promoters, albeit with differences in the order and spacing of the sites (Ref. 37, reviewed in Ref. 38).

The \(\epsilon\) promoter, like the \(\beta^A\) promoter, contains a non-canonical TATA element capable of binding GATA-1. However, our transient expression experiments did not demonstrate any difference between the \(\epsilon\) TATA and a canonical TATA in either promoter activity or promoter-enhancer interaction, unlike results from \(\beta^A\) expression (39, 40).

The CACCC sequence at \(-123\) to \(-131\) is a 9/9 match to the EKLF motif in the mammalian \(\beta\)-globin promoters, binds recombinant murine EKLF, and mediates enhancer activity. This is curious since, in mice, EKLF is a transcription factor essential for definitive erythropoiesis (41, 42) and may (43) or may not (41) be present in primitive erythroid cells. One possible explanation is that chicken EKLF has an embryonic developmental specificity. Alternate hypotheses are that (a) CACCC sites of embryonic genes bind embryonic-specific Krüppel family transactivators other than EKLF and/or (b) CACCC sites of embryonic genes bind definitive stage-specific Krüppel family members that act as repressors, for example due to Krüppel-associated box domains (44, 45). Our mutagenesis experiments with the chicken gene are not consistent with the last explanation. Elucidation of the actual mechanisms in the chicken and assessment of their generality requires characterization of chicken EKLF and of the proteins binding to the CACCC-like motifs that control embryonic globin expression in mammals.

**Comparison of \(\epsilon\)- and \(\rho\)-Globin Regulation**—The chicken \(\rho\)- and \(\epsilon\)-globins are expressed with the same developmental pattern and at similar levels. The proximal promoters and some of the exons and introns of these genes are very similar in sequence, probably due to gene conversion (23, 46). As expected
from the promoter (24), the $\beta\alpha$ element binds Sp1 and $\beta\beta$ is restricted to primitive erythroid elements and is not expressed in definitive or adult erythroid cells. Our data demonstrate that developmental stage-specific regulation of $\epsilon$ expression is affected through $\rho$ promoter/enhancer interaction independent of promoter strength; enhancer activity dropped from 185-fold in primitive cells to 16-fold in definitive cells. Enhancer activity is determined by factors binding to the promoter and to the enhancer, and by factors not binding directly to these elements. Developmental changes in any of these groups of proteins could account for the regulation of $\epsilon$. Two lines of evidence indicate that the mechanism is not regulation of a developmental stage-specific factor binding directly to the $\epsilon$ promoter. First, we were unable to detect stage-specific factor binding to the promoter. Second, no promoter mutant showed stage-specific regulation when lacking the enhancer. Moreover, our studies revealed no evidence of a silencer element. In contrast, regulation of the human $\epsilon$-globin gene does involve a silencer, which can be detected using transient expression (50).

Transient expression assays using competition between two promoters for an enhancer have provided evidence for a stage selector protein binding to the $\gamma$-globin genes in human $\gamma\beta$ switching (51, 52) and to the $\beta$-globin gene in the chicken (NF-E4; Refs. 28 and 53). Whether these or similar proteins are involved in developmental regulation of the chicken $\epsilon$-globin gene is unclear. Our results define a system in which developmental specificity can be examined in the absence of the confounding effects of a second promoter.

Previous experiments in transgenic mice and using transient expression have demonstrated that competition between promoters for an enhancer/LCR contributes to stage specificity. Since our reporter plasmids contained only a single promoter, competition cannot explain the observed developmental specificity. Moreover, since the $\beta\epsilon$ enhancer functions effectively in definitive erythroid cells to increase $\beta\alpha$-globin expression, an intrinsic, absolute stage specificity inherent in the enhancer can also be excluded. Rather, our results suggest that regulation of stage specificity occurs at the level of enhancer/promoter interaction, even in the absence of competition, and is not simply a property of the enhancer or promoter in isolation. This conclusion is consistent with a competition mechanism for the regulation of globin expression. Indeed, developmental regulation of the enhancer-$\epsilon$ promoter interaction is likely to be the basis for the decreasing expression of $\epsilon$-globin and the increasing expression of $\beta\alpha$-globin seen in competition assays and in vivo.

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REFERENCES
1. Stamatoyannopoulos, G., and Nienhuis, A. W. (1994) in The Molecular Basis of Blood Diseases (Stamatoyannopoulos, G., Nienhuis, A. W., Majerus, P. W., and Varmus, H., eds) pp. 107–155, W. B. Saunders, Philadelphia
2. Orkin, S. H. (1995) Eur. J. Biochem. 231, 271–281
3. Bruns, G. A. P., and Ingram, V. M. (1973) Philos. Trans. R. Soc. Lond. Ser. B Biol. Soc. 266, 225–305
4. Gruss, F., Blom van Assendelft, G., Grecaves, D., and Kollia, G. (1987) Cell 51, 975–985
5. Forrestor, W. C., Epner, E., Driscoll, M. C., Enver, T., Brice, M., Stamatoyannopoulos, G., and Groudine, M. (1990) Genes Dev. 4, 1637–1649
6. Reimart, M., Lee, E., Westphal, H., and Felsenfeld, G. (1993) Mol. Cell. Biol. 13, 3990–3996
7. Felsenfeld, G. (1992) Nature 355, 219–224
8. Choi, O.-R., and Engel, J. D. (1988) Cell 55, 17–26
9. Enver, T., Raich, N., Ebens, A. J., Papayannopoulos, T., and Costantini, F., and Stamatoyannopoulos, G. (1990) Nature 344, 309–313
10. Dillon, N., and Gruss, F. (1993) Trends Genet. 9, 134–137
11. Crossley, M., and Orkin, S. H. (1993) Curr. Biol. 3, 232–237
12. Peterson, K. R., and Stamatoyannopoulos, G. (1993) Mol. Cell. Biol. 13, 4836–4843
