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Bidirectional control of the chicken β- and ε-globin genes by a shared enhancer

(transcription/erythrocytes/transfection/developmental regulation)

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ABSTRACT **An enhancer specific to erythroid cells was identified previously in the 3′ flanking sequence of the chicken adult β-globin gene and shown to act on the β-globin promoter. This enhancer lies between the adult β-globin gene and the embryonic ε-globin gene, about equidistant from the two promoters. To determine whether this enhancer acts also on the ε-globin promoter, we constructed plasmids containing the enhancer and either the β- or the ε-globin promoter fused to the bacterial chloramphenicol acetyltransferase gene. Primary chicken erythrocytes of both primitive and definitive lineages were transfected with these plasmids. We show that the enhancer is able to stimulate expression from the ε-globin promoter as well as the β-globin promoter. Levels of expression change with the developmental stage of the cell in a way that is partially consistent with the observed developmental regulation of the β- and ε-globin genes *in vivo*. There appear to be no other enhancer elements either 5′ of the ε-globin gene or within 6 kilobase pairs of its 3′ end. Thus, the enhancer between the β- and ε-globin genes apparently serves to regulate both genes.**

The β-globin gene cluster of the chicken is a model system for studying developmental regulation of differentiated gene expression. Synthesis of each of the globins encoded in the cluster occurs only in specific erythrocytes at defined stages of embryogenesis (1–3). The primitive erythrocyte, which predominates in the circulation before day 5 of development, synthesizes the two embryonic globins ρ and ε. Subsequently the definitive red-cell line becomes the predominant population; cells of this line synthesize β-globin, the globin of the adult erythrocyte. The central question raised by these developmental events is how transcription of each gene of the β-globin cluster is confined to its specific cell lineage.

In this paper we describe the regulatory sequences that might be responsible for the switch in expression between the embryonic ε-globin gene and the adult β-globin gene during development. Attention is focused on the β and ε promoters and on an enhancer sequence that lies between the two genes, downstream of the β-globin gene and upstream of the ε gene (4). An osmotic-shock method is used to transfect erythrocytes with plasmids containing the ε or β regulatory elements. Primary red cells of both primitive and definitive lineages can be transfected by this method; they efficiently express genes introduced in this way (5). Previously, this transfection assay was used to show that the enhancer sequence functioned to stimulate β-globin expression 50- to 80-fold in a tissue-specific manner (4). We present evidence here that this enhancer sequence not only influences the developmental program of β-globin expression but also influences ε-globin expression in both primitive and definitive red-cell populations.

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METHODS

Construction of Plasmids. Construction of pAcat, pAcat-E50, and pAcatE55 has been described (4). The basic ε-globin vector contains the sequences from −1050 (*Bam*HI) to +45 (*Nco* I) in the structural gene (6). The ε-globin ATG translational initiation triplet was removed by mung bean nuclease digestion, *Bam*HI linkers were added, and the promoter fragment was subcloned 5′ to the chloramphenicol acetyltransferase (CAT) gene sequences in plasmid pUC18-catSV40 (4). From this basic ε-promoter–CAT fusion vector the other ε clones in Fig. 1 were produced by appropriate restriction digestions and ligations.

Osmotic Shock and Transfection of Primary Erythrocytes. Primary erythrocytes can take up and express transfected DNA efficiently if they are first osmotically shocked with NH_4Cl in the absence of DNA and then treated with a DEAE-dextran solution containing DNA (4, 5). Primitive and definitive erythrocytes differ morphologically; the larger volume of primitive cells affects their response to osmotic shock. For both major cell types it is important to ascertain optimal shock and transfection conditions such that the amount of CAT gene expression is directly proportional to the amount of DNA added to the cells. Only under such conditions can accurate quantitation of the transcription directed by each globin construct be assured. The amount of CAT expressed in each experiment must quantitatively reflect the interaction of DNA regulatory sequences (promoter and/or enhancer elements) with the cell's synthetic machinery and not be attributable to anomalies inherent in the DNA transfection itself, such as differences in extent of osmotic shock or in extent of DNA uptake.

The major variables in these transfections are cell density and DNA concentration. Variations in these parameters can affect cellular response, monitored as CAT activity (Fig. 2). pRSVcat (5, 8) was used as the test construct because it was efficiently expressed in all erythrocytes tested, regardless of developmental stage.

In the case of definitive cells, shocked in 0.25 M NH_4Cl for 40 min at 25°C, maximal response to a DNA concentration of 3 μg/ml is obtained at a cell concentration corresponding (4) to A_{412} values of 24–25 (1.9×10^8 cells per ml; Fig. 2*A*). The response is linear with cell number up to this maximum and drops off sharply beyond it; the amount of CAT synthesized per cell [expressed as % chloramphenicol monoacetylated (% CAT) per A_{412} unit] has an optimal value of 6.2. A corollary experiment is to increase the DNA concentration while maintaining the cell number constant. A cell density of 2.2×10^8 per ml (A_{412} = 28.6) is chosen because it is the highest cell concentration routinely transfected; results are shown in Fig. 2*B*. At this cell concentration there is a linear correlation between CAT synthesis and input DNA up to ≈5.5 μg/ml, at which point the maximal response per cell, expressed as % CAT per A_{412} unit, equals 7.2. These activities (6.2–7.2)

Abbreviation: CAT, chloramphenicol acetyltransferase.

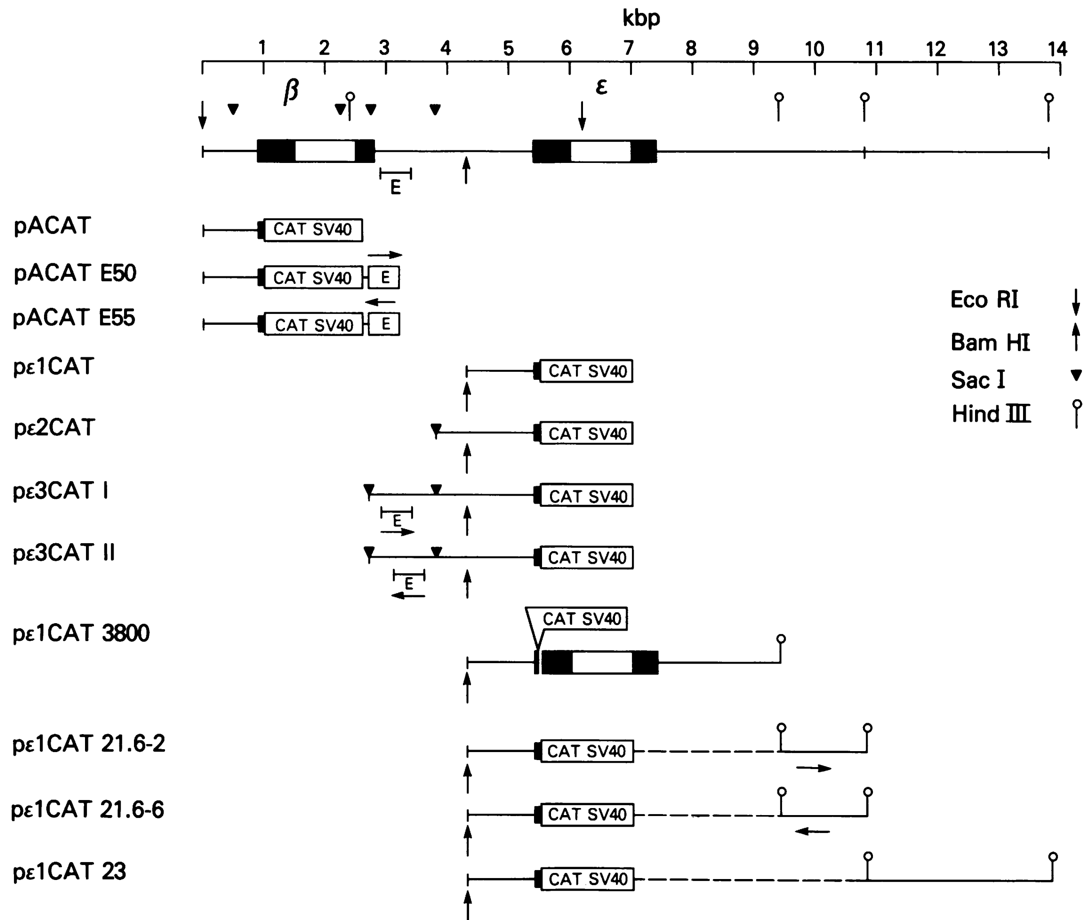

FIG. 1. Structure of globin–CAT fusion vectors. The β- and ε-globin genes are shown (exons in solid black; ref. 7) as well as the relative positions of the DNA fragments fused to pUC18cat (4) to generate the vectors used for transfections. The orientation of the enhancer region (E) within the vector is represented by an arrow. CAT SV40 represents the coding region of the CAT gene fused to splice and polyadenylylation sites of simian virus 40. kbp, Kilobase pairs.

appear to reflect the maximal response of the definitive erythrocytes to these particular transfection treatments.

Similar data showing relationships between number of primitive erythrocytes isolated from 4-day chicken embryos and concentration of DNA per assay are also presented in Fig. 2. For this cell lineage the optimal osmotic-shock conditions require treatment with 0.15 M NH_4Cl for 110 min at 25°C before exposure to DNA. In the experiment shown here, varying numbers of cells were titrated with increasing amounts of DNA. The results are plotted either as a red-cell concentration curve in which the individual lines represent different DNA concentrations (Fig. 2 *C* and *D*) or as a DNA concentration curve, with individual lines corresponding to different cell concentrations (Fig. 2*E*). The response to increasing the number of cells per reaction is linear up to $\approx 1.9 \times 10^7$ cells per ml ($A_{412} = 3$) at all but the highest DNA concentration analyzed. Conversely, for all cell concentrations assayed, CAT expression is linear with DNA concentration to 0.25 μg/ml. The value of CAT synthesis per cell (% CAT per A_{412} unit) is constant at any given DNA concentration in the linear range; at the highest DNA concentration in this range (0.25 μg/ml) this value is ≈45. Thus, these primitive cells have relatively high individual cell activity values compared to the definitives' value of 6.2–7.2. We do not know why the two cell types respond in such a quantitatively different way to transfection, but the definitive line requires, as a rule, about 8 times the number of cells and 8 times the amount of DNA to give a response equivalent to that from primitive erythrocytes. Regardless of this difference in response, within the ranges of cell and DNA concentrations established for each cell lineage one is able to compare directly the activity of CAT constructs containing different promoter sequences and, most importantly, to assess the effects of additional DNA sequences that may have possible regulatory functions.

RESULTS

Transfection of β- and ε-Globin–CAT Constructs into Definitive Erythrocytes. The constructs span the entire β- and ε-globin domains (Fig. 1). DNA titrations were carried out for each vector within the linear ranges previously established for each cell lineage, permitting direct quantitation of factors involved in regulation of the two globin genes in the two cell types we studied. Results of such DNA titrations in definitive cells from 9-day embryos are shown in Fig. 3. The constructs contain either the β- or the ε-globin promoter, with or without the enhancer fragment. The β promoter alone directs a very low, but measurable, amount of CAT activity. Addition of the enhancer fragment (E in Fig. 1) in either orientation increases the basal activity 50- to 80-fold (4). The vector with the inverted orientation (pAcatE55) consistently yields 60–80% of the activity of pAcatE50 with the enhancer in the correct orientation.

The plasmid containing the ε promoter alone (pε1cat) also directs a barely detectable level of CAT activity in these 9-day definitive erythrocytes (Fig. 3*B*). A second plasmid (pε3catI) contains additional sequences further upstream to the basic ε promoter, so that the enhancer is present with the genomic orientation and distance. This does stimulate the

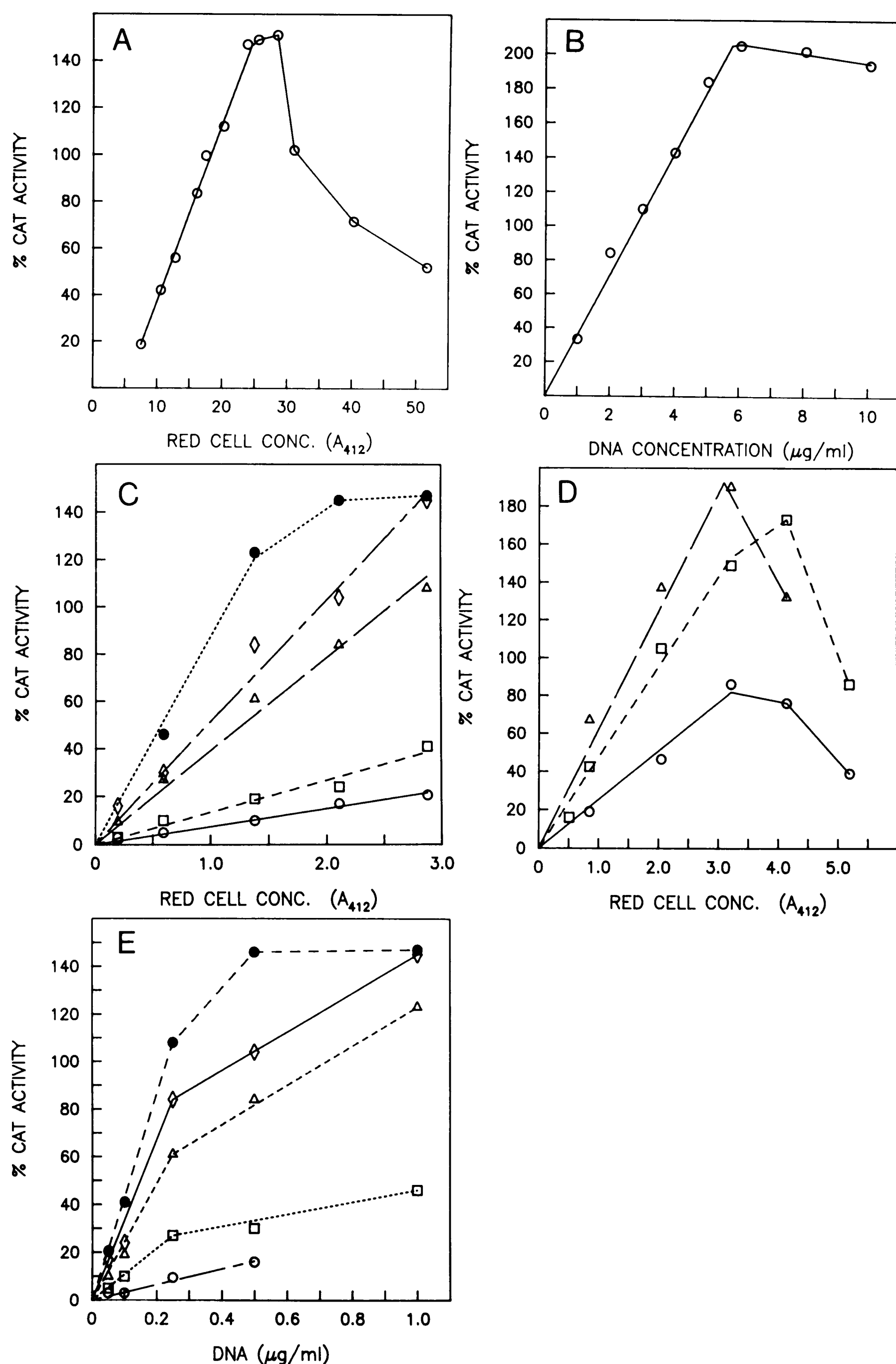

FIG. 2. Optimization of erythrocyte number and DNA concentration in osmotic-shock and transfection reactions. Levels of CAT activity resulting from each assay are plotted to show percentage of chloroamphenicol monoacetylated, as determined by direct cutting and scintillation counting of TLC plates. (*A*) Various numbers of definitive (9-day) erythrocytes were shocked (1-ml volume, 40 min, 25°C) with 0.25 M NH_4Cl and transfected with pRSVcat DNA (3 μg/ml). Definitive-cell number per ml equals A_{412} times 7.70×10^6. (*B*) Definitive erythrocytes (A_{412} units = 28.6) were shocked as in *A* and transfected with various concentrations of pRSVcat. (*C*) Various numbers of primitive (4-day) erythrocytes were shocked (1-ml volume, 110 min, 25°C) with 0.15 M NH_4Cl and transfected with pRSVcat DNA. DNA concentrations (μg/ml) were 0.05 (○), 0.1 (□), 0.25 (△), 0.5 (◇), and 1.0 (●). Primitive-cell number per ml equals A_{412} times 6.45×10^6. (*D*) Primitive cells treated as in *C* were transfected with pRSVcat at 0.125 (○), 0.25 (□), or 0.5 (△) μg/ml. (*E*) Various concentrations of pRSVcat were transfected into different numbers of primitive cells. A_{412} values (units) were 0.20 (○), 0.59 (□), 1.4 (△), 2.1 (◇), and 2.88 (●).

CAT response, but the increased activity consistently reaches a plateau value at input DNA concentration of 2–3 μg/ml. No other construct tested undergoes such a dramatic saturation at such low DNA concentrations. It suggests that some factor necessary for maximal ε expression is present in only limiting amounts in these 9-day definitive blood cells. Interestingly, this saturation can be overcome simply by inverting the 1200-bp *Sac* I fragment containing the enhancer region. In this plasmid (pε3catII) the enhancer is moved only slightly closer to the ε promoter, but the orientation of the fragment (including the enhancer) is opposite to its normal genomic position. This inversion completely abolishes the saturation effect of DNA concentration dependence seen with pε3catI and actually stimulates the ε promoter. Titrations of pRSVcat (Fig. 3*A*) provide assurance that the cells respond linearly to the transfection and also provide a reference gene that is useful in comparing activities in cells at different developmental stages (see *Discussion*).

Transfection of β- and ε-Globin–CAT Plasmids into Primitive Erythrocytes. Primitive erythrocytes isolated from 4-day chicken embryos were also transfected with the β- and ε-globin–CAT constructs. The β promoter alone (pAcat) gives a very low level of activity. Addition of the enhancer fragment stimulates transcription from the β promoter transfected into 4-day erythrocytes (Fig. 4*B*). Both pAcatE50 and pAcatE55 show linear responses to DNA addition resulting in ≈10-fold stimulation over the background level expressed by pAcat. The stimulatory effect of the enhancer on the β promoter in these cells is less than that seen in 9-day erythrocytes.

The ε promoter alone (pε1cat) gives a minimal level of activity similar to that of pAcat. Addition of upstream sequences containing the enhancer domain in the genomic orientation (pε3catI) stimulates expression ≈10-fold and the response is linear for the entire DNA range. The presence of the enhancer now makes the two promoters β and ε equally

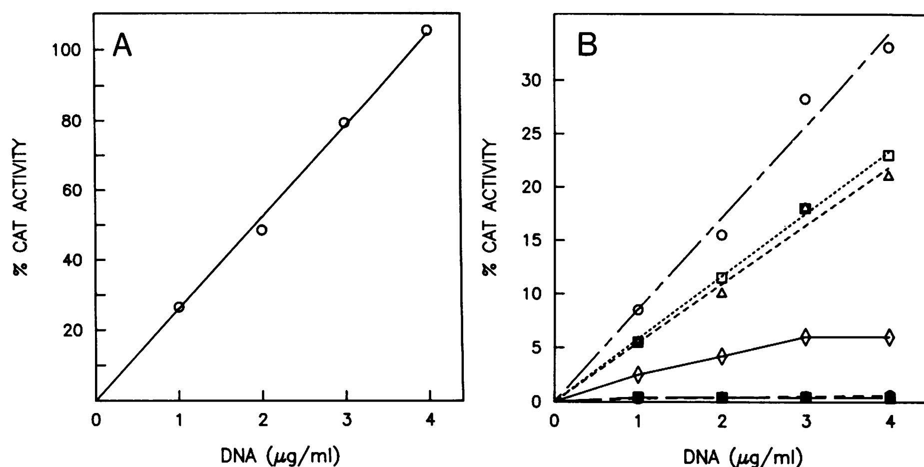

FIG. 3. Transfections of β- and ε-globin–CAT constructs into definitive erythrocytes. Cells (1 ml, 24–26 A_{412} units) were shocked as in Fig. 1A and transfected with various amounts of DNA. (A) pRSVcat. (B) ■, pAcat; ○, pAcatE50; □, pAcatE55; ●, pε1cat; ◇, pε3catI; △, pε3catII. Hemoglobin release during osmotic shock was 25%. CAT activity is given as % [^{14}C]chloramphenicol acetylated per 12.5 A_{412} units (10^8 cells) in 60 min.

powerful in these primitive cells. The greatest stimulation (30- to 40-fold) is exhibited by pε3catII, in which the enhancer-containing *Sac* I fragment is reversed in orientation. It is apparent that expression of pε1cat and pε3catI in primitive cells is lower than what might be anticipated for this embryonic globin gene, given that these cells are synthesizing ε-globin. This observation, combined with the fact that the β enhancer is located 3′ to its structural gene, provided some incentive for searching the remainder of the ε gene locus for possible enhancer function. We made use of ε constructs (Fig. 1), all of which contain the ε promoter coupled with various segments of either the structural gene itself or segments farther 3′ to the gene, encompassing a distance of 6 kbp. None of these sequences has any effect on expression of the ε promoter in these primitive cells (Fig. 4C). The scatter of the points is normal for such low levels of expression. Expression of the control pRSVcat is linear with increasing DNA concentration (Fig. 4A).

DISCUSSION

We have searched the β- and ε-globin gene loci for sequences involved in the stringent developmental control of the two genes. Particular attention was devoted to the enhancer element, which is located downstream of the β-globin gene and which had previously been shown to stimulate expression of that gene specifically in erythroid cells (4). This enhancer is located about 1.6 kbp 3′ of the β promoter and about 1.5 kbp 5′ of the ε promoter. An essential question was whether the enhancer could stimulate expression of the ε-globin gene as well as the β-globin gene, and whether such effects were dependent on the developmental stage of the erythrocytes used for transfection.

Table 1 lists the activities of the β- and ε-globin promoter constructs at each DNA concentration; activities are expressed relative to pRSVcat assayed in the same cell lineage. It is apparent that presence of the enhancer sequence stimulates expression from both β and ε promoters in the definitive cell lineage circulating in the 9-day embryo. In these definitive erythrocytes we observe partial, selective suppression of β gene expression: with the enhancer in the genomic orientation and at the genomic distance relative to the ε promoter, expression levels are only 20–25% of those observed with the β promoter. In studies of transcription within nuclei isolated from 9-day embryonic chicken erythrocytes, Landes *et al.* (3, 9) have found the same ratio of ε- to β-globin RNA production.

In addition to this quantitative difference in expression, the most dramatic difference between the response of the ε- and β-globin genes in 9-day cells is revealed by the DNA-concentration dependence of the embryonic ε-globin gene. The concentration dependence of CAT expression reaches saturation at quite low levels of the enhancer/ε-promoter construct, whereas other constructs maintain a linear dependence at much higher DNA concentrations (Fig. 3). This suggests that a factor essential for specific ε transcription is present in limiting amounts in these developmentally more mature erythrocytes, in which the endogenous ε-globin gene

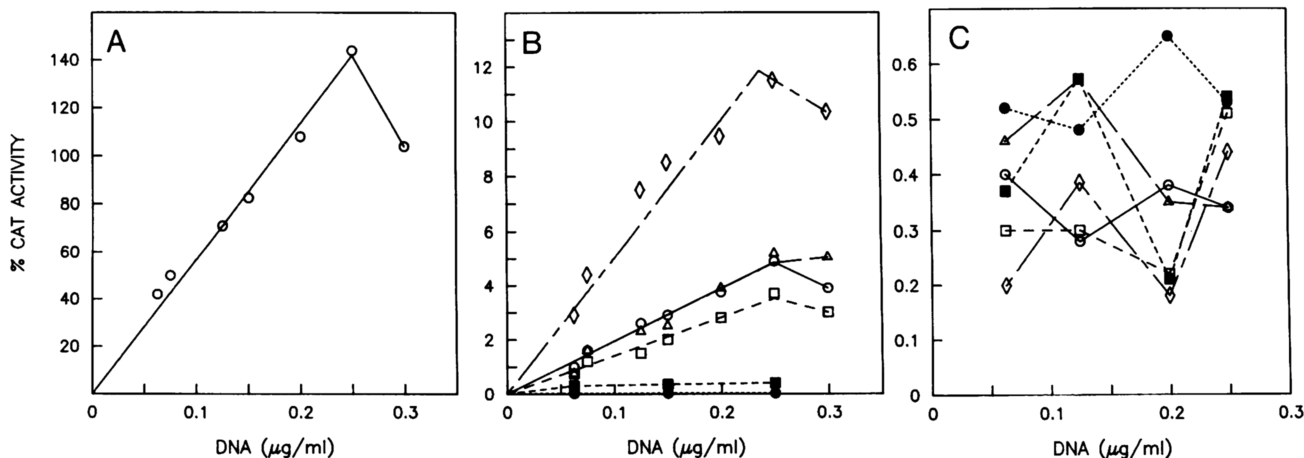

FIG. 4. Transfections of β- and ε-globin–CAT constructs into primitive erythrocytes. Cells (2–3 A_{412} units) were shocked as in Fig. 1C and transfected with increasing amounts of DNA. (A) pRSVcat. (B) ●, pAcat; ○, pAcatE50; □, pAcatE55; ■, pε1cat; △, pε3catI; ◇, pε3catII. (C) ○, pε1cat; □, pε2cat; △, pε1cat3800; ◇, pε1cat21.6-2; ●, pε1cat21.6-6; □, pε1cat23. Hemoglobin release during osmotic shock was 25%. CAT activity is given as % [^{14}C]chloramphenicol acetylated per 1.55 A_{412} units (10^7 cells) in 60 min.

Table 1. Activities relative to pRSVcat

DNA, ng	CAT activity, % pRSVcat value					
	pAcat	pAcat-E50	pAcat-E55	pε1cat	pε3-catI	pε3-catII
	Primitive cells					
31	—	4.4	2.1	—	3.0	10
38	—	3.6	2.6	—	3.5	8.8
62	0.22	4.4	2.2	0.20	3.3	—
75	—	3.5	2.5	—	3.1	9.3
100	0.20	3.7	2.9	0.21	3.4	9.4
125	0.22	3.5	3.2	0.26	3.6	12
150	—	4.1	3.2	—	4.6	9.1
	Definitive cells					
500	0.81	31	20	0.59	8.3	21
1000	0.83	30	21	0.58	7.9	20
1500	0.79	33	22	0.59	7.4	23
2000	0.87	32	22	0.65	6.0	20

Activity of each plasmid is expressed as a percentage of CAT activity produced by the same cells in response to a comparable amount of pRSVcat DNA. All numbers are averages of at least five different experimental transfections.

is not expressed. The DNA-titration methods described here are essential for the detection of such effects.

Surprisingly, the inversion of the 1.2-kbp *Sac* I fragment containing the enhancer abolishes this saturation effect. The inversion also results in 2- to 3-fold stimulation of ε-globin expression. The inversion places the 500-bp sequence at most 1 kbp closer to the ε promoter, without disturbing the intervening 1.5-kbp DNA segment containing the promoter itself. The abolition of the dependence on a putative limiting factor, and the increase in activity, may result from the slightly altered separation between enhancer and ε promoter or may reflect the altered orientation of the ε promoter relative to domains in the inverted enhancer. Within the enhancer fragment, we previously defined at least five domains capable of binding protein factors from erythrocytes (10). Functional deletion analysis across the enhancer revealed that the 150-bp domain at the 3′ end of the enhancer contains an inhibitory element; deletion of this 3′ region results in a 2-fold increase in expression relative to intact enhancer when assayed with the β-globin gene in definitive erythrocytes. Perhaps it is the removal of this 3′ domain from a position between the promoter and the body of the enhancer that accounts for the stimulatory effect. A similar effect is observed with the β promoter; pAcatE50 activity is 1.5 times that of the β-globin construct with the inverted enhancer, pAcatE55, in which the 3′ inhibitory domain is between the enhancer and the β promoter.

In the developmentally earlier primitive erythrocytes, the enhancer is again required for maximal expression of both β- and ε-globin. However, in these cells both genes give quantitatively equivalent responses, and neither gene is a very efficient transcriptional unit. This suggests that some additional level of control not conferred by DNA sequence alone must be necessary for correct differential expression. The most obvious possibility is that chromatin structure, not present in the transfected plasmids, is an important element in proper regulation of expression. For this reason it is quite interesting that the most active ε construct carries the enhancer in an inverted orientation. As described above, in this inversion the individual domains within the enhancer are oriented differently with respect to the ε promoter; perhaps a similar alignment of sequences by bending or looping in chromatin can contribute to differential expression of the ε-globin gene in development.

The low level of ε-globin expression that we observe is nonetheless consistent with data from *in vitro* transcription of nuclei isolated from 5-day chicken embryo erythrocytes (11). The ρ gene, which is another embryonic β-globin gene, is significantly (5-fold) more active as a template than the ε gene in these nuclei. This is perhaps related to the known ≈2.5-fold ratio of ρ-globin to ε-globin protein abundance in primitive cells (12). These results suggest that the ε gene is an innately inefficient template. In the case of our transfection studies, however, it seemed possible that some DNA element important for ε gene expression had been omitted from the constructs. The search for such elements, capable of augmenting ε response in 4-day cells, was unsuccessful. Analysis of the ε structural gene itself and beyond it in the 3′ direction for a distance of 6 kbp revealed no sequences capable of stimulating expression from the ε promoter in our assay. The β enhancer is thus the only element near the ε promoter that stimulates that promoter.

This discussion has largely compared β- and ε-globin gene expression within cells of the same lineage, either primitive or definitive erythrocytes. To compare expression between lineages, it would be necessary to assume that the expression of the reference gene, in pRSVcat, is a measure of general transcriptional response of the cell. This point of view is supported by the fact that when primitive erythrocytes are stored in saline longer than normal before osmotic shock, a lower response to pRSVcat is observed but all other constructs yield correspondingly lower values, so that the activity of these constructs relative to the activity of pRSVcat remains invariant (data not shown). Nonetheless, some caution must be exercised in interpreting, for example, the relative amounts of β-globin expression observed in definitive and primitive cells.

The analysis we have described here involves combinations of the β-globin enhancer with the ε and β promoters. The results show that relative levels of expression from constructs containing these elements are modulated in primitive and definitive embryonic erythrocytes in a way that is at least consistent with the observed developmental regulation of the β- and ε-globin genes *in vivo*. Our search did not reveal any stimulatory element in the neighborhood of, or within, the ε-globin gene other than the β enhancer. It therefore seems likely that this enhancer functions bidirectionally to control expression from both β- and ε-globin genes, in a developmentally regulated manner. Methods like those described here should make it possible to identify enhancer and promoter sequences, and protein factors, responsible for that behavior.

We thank Dr. H. Martinson for supplying subclones of the ε-globin structural gene and adjacent downstream sequences.

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