Developmental modulation of protein binding to β -globin gene regulatory sites within chicken erythrocyte nuclei

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We describe the interaction of two adjacent binding sites in the chicken β -globin gene promoter with regulatory factors present in erythroid cells. One of these sites is a palindromic sequence (Pal) that binds a member of the nuclear factor 1 family; the other is the CACCC sequence found in most adult β -globin promoters. Transfection of primary erythrocytes with plasmids carrying the gene coupled to truncated promoters reveals that the Pal site inhibits and the CACCC site stimulates expression. Nuclease protection experiments on intact nuclei show that at early stages of embryonic development, the CACCC site is occupied and the Pal site is vacant, but as development progresses, the Pal site is filled gradually and the CACCC site loses its bound protein. Beyond day 15 of development, Pal is completely occupied and CACCC is empty *in vivo*. Parallel DNase I footprinting and gel retardation studies *in vitro* show that nuclear extracts contain sharply increasing Pal-binding activity as development proceeds, but CACCC-binding activity falls off only slightly. We show that the two factors bind to their sites *in vitro* in an anticooperative manner and conclude that this could account for the observed changes in site occupancy *in vivo*. Our results suggest that the Pal factor may play a role in the shutdown of adult β -globin expression late in erythroid development.

[*Key Words:* β -Globin; erythrocyte; Pal site]

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For many years the family of globin genes has been analyzed as a model system for the study of developmental regulation of eukaryotic gene expression (Bruns and Ingram 1973; Collins and Weissman 1984). Only recently has it become possible to explore how the entire globin family, as well as individual members, are switched on and off during development. We have been asking such questions about the control of the α - and β -globin genes of chicken, exploiting the capacity to isolate and transfect primary erythrocytes at any developmental stage (Hesse et al. 1986; Emerson et al. 1987; Lieber et al. 1987; Knezetic and Felsenfeld 1989). In the course of this work, we identified one DNA sequence motif present near every member of the α - and β -globin families (Kemper et al. 1987; Evans et al. 1988; Knezetic and Felsenfeld 1989), and we have cloned and characterized the corresponding erythroid-specific transcription factor (Evans and Felsenfeld 1989). This protein is present at every stage of erythroid development and could serve as part of a general activation signal for all members of the globin family.

It is not possible for such a factor to modulate expression of individual members of the globin family; there must be other factors that provide additional, stage-specific control. In this paper we examine in detail elements present in the promoter of the β -globin gene and

describe a factor that appears to be involved in its temporal regulation.

The earliest analysis of the β -globin promoter revealed the presence of sequence-specific binding factors in the nuclease-hypersensitive domain extending from nucleotide -40 to -240 (Emerson et al. 1985). Footprints obtained *in vitro* with erythrocyte nuclear extracts, as well as intranuclear footprints (Jackson and Felsenfeld 1985), defined two regions of bound protein at a low level of resolution (Fig. 1). One of these regions consists of a string of 16 G residues; it binds a protein, BGP1, that has been described in detail elsewhere (Lewis et al. 1988). The second footprinted region contains a palindromic sequence (Pal), 25 bp long, ending in the sequence TGAGCC. The last two nucleotides also constitute the beginning of a perfect consensus match (Dierks et al. 1983; Myers et al. 1986) to a 10-bp element, common to most adult β -globin promoters. This 'globin consensus sequence' continues with the sequence CCACCCTG, often referred to as the CACCC box, shown previously (Dierks et al. 1983; Myers et al. 1986; Cowie and Myers 1988) to act as a positive regulator in the promoters of the rabbit and mouse β -globin genes.

We analyze here the function of the Pal sequence and the CACCC sequence in β -globin gene transcriptional regulation. We show that there is a marked change in

Figure 1. Sequence of the adult β -globin promoter region indicating the sequence elements analyzed in this study (see text).

the pattern of protein binding to these sites *in vivo* during erythroid development and that this can be accounted for in terms of changes in the intranuclear concentrations of the proteins and their interactions.

Results

Patterns of binding to DNA within the nucleus

We began by measuring the binding to the endogenous β -globin promoter (Fig. 1) within the chicken erythrocyte nucleus, as a function of developmental stage. First, we mapped DNase I digestion patterns at low resolution to confirm the behavior of the hypersensitive domain. Cells were lysed, and the nuclei digested with appropriate amounts of DNase I (see Methods) directly in the lysate, to minimize perturbation of binding patterns within the nucleus. The resulting DNA was analyzed by Southern blotting after digestion with *Hind*III. The probe used in these experiments lies just 5' of the *Hind*III site at +1000 and will detect hypersensitive regions upstream of this site. For example, an analysis of digested DNA isolated from adult cell nuclei is shown in Figure 2a. As reported previously (McGhee et al. 1981), the region of the promoter, including ~200 bp upstream from the cap site, is hypersensitive to the nuclease in these cells. In contrast, the region is not hypersensitive to DNase I in 5-day embryonic nuclei (see below).

After the DNA was fractionated to enrich for β -globin sequences (Methods), the samples were hybridized to an end-labeled, promoter-specific probe, digested with mung bean nuclease, and electrophoresed on polyacrylamide gels (Jackson and Felsenfeld 1985) to obtain a high resolution map of the promoter's DNase I cleavage pattern within the nucleus.

Comparison of the DNase I digestion patterns of erythrocyte nuclear DNA with the pattern obtained with protein-free genomic DNA reveals the presence of factors bound *in vivo* to promoter region sequences (Fig. 2b).

In nuclei from 5-day embryonic cells (lanes 5), in which the β -globin gene is inactive, the entire upstream domain is ~20-fold more resistant to DNase I than it is in erythrocytes from later stages in which β -globin is expressed. Within this region in 5-day cells, the pattern of digestion is quite similar to that of protein-free DNA, with no relatively sensitive or protected sequences. This is consistent with the presence in 5-day cells (primitive lineage) of a nucleosome covering this region (Emerson et al. 1985; Jackson 1986). The nucleosome is no longer present and the DNA is exposed at later stages of devel-

opment (definitive lineage cells from 10- or 15-day embryos or adults), but there are several regions within the domain that are resistant to DNase I attack. Sites protected against DNase I cleavage at some stage of erythrocyte development are indicated by arrowheads. The diagram at the right side of Figure 2b shows the positions of the binding sites *in vitro* for comparison, as determined by earlier footprinting studies (Emerson et al. 1985; Jackson and Felsenfeld 1985).

It is apparent from the data in Figure 2 that within the nucleus there is very little protection of the Pal site at day 11 of development and almost complete protection in adults; at day 15, there is an intermediate level of protection. The inverse pattern is seen at the CACCC site, which is about one-third occupied at day 11 and is vacant in adults. The BGP1 site is occupied from day 11 through adulthood (see legend to Fig. 2). Densitometry of selected lanes from Figure 2b (see, e.g., Fig. 2c) provides a more quantitative measure of site occupancy, summarized in Table 1.

Patterns of binding to DNA in vitro

The *in vivo* footprinting data show that within definitive erythrocyte nuclei there is a gradual replacement of protein bound to the CACCC site with protein bound to the Pal sequence as erythrocyte development proceeds. To study the binding process further, we compared the above nuclear footprints to DNase I footprints obtained *in vitro* with nuclear extracts (see Methods) and DNA restriction fragments containing the promoter region. The same samples of lysed erythrocytes that were used for genomic footprinting were also used as a source of nuclear extracts for the *in vitro* footprinting. The results obtained with extracts from 5-day embryonic and adult cells and from 11- and 15-day cells are shown in Figures 3 and 4, respectively. The shifting pattern of CACCC and Pal protection resembles that seen within erythrocyte nuclei. The Pal-binding site is increasingly occupied, and the CACCC site less occupied by factors present in nuclear extracts from embryos of increasing age.

Gel mobility assays

Gel retardation assays (Fried and Crothers 1981; Garner and Revzin 1981) were used as a method of measuring the abundance of the Pal- and CACCC-binding activities in nuclear extracts. Oligonucleotide duplexes, mutated so that only Pal or CACCC proteins would bind (S. Clark, unpubl.), were mixed with nuclear extracts from

Figure 2. Analysis of sequences upstream of the chicken β -globin gene bound by proteins in erythrocyte nuclei at various stages of development. (a) Nuclei were digested with DNase I, and genomic DNA was processed as described in Methods. Shown is a representative autoradiograph of a Southern blot of DNA prepared from nuclei of adult erythrocytes, after digestion with *Hind*III. In this indirect end-labeling experiment, increasing amounts of DNase I (left to right) result in the appearance of a group of closely spaced bands (bracket), mapping nuclease cleavage to the promoter region of the gene. (b) Samples from 5-, 11-, or 15-day embryonic nuclei, from adult nuclei (AD), or from DNA deproteinized prior to DNase I digestion (DNA) were analyzed by hybridization to end-labeled probe, followed by mung bean nuclease digestion [see Methods; Jackson and Felsenfeld 1985, 1987]. Because the probe is end-labeled from position -279, it analyzes sequences on the genomic strand complementary to that shown in Fig. 1, in the orientation indicated by the vertical arrow at far right. Lanes M contain marker genomic restriction digests that were combined and analyzed with the experimental samples. Samples from each group were digested with increasing amounts of DNase I, from right to left. Arrowheads alongside the adult samples indicate bands that, when absent, are diagnostic of bound protein at the three promoter elements diagrammed at the right. These elements are the Pal- and CACCC-binding sites, analyzed in detail in the text, and the G-string binding site (G). The latter site has been shown to bind the factor BGP1 (Lewis et al. 1988). The pattern of protection in day-5 nuclei is consistent with the presence of a nucleosome occupying the promoter (Jackson 1986). (c) Densitometer tracings of selected lanes of b. [Solid line] Adult lanes AD in b; [dotted line] day 15, lanes 15 in b; [dashed line] day 11, lanes 11 in b. These tracings have not been corrected for baseline variation or for small differences in amounts of DNA loaded.

Table 1. Stage-specific variation of occupancy of promoter-binding sites within the nucleus

Stage	Occupancy (%)	
	palindrome	CACCC
11-day embryo	7	36
15-day embryo	23	13
Adult	83	5

various developmental stages and electrophoresed as shown in Figure 5. To assess the integrity of the extracts, each was also tested for the presence of the widely distributed transcription factor Sp1 (Kadonaga et al. 1986). This assay ensures that gross proteolysis has not occurred and allows us to control for differences in yield during the extraction of nuclear proteins. The quantitative analysis of the results is shown in Table 2. These experiments differ from the *in vitro* footprinting studies, because the Pal- and the CACCC-binding sites are carried on separate molecules and there can be no interactions between bound proteins (see below). For this

reason, the gel retardation assay provides a more reliable assay for the actual abundance of individual factors.

As determined by the gel mobility assay, the relative abundance of the Pal factor as a function of developmental stage parallels the changes in site occupancy observed *in vivo* (Table 1). On the other hand, although CACCC occupancy *in vivo* falls off during development, the gel-binding studies do not show a correspondingly large decrease in the amount of CACCC-binding activity present.

The results obtained using the CACCC probe are somewhat complicated because two separate binding activities compete for the sequence. Competition experiments (not shown) demonstrate that the faster mobility complex (small arrowhead, Fig. 5) is specific to the CACCC site and the slower mobility complex formed with this probe (large arrow) is the result of interaction with Sp1 (see Lewis et al. 1988); the CACCC site binds Sp1 much more weakly than does a canonical Sp1 site. The interaction of the CACCC site with Sp1 does not affect our measurements of the relative abundance of the principal CACCC-binding activity, because the concentration of DNA is the same in all lanes of Figure 5 and is in vast excess. Under these conditions, the amount of specific complex formed is proportional to the total CACCC-specific protein present. We also note that a roughly constant quantity of Sp1 is present at each developmental stage, as judged by the amounts of Sp1-binding activity detected using either the Sp1 probe or the CACCC probe (Fig. 5, large arrows). We have seen no evidence that proteolysis occurs during the time necessary for our experiments. Also, there is no appreciable redistribution of binding activities between nucleus and cytoplasm: When nuclear extracts and cytoplasmic supernatants from 11- or 16-day embryonic erythrocytes were analyzed for Pal-binding activity, 80% of the activity separated with the nuclei.

Figure 3. *In vitro* footprints of the β -globin promoter region using protein extracts from 5-day embryonic or adult (Ad) chicken erythrocyte nuclei. The probe was 3'-end-labeled at an *Eag*I site at position -4. It was incubated with increasing amounts (right to left) of extract prior to digestion with DNase I. (Lanes 0) Control reactions incubated only with buffer D. Lanes GA and CT contain sequencing reactions; the protected elements are shown at left. Binding to the G-string is observed with both 5-day and adult nuclear extracts.

Interaction between bound Pal and CACCC proteins

The gradual replacement of bound CACCC protein by Pal protein *in vivo* during development, together with the proximity of the two sites, suggests that binding of protein at one site may have a negative effect on binding at the other. We have carried out a series of experiments to determine whether such anticoooperative binding occurs *in vitro*:

1. Direct examination of DNase I footprints *in vitro* as a function of increasing protein concentration shows that it is possible to saturate Pal and CACCC sites simultaneously with large excesses of adult cell extract (Emerson et al. 1985; data not shown). However, footprinting experiments with 11- and 15-day embryonic extracts provide evidence of some competition between the two factors. As shown in Figure 4, 11-day extracts (lane 3, left) protect the Pal region partially and protect the CACCC site completely. On the other hand, 15-day extracts (lane 3, right) protect Pal completely and CACCC only partially. The amounts of extract used

Figure 4. In vitro footprints with protein extracts from 11- or 15-day embryonic erythrocyte nuclei. The probe was as in Fig. 3. Lanes CT, AG, and 0 are as in Fig. 3; samples in lanes 1–3 were incubated with increasing amounts of protein extract. The remaining samples contained the same amount of protein as used for the sample in lane 3 but also included increasing amounts (left to right) of unlabeled duplex oligonucleotide competitor containing either the CACCC- or the Pal-binding site. Arrows indicate bands that demonstrate more complete binding of Pal protein as the CACCC protein is removed by cold competitor.

have about the same amounts of CACCC protein, but the 15-day sample has considerably more Pal protein (see below and Table 2). We conclude that the binding of Pal protein reduces the affinity of CACCC protein for its site (and vice versa) but that the effect is not strong.

2. As shown in Figure 4, either the Pal or CACCC protein can be specifically competed by an appropriate unlabeled oligomer containing only one of the two

sites. Note that when competitor DNA containing the CACCC site is added together with 11-day extracts (right-most lane of the 11-day + CAC series), Pal protein binding increases somewhat. This is particularly evident in the disappearance of the strong bands at the bottom of the Pal region (arrows). A similar (though very weak) response is seen for CACCC binding with 15-day extracts when cold Pal competitor DNA is added. These results

Figure 5. Quantitative analysis of the abundance of Pal- and CACCC-binding activities at various stages of erythroid development. Gel mobility-shift assays were performed using the probes indicated and extracts from 5-, 11-, 15-day embryonic, or from adult (Ad) nuclei. Lanes 0 contain probes incubated only in buffer D; in addition, each probe was incubated in the presence of 1, 2, or 4 μ l of extract from each developmental stage (left to right). The Sp1 probe was used as a control to measure the amount of chicken Sp1, which is expected to be present at all stages in comparable amounts. Sp1 binds to both the Sp1 and the CACCC probes (large arrows), whereas the CACCC factor (arrowheads) binds only weakly to the Sp1 probe. The extent of complex formation was quantified by scanning densitometry and normalized relative to Sp1 abundance to obtain estimates of factor binding abundances given in Table 1.

Table 2. Stage-specific variation of specific promoter-binding activity *in vitro* from gel mobility shift assays

Stage	Relative abundance	
	palindrome	CACCC
5-day embryo	1.0	1.0
11-day embryo	6	1.2
15-day embryo	23	1.0
Adult	170	0.44

are also consistent with weakly anticooperative binding to the Pal and CACCC sites.

Tissue specificity of DNA binding factors

Nuclear extracts from either blood or brain tissue of 10-day chicken embryos were used in the footprinting experiment shown in Figure 6 to determine whether any of the activities that bind to the promoter region are re-

stricted to erythrocytes. The brain extract reveals binding activities to both the G-string and the Pal site but shows no binding to the CACCC. We do not yet know whether the brain factors are identical to those present in erythroid nuclei. In the case of G-string binding, we have previously reported that BGPI activity is absent from a number of other nonerythroid tissues (Lewis et al. 1988).

Binding at the Pal site in the 10-day brain extract is much more abundant than in the extract from 10-day erythrocytes, comparable to activity detected from adult erythrocytes. Additional binding activities are detected only in the brain extract around positions -105 and -75 [and also in extracts from adult erythrocytes (Fig. 3)]; these have not yet been investigated further.

A more quantitative analysis comparing binding activities present in the different tissues was carried out by the gel mobility-shift assay, shown in Figure 7. Control oligomers reveal approximately equal amounts of binding activity in both tissues, either to an Sp1 probe or a nonspecific probe that does not contain sequence from the promoter region (NS). Both extracts also form a similar complex to a probe containing the β -globin promoter CAAT sequence; the binding activity appears to be about twofold more abundant in the case of the erythrocyte extract. In agreement with the footprinting data, binding activity to the G-string is comparable in the two tissues, but that to the CACCC is much more abundant in the erythrocyte extract. Also in agreement, binding to Pal is much more abundant in brain extracts than in the 10-day erythrocyte extract, although the mobility of the complex appears to be different. This altered mobility would suggest that the Pal protein that becomes abundant in later stage erythrocytes is somehow different from that present in 10-day brain (see Discussion). Using a probe for the erythroid-specific factor Eryf1 (Evans and Felsenfeld 1989), we can show that binding activity seen in brain extracts is not the result of significant blood contamination.

Function of the upstream binding sites

To determine what role the Pal- and the CACCC-binding sites play in regulation of globin gene expression, we studied the effect of progressive 5' deletions on transient expression in primary chicken erythrocytes (Hesse et al. 1986; Lieber et al. 1987; Nickol and Felsenfeld 1988). Plasmid constructions (Fig. 8) contained the chicken β -globin gene with an insertion in the 5'-untranslated sequence that allow detection of transcripts in S1 nuclease protection assays. The plasmid pUC18ABC containing the entire upstream promoter sequence, starting at -1050 , served as a reference gene in each transfection. Mutant plasmids carried successive deletions from the 5' side of the promoter.

It is clear from the results in Figure 8 that deletions up to nucleotide -165 [and including the G-string protein binding site (Lewis et al. 1988)] have no effect on transcription of the β -globin gene. However, deletion of the Pal-binding site results in a three- to fourfold increase in

Figure 6. Analysis of β -globin promoter-binding activities in a nonerythroid tissue. Extracts were prepared from 11-day erythrocytes (RBC) or brain tissue (BRAIN) and used in a DNase I footprint as in Fig. 4. The probe was incubated with increasing amounts of protein (*left to right*) for each extract. Note that two additional binding sites near positions -105 and -75 , occupied in footprints with extracts from brain (as well as from adult erythrocytes; see Fig. 3), both contain a T/AGCCA sequence that could represent half-sites for the binding of Pal protein or other factors with NF1-binding specificity.

Figure 7. Quantitative comparison of the abundances of binding activities present in erythroid or nonerythroid tissues. Gel mobility-shift assays were performed as in Fig. 5, using various probes as indicated. Each probe was incubated in buffer alone (lanes 0), or in the presence of 2, 4, or 6 μ l (left to right) of protein extract prepared either from nuclei of 11-day embryonic erythrocytes (E) or from 11-day embryonic brain tissue (B). The Sp1 and NS (nonspecific) probes are controls to indicate the equivalence of extract preparation. Although not obvious here, the mobility of the complexes formed with the Pal probe in the presence of brain extract are distinct from those formed using the erythroid extract.

transcript abundance (lane -142), and a larger deletion that also removes the CACCC site results in a return to slightly greater than baseline transcription levels (lane -122). Further deletions to nucleotide -58 maintain this same level of activity, but transcription is completely abolished by deleting down to nucleotide -39.

These results suggest that the Pal-binding region exerts a negative regulatory effect on transcription, whereas the CACCC site has a positive effect.

Discussion

We have shown here that the binding of certain factors to the promoter of the chicken β -globin gene is modulated during development, indicating that the factors play a role in the developmental regulation of the gene.

It is apparent from the data in Figure 8 that the basal activity of the promoter does not require the Pal or CACCC regions but only sequences downstream of nucleotide -58 (in addition to the erythroid-specific enhancer sequences also required in these constructions). However, the data also show that this basal activity can be modulated by factors interacting with Pal and CACCC. A deletion from the 5' side that removes the Pal sequence results in a fourfold stimulation of transcription; a further deletion, removing the CACCC site, restores a slightly higher than baseline transcriptional activity. This result is consistent with a negative regulatory role for the Pal sequence element and a positive role for the CACCC site. Furthermore, *in vitro* transcription studies carried out with the same deletion mutants reveal quite similar behavior (Emerson et al. 1989). The CACCC sequence has already been shown to serve as a positive element in other β -globin promoters (Grosveld et al. 1982; Dierks et al. 1983; Charney et al. 1985; Myers et al. 1986; Cowie and Myers 1988).

The stimulation of globin transcription by the CACCC site probably reflects the action of the strongly bound factor revealed in gel mobility-shift experiments. Factors (other than Sp1) that bind to the CACCC site

can be found in many kinds of cells. They are present, for example, in non-globin-expressing brain tissue (Fig. 7) but are much less abundant than in globin-expressing erythrocytes from the equivalent developmental stage. The general positive regulatory factor Sp1 also binds to this site and may affect globin gene expression. The CACCC factor is distinguished from Sp1 both by the pattern of complexes it forms in gel mobility shift assays and by its preferential binding to the CACCC site in competition with Sp1 sequence (data not shown). The experiments in Figure 5 show that under the conditions of relative abundance present in nuclear extracts, the complex with the CACCC factor is somewhat more abundant than that formed with Sp1.

Several CACCC-specific binding activities (CACCC-box-binding factors) have been identified previously in human erythroleukemia cells (Gumucio et al. 1988; Mantovani et al. 1988) and chicken erythrocytes (Plumb et al. 1986; Rupp et al. 1988). In addition, a binding activity to a CACCC element has been shown to act cooperatively with a mouse mammary tumor virus glucocorticoid receptor site to mediate transcriptional induction by steroid hormone (Schüle et al. 1988). Some of these activities appear to be ubiquitous, whereas others are more cell-type restricted; therefore, it is unclear what the relation of each is to the CACCC-binding factors being studied.

For example, Xiao et al. (1987) pointed out that the CACCC site is very similar to the GT-I motif of the SV40 enhancer. They showed that both the enhancer element and the CACCC site in the murine major β -globin gene bind apparently related factors from a variety of cells. Similar to our results concerning the chicken β -globin CACCC site, at least one binding activity is Sp1. A cell-type-specific binding activity to the GT-I motif (TEF2) is distinct from Sp1, and the presence of this latter activity correlates well with the positive transcriptional effect of the *cis*-acting element (Nomiya et al. 1987; Xiao et al. 1987; Davidson et al. 1988). We do not yet know whether the erythrocyte

Figure 8. Functional deletion analysis of the chicken β -globin gene promoter. (A) Map of deletions. Deletion constructs contained various lengths of promoter sequence, the entire structural gene including a 35-bp insert (A', denoted as a bipartite box), and sequences extending downstream for $\sim$ 1500 bp (BamHI site). The restriction enzymes used to generate the 5' boundary of each deletion are shown on the map. The number of each deletion refers to the length of its promoter from +1. (B) S1 analysis. The reference gene pUC18ABC spanned the entire globin locus and contained a shorter (17 bp), but overlapping, A insert (dotted box). It was cotransfected with each deletion or wild-type gene, PUC18A'BC, into 9- or 10-day embryonic erythrocytes. Total cellular RNA was isolated and hybridized to a DNA probe that extended from -214 to $+83$ (NcoI) and contained the longer 35-bp (A') insert. This one probe, labeled at its 5' end at $+83$, yielded different S1 digestion products whose lengths depended on the template source of the transcript, as listed. (C) Gel analysis. Products of S1 digestion were electrophoresed on denaturing gels (6% acrylamide/8.3 M urea). Transcription from each deletion mutant and the wild-type gene resulted in a protected fragment of 118 nucleotide (A'), compared to the 54 nucleotide fragment (A) from the reference gene. The autoradiograph was scanned to quantitate the level of transcription driven by each mutant. RTL is defined as (PUC18A'BCA/PUC18ABC)/(PUC18A'BC/PUC18ABC) and is shown for each deletion in A.

factor that is distinct from Sp1 is the same as or related to TEF2.

The palindrome site has considerable sequence similarity to the binding site for human nuclear factor 1 (NF1) (see Fig 1; Rosenfeld and Kelly 1986; Jones et al. 1987). Sippel and his collaborators (Leegwater et al. 1986) have reported that chicken tissues contain at least one factor that binds to a consensus sequence like that of NF1 and have shown that chicken erythrocytes contain a similar factor. It was shown some time ago (Emerson et al. 1985) that human NF1 does not bind tightly to the β -globin Pal sequence. However, we find in the reciprocal experiment that the principal adult erythrocyte factor binds somewhat more tightly to a canonical human NF1 site than it does to Pal. Furthermore, the purified erythrocyte protein contains amino acid sequences common to other members of the NF1 family (C. Lewis, unpubl.). Gel retardation studies with the purified protein strongly suggest that it is a distinct, apparently erythroid-specific binding species, although some evidence to the contrary has been reported (Rupp et al. 1988).

The results reported in this paper show that the Pal binding factor is stage specific. Pal-binding activity is quite low or absent in extracts from 5-day embryonic erythrocytes of the primitive lineage, as judged both from gel retardation and footprint assays (Table 2). Between 6 and 10 times more activity is observed in circulating erythrocytes from 11-day embryos (which are of the definitive lineage), and the activity increases nearly another order of magnitude in the circulating erythrocytes of the adult. Consistent with these observations, the palindrome site within 11-day erythrocyte nuclei is found to be only weakly occupied (in 5-day nuclei of the primitive lineage the site is apparently occupied by a nucleosome). The site is increasingly filled in the nuclei from 15-day cells and from adults. The increasing abundance of palindrome-binding protein measured *in vitro* thus parallels increasing occupancy of the site *in vivo*.

The behavior of the CACCC-binding factor is somewhat different. There is a small decrease in its concentration in nuclear extracts as development progresses (Table 2), but there is a larger decrease in occupancy of the site within the nucleus (Table 1). Although this might reflect a difference between conditions *in vitro* and within the nucleus, the most obvious possible explanation is that the binding of Pal protein interferes with the binding to the CACCC site, which is immediately adjacent to it.

Other negative regulatory mechanisms of this kind, which depend on displacement of a positive factor, have been reported. Barberis et al. (1987) described a factor (CDP) that inhibits tissue-specific expression of sea urchin histone H2B by displacing a protein bound to an overlapping CCAAT sequence. A vertebrate homolog, present in a variety of cell types, binds *in vitro* to the CCAAT region of the human A_{γ} -globin gene (Superti-Furga et al. 1988). Goodbourn and Maniatis (1988) suggested that a similar displacement mechanism may be involved in regulation of the human β -interferon gene.

Binding of Pal and CACCC proteins is clearly not mutually exclusive *in vitro*. Footprinting experiments (Emerson et al. 1985) show that it is possible to saturate both sites simultaneously. On the other hand, the footprints in Figure 4 suggest that the binding of the Pal and CACCC proteins is somewhat anticooperative. Can these *in vitro* data be reconciled with the observation of rather strong apparent mutual exclusion between Pal and CACCC proteins *in vivo*? Our data are consistent with the following model. (1) The CACCC protein binds to its site with an affinity somewhat greater than that of the Pal protein for its site. At moderate Pal and CACCC protein concentrations, the CACCC site is occupied and the Pal site is partly or completely empty. (2) The binding of either protein to its site is reduced but not completely blocked when the other site is occupied. (3) The concentration of Pal protein increases late in development sufficiently to force occupancy of the Pal site and displacement of the CACCC protein.

We have carried out calculations (see Appendix) in which we assigned reasonable values to the concentrations of the various components and to their binding constants, consistent with the data and the assumptions outlined above. The calculated results can be made to reproduce quite well the observed experimental behavior of the system.

Our results appear to establish a negative regulatory role for the Pal protein when it is bound to the Pal site. Deletion of the Pal sequence alone increases globin gene transcription rates. Because deletion of both Pal and CACCC returns the transcription rate to its baseline level, it seems likely that Pal protein functions primarily by reducing binding to the CACCC site. It should be pointed out that our model is also consistent with the footprint results of Emerson et al. (1989), who concluded that the Pal and CACCC factors bind independently (these workers referred to CACCC as CON). Our explanation of the data is different. As shown above and in the Appendix, simultaneous occupancy of both the CACCC and Pal sites can occur at sufficiently high factor concentrations even though CACCC and Pal proteins bind anticooperatively. Our footprint results and those of Emerson et al. (1989) therefore are not inconsistent with the anticooperative model, which our other data support. The further observation of Emerson et al. (1989) that addition of a protein (human CTF) displacing the Pal protein also relieves inhibition suggests, as one possibility, that CTF does not interfere with CACCC protein binding.

The Pal factor is absent from erythrocytes of the early embryo, and it (or, more precisely, its binding activity) is in greatest abundance in circulating erythrocytes of the adult. This, together with our observation that the Pal element has a negative effect on transcription, led us to suggest that the Pal factor functions during erythrocyte development to help shut down expression of the β -globin gene as cells of the definitive lineage reach maturity, though the magnitude of this effect in developing chickens remains to be determined. It is also possible that the Pal factor acts as a repressor in the regulatory

domains of other genes. We note that there is a Pal-binding site in the promoter of the β -hatching globin gene (Plumb et al. 1986; Rupp et al. 1988), which must shut off expression as embryonic development comes to a close. Furthermore, there is a second binding site for an uncharacterized protein immediately adjacent to the Pal site. The hatching gene Pal site is of higher affinity than that of β -globin and should become occupied at lower Pal protein concentrations (hence, earlier in development).

In any case, the binding of the Pal protein to the NF1-binding site in the β -globin promoter appears to be an example of a mechanism that operates on only a subset of the globin gene family. This class of mechanisms is distinctly different from that involving the factor Eryf1, a more universal activator protein that can serve as a positive signal for all members of the globin family (Evans et al. 1988). Given the existence of such a general erythroid-specific factor, it seems reasonable that other factors must exist to modulate expression of the individual genes. The Pal protein appears to be one such factor.

Methods

Protein and genomic DNA sample preparation

Nuclei were prepared and used simultaneously either for digestion of genomic DNA (in vivo footprints) or for preparation of nuclear protein extracts (in vitro footprints and binding assays). This was intended to minimize artifactual differences between protein samples extracted from nuclei and samples analyzed directly for the presence of bound proteins in vivo.

Erythrocytes were obtained by bleeding 11- or 15-day chicken embryos or adult hens. To obtain red blood cells from 5-day embryos, whole embryos were bled in PBS by gentle agitation in a Waring Blendor operated at low speed. After filtering through cheesecloth, erythrocytes were pelleted twice through lymphocyte separation medium (LSM, Organon Teknika). Nuclei were then prepared by hypotonic lysis as described (Evans et al. 1988). One-half of each sample was pelleted for 20 min, after which nuclear proteins were extracted, as described (Evans et al. 1988), and dialyzed into buffer D: 20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μ g/ml leupeptin, and 0.7 μ g/ml pepstatin A (Dignam et al. 1983). While the nuclei were being pelleted, the other half of the sample of lysed cells was divided into aliquots and digested with one-seventh volume of twofold dilutions of DNase I (Pharmacia) in lysis buffer plus 7 mM CaCl_2 . Control samples were incubated without DNase I. After 20 min of digestion at 0°C, the reaction was stopped and DNA was prepared as described (Jackson and Felsenfeld 1987).

The DNase I concentrations required to achieve sufficient cleavage within the β -globin promoter region varied, depending on the tissue being digested. The ranges of nuclease required for the digestions shown in Figure 2 are as follows: 5-day erythrocytes, 175–1400 units of DNase I per milligram of genomic DNA; 11-day erythrocytes, 22–175 U/mg; 15-day erythrocytes, 6–45 U/mg; adult erythrocytes, 6–90 U/mg; protein-free DNA, 0.26–4 U/mg.

Proteins were prepared from 11-day embryonic brain tissue by manual dissection of brains after the embryos had been thoroughly bled. Erythrocytes were further washed from the

tissue by mincing the brains and allowing the fragments to settle several times in fresh PBS. Tissue was broken up by several gentle strokes of a Teflon Dounce homogenizer in PBS, and cell number was estimated prior to extract preparation by lysing a small sample in 0.1% SDS followed by measurement of A_{260} to determine the nucleic acid concentration.

Genomic footprints

To assay the extent of DNase I digestion in the region of the β -globin gene, a portion of each DNA sample was digested with *Hind*III and analyzed by Southern blotting. The blots were probed with a fragment that extends from the *Hind*III site at position +1062 to the *Bgl*II site at position +582. This makes it possible to monitor both the disappearance of the full-length *Hind*III fragment containing the promoter region and the appearance of discrete subfragments caused by digestion in the hypersensitive promoter region (see McGhee et al. 1981). Samples that showed similar amounts of digestion (several from each age group) were then analyzed at high resolution. Because this region is not hypersensitive to DNase I in 5-day nuclei, the loss of the full-length *Hind*III fragment was used as an indicator of the level of digestion in these cells.

Samples in which the DNase I cleavage sites were to be mapped at high resolution were purified further. This purification increases the abundance of globin sequence about sevenfold and consequently increases the sensitivity of the high resolution mapping. Each sample was digested to completion with *Sac*I (which isolates the promoter region on a 1.47-kb *Sac*I fragment) and electrophoresed through a 1.2% agarose gel. DNA smaller than ~3 kb was electrophoresed onto DE81 paper (Whatman) and eluted by incubation in a 3 M NaCl solution. Genomic DNA that had not been digested with DNase I was also processed in this manner and then was used to generate restriction digest size standards. After dialysis to remove the NaCl, samples were concentrated by butanol extraction, precipitated with isopropanol, and resuspended in TE, and the yields quantitated by measurement of A_{260} . Fifteen micrograms of each sample was then digested to completion with *Bst*NI (which cuts just outside of the region to be analyzed) to decrease the size of the genomic DNA and minimize displacement of probe by genomic DNA during subsequent hybridization.

Cleavage sites within each sample were then mapped, as described by Jackson and Felsenfeld (1985, 1987), by hybridization to an end-labeled, single-stranded probe followed by mung bean nuclease digestion. The probe used in these hybridizations was end-labeled and extended on a single-stranded β -globin/M13 recombinant phage DNA template from a 15-nucleotide synthetic oligonucleotide primer starting at position -279 (relative to the β -globin mRNA cap site) and extending to a *Bgl*II site at position +126; it contained ~40 nucleotides of [α - 32 P]dNTP (3000 Ci/mmol) at the 5' end.

In vitro footprinting

Restriction fragments were end-labeled and purified from preparative polyacrylamide gels by standard techniques (Maniatis et al. 1982). Probes (0.5–1.0 ng) were incubated on ice for 10 min with 1.0 μ g poly[d(I-C)] (Boehringer-Mannheim) and various amounts of protein extract in 50 μ l at a final concentration of $0.5 \times$ buffer D containing 10 mM MgCl_2 and 5% PEG 8000. A 50 μ l sample of 10 mM MgCl_2 and 5 mM CaCl_2 was added immediately prior to addition of 1 μ l of diluted DNase I stock. The appropriate DNase I concentration was determined empirically and was usually in the range 0.04–0.3 U/ μ l. After a

1-min digestion at room temperature, the reactions were stopped by addition of 100 μ l of 200 mM NaCl, 20 mM EDTA, 1% SDS, and 250 μ g/ml yeast tRNA. Samples were extracted twice with 1:1 phenol/chloroform-isoamyl alcohol (24:1) and once with chloroform-isoamyl alcohol alone, and ethanol-precipitated. DNA was then resuspended in 80% formamide loading buffer and electrophoresed through 6% urea-polyacrylamide gels, alongside sequencing ladders from undigested material (Maxam and Gilbert 1980).

Mobility-shift assays

Gel mobility-shift assays were performed as described (Evans et al. 1988). For most competition experiments, the unlabeled duplex was added prior to addition of protein.

The sequences of the oligonucleotide probes used in this study are as follows, with the relevant binding site underlined (only the top strand is shown):

```
Sp1: AATTGCAGAGCTGGGAATCCTAACTGGGCGGAGTTATGCTGGTGGTGG
CACCC: GGCGCGGATCACCGACCTTGGCTGAGCCCCACCTGATGA
Pal: GGCGCGGATCTGGGACCTTGGCTGAGCCACCGCTATTGCA
Pal/CACCC: GGCGCGGATCTGGGACCTTGGCTGAGCCCCACCTGATGA
CAAT: GGGGTGCCAAGGCTGGGGCCCTCCGGAGATGCAGCCAATTGCGGGTGGCC
G: AATTGCAGAGCTGGGAATCGCGGGGGGGGGGCAAGGTGGTGGTGGTGG
ERYII: AGCTTCGGTTGCAGATAAACATTGAATTCA
NS: AATTGCAGAGCTGGGAATCCTAACTGGAAGTGAAGTGGTGGTGGTGG
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Construction of deletion mutants

For accurate quantitation of RNA synthesis from the deletion mutants, it is necessary to distinguish RNA transcribed off the transfected DNA from the high background of endogenous globin mRNA. To accomplish this, a 35-bp insert (A') was cloned into the β -globin structural gene at the *Rsa*I site (+46) in the center of the transcribed but untranslated leader sequence. The resulting plasmid, pUC18A'BC, contains the upstream promoter (from the *Eco*RI site at -1050), the structural gene with the 35-bp A' insert, and 1.5 kbp of 3'-flanking sequences extending downstream to the *Bam*HI site, thereby encompassing the globin enhancer. The 5' deletions were generated from the parent pUC18A'BC by digestion at the appropriate restriction sites within the promoter region (Fig. 8A), blunting the overhanging ends with T4 polymerase or Klenow fragment, and ligating *Eco*RI linkers so that adjoining plasmid sequences are identical. The promoter regions of all deletion constructs were confirmed by DNA sequencing. These are the identical plasmids used in the *in vitro* transcription studies of Emerson et al. (1989).

A shorter insert (A), which shares the same 3'-flanking 17 bp with the longer A' insert, was cloned into the wild-type globin gene at the same site as the insert in pUC18A'BC; all other sequences within the two test genes were identical. The resulting construction, pUC18ABC, was used as a reference gene and was cotransfected with each deletion mutant. The overlapping design of reference and test globin genes enabled us to use the same labeled probe in nuclease S1 assays to quantitate both deletion and wild-type promoter activity in the same cell population.

Primary erythrocyte transfections

Definitive erythrocytes isolated from the peripheral circulation of 9- or 10-day chicken embryos were osmotically shocked and transfected as described previously (Hesse et al. 1986), except that the volume for each reaction was increased 10-fold to facilitate subsequent RNA extractions. In a standard reaction, erythrocytes (an original 5-ml volume; 3.5×10^8 to 3.8×10^8 cells/ml; 23–25 A_{412} /ml) were pelleted and osmotically

shocked in 10 ml of 0.25 M NH_4Cl for 40 min at 25°C [a release of 25% of total hemoglobin]. The cells were transfected with 5 ml of a solution containing both deletion and reference gene DNA each at 1.5 μ g/ml. The total DNA concentration (3 μ g/ml) was within the linear range for response of RNA synthesis to input DNA. After transfection, the cells were washed with 10 ml L-15 media without serum and were finally resuspended in 3 ml L-15 media containing 30% chicken serum for 48 hr of incubation at 37°C.

RNA isolation and S1 analysis

After incubation, erythrocytes were pelleted and resuspended in 1 ml 50% guanidinium chloride/imidazole solution, and total cellular RNA was isolated by pelleting through a CsCl cushion as described (Stevens et al. 1987). The RNA pellets were resuspended in 50 mM sodium acetate (pH 5.2), 0.5% sodium dodecyl sulfate, 5 mM EDTA, heated at 65°C for 10 min, extracted with phenol/chloroform, and ethanol-precipitated. For the S1 nuclease protection assays, one-half of each total RNA sample was annealed to a 332-bp DNA fragment (-214 to +83) that contained the 35-bp A' insert at +46 of the β -globin gene. After hybridization at 42°C for 6 hr, the samples were digested with S1 nuclease (250 μ /ml, 37°C, 30 min). Conditions and buffers for hybridization and S1 digestion were as described (Maniatis et al. 1982). The three different products of the nuclease digestion are shown in Figure 8B. RNA synthesized from the deletion mutants spans the full length of the probe from the cap site to +83 (110 nucleotides), whereas transcripts directed by the reference gene are complementary to the labeled A' probe for only 54 nucleotides, and the β -globin endogenous mRNA yields an even shorter 37-nucleotide-long product. Transient expression experiments have been performed several times to confirm the results of Figure 8; the relative transcription (RTL) values in Figure 8a are from the particular experiment shown in Figure 8c.

Appendix

To understand better the protein-DNA interactions described above, we analyzed a simple model system in which palindromic protein (P) and CACCC protein (C) bind to a DNA molecule (D) containing sites for both proteins. To make this calculation, we chose 'reasonable' values for the binding constants and intranuclear concentrations of the components: The specific and nonspecific binding constants and the concentrations of factors we used are in the range found in eukaryotic cells (see, e.g., Emerson et al. 1985). However, the purpose of this calculation is to show that the experimentally observed behavior is qualitatively consistent with our model and that some behavior that might seem paradoxical is to be expected. The exact values we assume or obtain from the calculation are not to be taken literally; many other sets of values we could have chosen are consistent with the general behavior described here.

The reactions and their equilibrium constants to be considered are as follows:

where DP, DC, and DPC are the three kinds of complexes that can form.

We assign reasonable values to the specific binding constants, setting $K_P = 0.9 \times 10^{11}$ and $K_C = 1.5 \times 10^{11}$. (The use

of slightly unequal values for these two constants gives somewhat better results.) The experiments show that there is interference between binding of *P* and *C* to the same molecule of *D*. This is equivalent to saying that $K_{PC} < K_C$ (i.e., the affinity constant for binding *C* to a molecule already carrying *P* is less than the constant for binding *C* to a naked site). It must then also be true that $K_{CP} < K_P$; fixing the first three constants also fixes the fourth. We assume here that $K_{PC} = 0.0005 K_C$, corresponding to moderate interference.

When considering binding within the nucleus, it is also necessary to consider nonspecific binding, which sequesters most of the protein. We assume that the nonspecific constant is 10^4 for both *P* and *C*.

We used these equations to calculate the expected change in Pal site occupancy within the nucleus as *P* concentration changes during development (Fig. 9). For simplicity, we have kept *C* concentration constant; the results would be even more striking if *C* were allowed to decrease somewhat, as it does in vivo. The results show that as *P* concentration rises during development, the Pal site becomes filled and the CACCC site becomes vacant, consistent with the intranuclear footprint data (Fig. 9).

The results of footprinting experiments in vitro are somewhat different. In Figure 10, we introduce the much larger specific DNA concentrations that might be used in such an experiment. Two kinds of results are obtained. If the concentration of one component (in this case, *C*) is held constant and the other (*P*) is increased, a displacement reaction is observed (Fig. 10A), somewhat like that seen in Figure 9, except that at high *P* concentrations the occupancy of CACCC does not drop to zero, but approaches a lower limit. (The exact value of this limit depends on the numerical values of the constants.) This type of behavior is observed experimentally. The second type of behavior is seen if the concentrations of Pal and CACCC protein are kept equal to one another and allowed to increase. In that case (Fig. 10B), both sites becomes saturated, as observed for in vitro footprints obtained at high concentrations of nuclear extract.

Figure 9. Effect of increasing Pal protein concentration on Pal and CACCC site occupancy within the nucleus. The assumed values of the binding constants are given in the Appendix. Intranuclear concentrations of specific and nonspecific binding sites were calculated, assuming a nuclear diameter of 5×10^{-4} cm. The concentration of CACCC protein was fixed at 2.5×10^{-7} M (~ 5000 molecules per nucleus). Pal protein concentration has been allowed to vary in a way consistent with the observed increase in its intranuclear abundance during development [see Discussion].

Figure 10. Effect of varying concentrations of binding proteins on site occupancy under in vitro footprinting conditions. (A) Binding site and CACCC protein concentrations are fixed at 10^{-7} M. Pal protein concentration is allowed to vary. (B) Binding site concentration is the same as in A, but both CACCC and Pal protein concentrations are allowed to vary (Pal- and CACCC-binding protein concentrations are kept equal to one another).

The final calculation concerns the behavior of transfected plasmids. It seemed paradoxical that the deletion of the Pal region (Fig. 8) should have an effect on transcriptional activity in 9-day cells, which do not have detectable amounts of the protein bound at that site within the nucleus. Calculation shows, however, that when the nuclear equilibrium is perturbed by the introduction of large amounts of specific binding site, enough Pal protein is bound to permit an effect on transcription. Thus, we predict that if 4000 copies of the test plasmid are introduced into each cell and if the nucleus also contains that number of Pal and CACCC protein molecules, deletion of the Pal site will result in a 50% increase in the occupancy of the *C* site. This is somewhat lower than the fourfold increase reported in Figure 8; we could come closer to the experimental value by adjusting parameters further, but we present this calculation only to show that all of the observed behavior can be explained by simple assumptions about the reactions that occur.

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