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A developmentally stable chromatin structure in the human β-globin gene cluster

(ε-globin/DNase I-hypersensitive site/hemoglobin switching)

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ABSTRACT **The DNase I-hypersensitive sites in the human embryonic β-globin gene region have been mapped in erythroid-enriched fractions of disaggregated fetal livers, in adult nucleated red blood cells, and in fetal brain tissue. Our analysis of a region extending 11 kilobases (kb) 5′ of the ε-globin gene reveals many minor nuclease-hypersensitive sites and one major site located 6.1 kb upstream of the ε-globin gene. All of these hypersensitive sites are erythroid-specific, and the major site is stable throughout erythroid development. As assayed by nuclear runoff transcription, little or no ε-globin gene expression is detectable in fetal or adult erythroid cells. Thus, the presence of the major hypersensitive site 5′ of the ε-globin gene in both fetal and adult erythroid cells demonstrates that this site is not specifically correlated with transcription of the gene or with a particular stage of development. Rather, this site may reflect an early event in erythroid differentiation. In addition, DNase I has been used to probe the overall sensitivity of ε-globin chromatin in fetal erythroid cells. Our findings indicate that the ε-globin gene as well as the other genes in the β-globin cluster reside within the chromatin domain that is more DNase I-sensitive than "bulk" chromatin.**

The human β-globin gene cluster contains six β-type-globin genes. Five of these are differentially activated during development and one is a nonexpressed pseudogene (for review, see refs. 1 and 2). The fetal ($^{G}\gamma$, $^{A}\gamma$) globins and the adult (δ, β) globins are active within well-characterized periods during development. The fetal globins are the predominant β-type chains from 7 weeks after fertilization until birth and the adult β-type globins, primarily β itself, are the major β chains present in postnatal life. The embryonic β-type globin (ε) gene is expressed only during a short period very early in development and as a result is difficult to study. Maximal ε-globin-gene expression occurs within the primitive erythroid lineage at a time, prior to 42 days, when erythropoiesis takes place in the yolk sac (3–5). In this environment, erythroid stem cells differentiate into embryonic erythroid cells which primarily synthesize the embryonic (Gower I [$\zeta_2\varepsilon_2$] and Gower II [$\alpha_2\varepsilon_2$]) hemoglobins. The switch from embryonic (ε) to fetal (γ) globin synthesis occurs concomitantly with the migration of erythropoiesis to the fetal liver. However, based on globin chain analysis in young (6- to 8-week-old) embryos, it appears that the fetal liver contains a small percentage of the primitive erythroid lineage which continues to express ε-globin (3).

Previous studies have defined changes in the chromatin structure of the β-globin locus associated with the switch from fetal to adult β-globin gene expression. For example, DNase I-hypersensitive sites located 200 base pairs upstream of the transcriptionally active $^{G}\gamma$ and $^{A}\gamma$ genes in fetal liver erythroid cells are lost in adult bone marrow erythroid cells, in which these genes are transcriptionally silent (6). In addition to these types of hypersensitive sites, which are correlated with gene transcription, it has been suggested from the analysis of the avian β^{A}-globin locus that stable chromatin structures, also identifiable as DNase I-hypersensitive sites, may be "footprints" of determinative events that occur during development (7).

The experiments presented here involve the localization of DNase I-hypersensitive sites around the ε-globin gene in fetal erythroid cells, adult nucleated red cells, and in nonerythroid tissue. Our results demonstrate the presence of two classes of erythroid-specific hypersensitive sites: (*i*) a number of minor sites specific to fetal erythroid cells and (*ii*) a major site, located far upstream of the ε-globin gene, that is found in fetal and adult erythroid cells. In addition, the ε-globin gene is in a DNase I-sensitive conformation in fetal erythroid cells. We assayed nuclear runoff transcription to determine if either the minor or major hypersensitive sites or the DNase I-sensitive conformation were correlated with ε transcription. Our results show that the ε-globin gene is transcriptionally silent in the youngest (54-day) fetal erythroid cells examined, as well as in older fetal and adult erythroid cells, suggesting that these alterations in chromatin structure are not specifically associated with ε expression. The developmental stability of the major hypersensitive site, 5′ of the ε gene, indicates that this structure is unaffected by the events that regulate globin-gene switching. Therefore, this site may define a region, 5′ of the entire β-gene cluster, that is specifically activated during the early stages of erythroid development.

MATERIALS AND METHODS

Cell Preparations. A detailed description of the erythroblast preparations will be published separately. Erythroid fractions from fetal livers and adult bone marrow routinely contained >70% nucleated erythroid cells.

Isolation of nuclei, DNase I digestion, and blot-hybridization conditions were as described (6).

Nuclear Runoff Transcription. The *in vitro* transcription assays were performed as described (8).

Globin Probes. Probes were derived from recombinant cosmid clones generously provided by F. Grosveld (National Institute for Medical Research, UK), the 5′ ε-globin [1.3-kilobase (kb) *Bam*HI–*Hin*dIII] probe was provided by P. Powers and O. Smithies (University of Wisconsin), and the 3′ β-globin (1.1-kb *Eco*RI fragment in pBR322) probe was provided by R. Kaufman (Duke University). The restriction maps were derived from the published nucleotide sequence of the ε-globin gene and its 5′ flanking region (9–13).

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Abbreviation: kb, kilobase(s).

RESULTS

DNase I-Hypersensitive Sites in the ε-Globin Region. To investigate the chromatin structure of the ε-globin region, fetal erythrocyte nuclei were isolated from 54- to 120-day-old fetal livers and treated with various amounts of DNase I. The DNA purified from these DNase I-treated nuclei subsequently was digested with restriction enzymes, electrophoresed in agarose gels, blotted, and probed by the indirect end-labeling method (14). This technique allows one to accurately position any double-strand cleavages, introduced by the nuclease, within a particular restriction fragment. Results of a typical experiment with fetal erythroid cells are shown in Fig. 1*A*. The DNA from these DNase I-treated nuclei was digested with *Kpn* I and hybridized with a ^{32}P-labeled 1.3-kb *Bam*HI–*Eco*RI fragment containing the 3′ end of the ε-globin gene. This digest was chosen because it allows one to survey a relatively large section (13.8 kb) of chromatin that includes the ε-globin gene.

We have mapped the upstream sites seen in Fig. 1 more precisely by using restriction enzymes that produce subbands within a size range that can be resolved accurately. This was accomplished by the indirect end-labeling of a 6.3-kb *Eco*RI fragment (Fig. 2*C*). Probes specific for opposite ends of this upstream-ε fragment provided two sets of data by which hypersensitive sites could be mapped. The results of this analysis are shown in Fig. 2 *A* and *B* and are summarized in Fig. 2*C*. The dominant hypersensitive site, which is 6.1 kb 5′ (−6.1 kb) of the ε-globin gene, is flanked by five less hypersensitive sites, which are located 7.5, 6.7, 6, 3.9 and 3.1 kb 5′ of ε. In a similar analysis of adult nucleated erythroid cells, none of these minor hypersensitive sites were observed (data not shown). Subbands that appear in Fig. 2 *A* and *B* but are not indicated in *C* presumably result from multiple DNase I-induced cleavages on the same molecule.

The Nuclease Hypersensitive Site at −6.1 kb Is Developmentally Stable and Tissue-Specific. Adult nucleated red cells, fetal erythroid cells, and fetal brain cells were prepared as described (6) and also by counterflow centrifugation (to be described elsewhere). The results in Fig. 3*A* illustrate the similarities and differences among the three tissue types used. The adult and fetal erythroid cells display the −6.1-kb hypersensitive site of ε, whereas no such site is detectable in brain cells. The same brain DNAs used in this experiment displayed hypersensitive sites when hybridized to an actin-gene probe, confirming the quality of the DNase I digestion in this series (data not shown). Similarly, the −6.1-kb site is not observed in Manca, a lymphoblastoid cell line (15), or in HL-60, a cell line of malignant promyelocytes derived from a patient with acute promyelocytic leukemia (16).

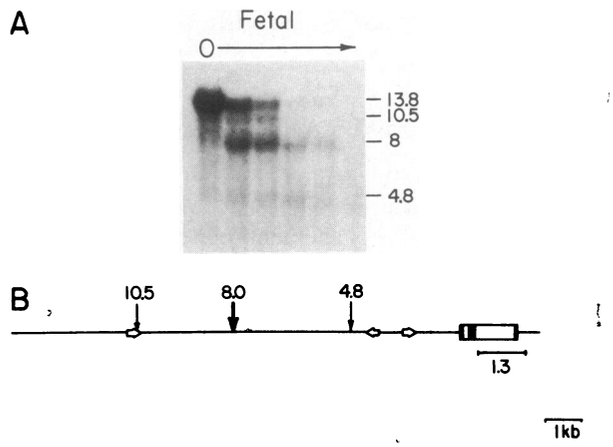

FIG. 1. DNase I-hypersensitive sites adjacent to the ε-globin gene. (*A*) After treatment of fetal erythroid cells from a 90-day fetal liver with increasing amounts of DNase I (indicated by the arrow above the figure), the DNA was purified, digested with *Kpn* I, and blot-hybridized to an ε (intron 2) 1.3-kb *Bam*HI–*Eco*RI probe. The sizes (in kb) of the DNase I-induced sub-bands are indicated at right. (*B*) The diagram represents the 13.8-kb *Kpn* I fragment and the positions of the DNase I-hypersensitive sites shown in *A*. The ε-globin gene is shown as a boxed area on the right. The numbering is relative to the *Kpn* I site located ≈300 base pairs 3′ of the gene.

The fetal erythroid cells as well as the adult nucleated red cells used in this analysis may be contaminated with some nonerythroid cells. Thus, these samples were hybridized as well to an adult β-globin probe whose nuclease-hypersensitivity pattern and erythroid-specificity have been described (6). The yield of the β-globin sub-band should be directly proportional to the number of erythroid cells in these samples. The fetal and adult erythroid cells display the −6.1-kb ε sub-band in a yield identical to that observed for the β sub-band (Fig. 3*B*), indicating that the 5′ ε-globin sub-band most likely arises from the erythroid rather than the nonerythroid population. In addition, similar analyses of purified fetal hepatocytes and nonerythroid adult bone marrow cells (a mixture of immature polymorphonuclear leukocytes, monocytes, and lymphoid cells), the likely contaminants in these preparations, have failed to reveal these sub-bands (data not shown). Thus, we conclude that the −6.1-kb hypersensitive site is erythroid-specific and not correlated with a specific stage of erythroid development.

The β-Globin-Cluster Chromatin Structure in Fetal Erythroid Cells. The overall DNase I sensitivity of the β-globin gene cluster was assayed in erythroid fractions of fetal livers hybridized to a mix of ε-, ^{G}γ-, ^{A}γ-, δ-, and β-globin-specific probes. For comparison with an inactive-gene control, the same blot was rehybridized to an α_2(I) procollagen genomic subclone (6). These experiments afford a comparison of the DNase I sensitivities of the different globin genes as well as an assessment of the relative sensitivities of the globin genes and the procollagen gene. The DNA from DNase I-treated nuclei from erythroid cells of a 130-day-old fetus was digested with *Eco*RI, electrophoresed, and blotted. The bands resulting from this multi-probe hybridization were compared with an internal gene control, and the relative sensitivities were determined. The results (Fig. 4) demonstrate that all of the globin genes are more sensitive to DNase I than is the procollagen gene. Unfortunately, the 5′ ε probe, which hybridizes to the largest (10.5-kb) fragment in the erythroid fraction, contains a DNase I-hypersensitive site, preventing an accurate assessment of this region's DNase I sensitivity. The 3′ β probe as well as the β- and ^{A}γ-globin probes were of low specific activity and as a result the corresponding fragments disappear quicker than other bands due to signal intensity rather than enhanced DNase I sensitivity. Fig. 4*C* shows a direct comparison of the DNase I sensitivities of the ε-globin gene region and a procollagen *Eco*RI fragment. From this comparison, it is clear that the ε-globin gene is in a conformation that renders it more sensitive to DNase I than the procollagen gene.

To determine the contribution of any contaminating hepatocytes to these results, as well as the relative DNase I sensitivity of the β-cluster in a nonerythroid tissue, we prepared the hepatocyte fraction from fetal livers. These DNase I-treated samples were digested with *Eco*RI and probed as described above. When the globin genes are compared with the inactive procollagen control in hepatocytes (Fig. 5) no preferential sensitivity of the β-globin cluster is observed. This is most easily seen by comparing the same ε-globin and procollagen fragments as in Fig. 4. The ε-globin gene is sensitive in the erythroid fraction and insensitive in the hepatocyte fraction when compared to the procollagen gene. The conclusion from this experiment is that in fetal erythroid cells, the entire β-globin cluster is in a conformation that renders it more sensitive to DNase I than bulk

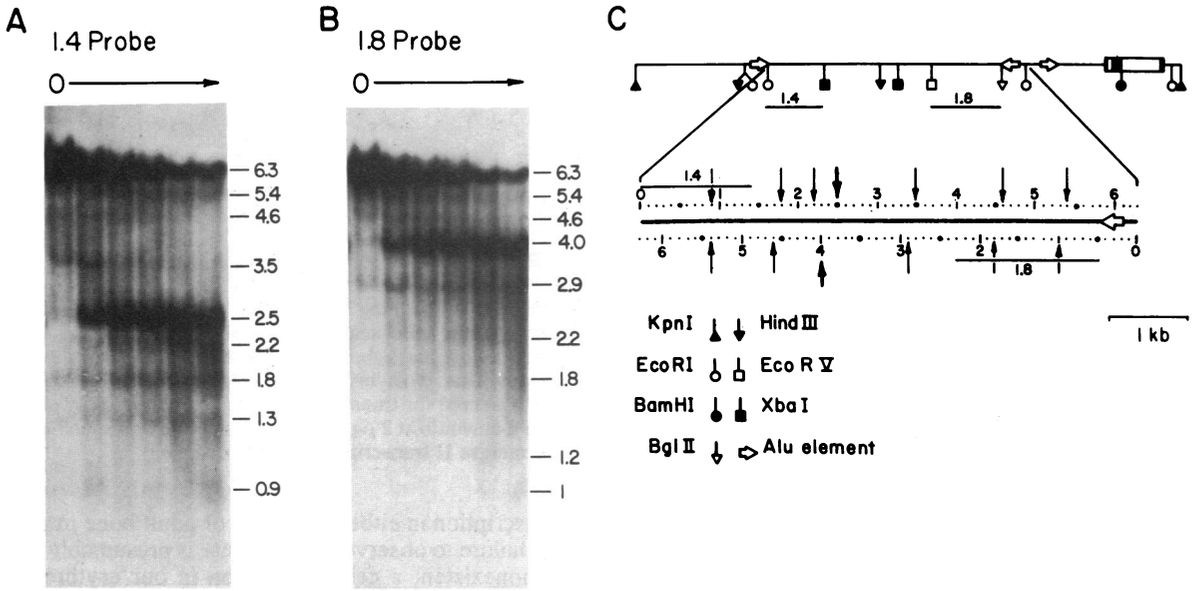

FIG. 2. Mapping the DNase I-hypersensitive sites in the ε-globin upstream region. DNA from DNase I-treated erythroid nuclei from a 90-day fetal liver was digested with *Eco*RI and the fragments were separated in a 1% agarose gel and transferred to nitrocellulose. (*A*) Hybridization to a nick-translated 5′ ε 1.4-kb *Eco*RI–*Xba* I fragment that indirectly end-labels a 6.3-kb *Eco*RI fragment at the 5′ end. (*B*) Hybridization to a 1.8-kb *Eco*RV–*Bgl* II probe from the ε-gene-proximal end of the same 6.3-kb *Eco*RI fragment at different ends, providing two complementary views of the same region. The sizes of the parent (6.3-kb) fragment and of the DNase I-produced sub-bands are given in kb to the right of the autoradiographs. (*C*) A summary of the DNase I-hypersensitive sites in this upstream ε region. The map coordinates correspond to the particular end of the 6.3-kb fragment "end-labeled." The gene-proximal *Eco*RI site of the 6.3-kb fragment is 2.0 kb 5′ of the ε cap site.

chromatin, even though it has been reported that no steady-state ε-globin mRNA or protein is found in erythroid cells from this gestational age (130 days) (3, 18).

Transcriptional Analysis of the ε-Globin Gene. The presence of the −6.1-kb hypersensitive site, the several minor hypersensitive sites, and the preferential sensitivity of the ε-globin gene compared to the procollagen gene in fetal erythroid cells could reflect a chromatin configuration that correlates with the expression of this gene. If this were the case, one would expect to see transcription of the ε-globin gene in fetal erythroid cells. The diminishing amounts of ε protein (2) and stable ε mRNA (18) in fetuses older than 8 weeks might reflect a posttranscriptional type of regulation, such as differential messenger stability. For example, it has been shown that in murine erythroleukemia cells induced to differentiate, a number of mRNAs such as tubulin were destabilized following induction while the endogenous adult globin mRNAs remained stable (19). Therefore, to ascertain the transcriptional state of the ε-globin gene in different erythroid lineages, we performed transcriptional runoff assays on isolated nuclei. Labeled RNAs purified from these nuclei were then hybridized to a panel of cloned DNAs immobilized in nitrocellulose filters. The results (Fig. 6) demonstrate the transcriptional activity of the γ- and β-globin genes in both a 54- and a 120-day-old fetal liver, as well as in adult (erythroid) bone marrow cells. The ε-globin gene appears to be devoid of any actively transcribing polymerase molecules even in our earliest samples. It is noteworthy that in the 54-day fetal liver, γ appears to be the predominant β-type globin gene being transcribed, whereas in the 120-day-old fetal liver, the γ and β genes are equally active, reflecting the transition to the adult erythroid program. In the adult bone marrow nuclei, little or no ε or γ expression was found. In contrast, the strong β-globin signal attests to the completed adult switching process. The minor ε and γ signals observed in the adult bone marrow sample are absent when the pBR control shows no hybridization, and most likely reflect background variation in the assay.

DISCUSSION

These studies were initiated in order to ascertain any structural features of ε-globin chromatin that are correlated with its expression or with events specific to the erythroid lineage. Previous work on the chromatin structure of the fetal and

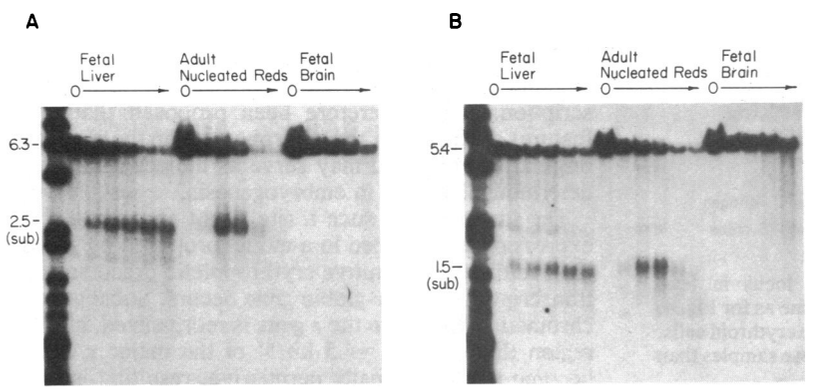

FIG. 3. The major hypersensitive site in the ε-globin region is tissue-specific and developmentally stable. Nuclei from fetal erythroid cells (from liver of a 90-day fetus), adult nucleated red cells, and fetal brain cells were treated with DNase I, and the DNA was digested with *Eco*RI. (*A*) The blot was hybridized with the 5′ ε 1.4-kb *Eco*RI–*Xba* I probe (see Fig. 2 legend). (*B*) The adult β-globin 0.98-kb *Bam*HI–*Eco*RI (intron 2) probe was used. This probe indirectly end-labels a 5.4-kb *Eco*RI fragment and demonstrates the presence of a known hypersensitive site (5). Left-most lane in *A* and *B*: labeled *Hin*dIII- and *Hae* III-digested λ and φX174 DNA, respectively.

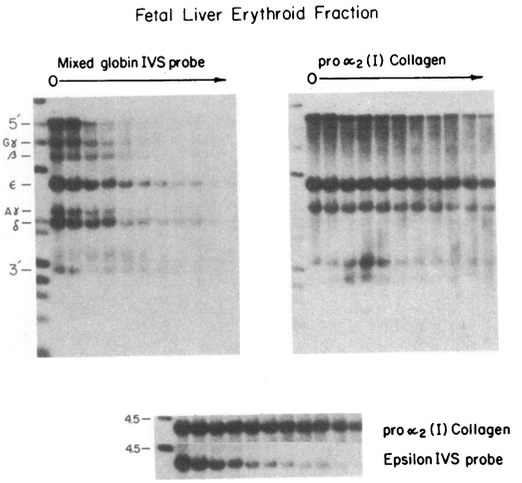

FIG. 4. Chromatin structure of the β-globin locus in fetal erythroid cells. (*A*) DNA from DNase I-treated fetal erythroid nuclei (from a 130-day-old fetus) was restricted with *Eco*RI and blot-hybridized to a combination of previously described β-type globin gene intervening sequence (IVS) probes (17). The 5′ ε probe is a 1.3-kb *Bam*HI–*Hin*dIII fragment located 14.3 kb upstream of the ε gene, and the 3′ β probe is a 1.1-kb *Eco*RI fragment located 17 kb downstream of the β gene. (*B*) These probes were removed and the blot was rehybridized to an α_2(I) procollagen probe. (*C*) Direct comparison of the ε-globin 3.7-kb *Eco*RI fragment and a procollagen fragment of similar size (4.2 kb). The 4.5-kb size marker (from *Hin*dIII-digested phage λ DNA) is indicated at left.

adult globins in humans has identified conformational properties as well as methylation patterns that may reflect controls operating during the switching process (4, 6). Studies in very young embryos have shown that the ε-globin region also contains methylation patterns that correlate with its expression (4). It is presumed that conformational changes accompany ε expression as well, although it has been difficult to obtain sufficient quantities of ε-expressing erythroblasts to perform chromatin analyses. Others have described hypersensitive sites in the −200 region of the ε-globin gene in cell lines that express this gene (20, 21). Our attempts to locate these hypersensitive sites in very young (54-day) fetal erythroid cells have been unsuccessful (data not shown). Since our nuclear runoff-transcription analysis revealed no ε

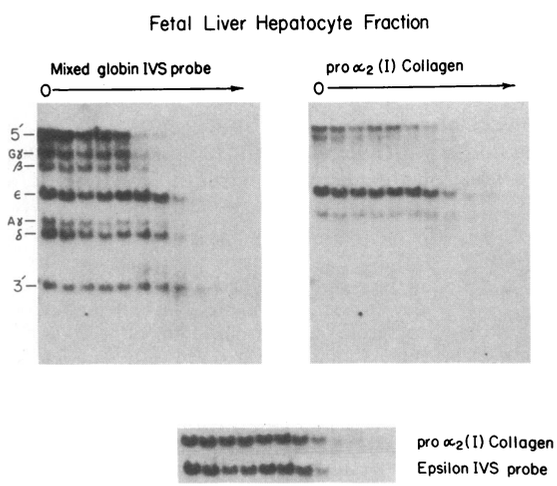

FIG. 5. Chromatin structure of the β-globin locus in fetal hepatocytes. The procedure and probes were the same as for Fig. 4, except that fetal hepatocytes were used instead of the erythroid cells. The DNase I digestion proceeded more rapidly in these samples than in the erythroid fraction shown in Fig. 4.

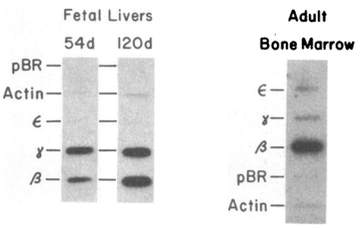

FIG. 6. Globin-gene transcription in fetal (54 and 120 days of gestation) and adult erythroid cells. Plasmid DNA (5 μg) carrying either the intervening sequence of the ε-, $^{G}\gamma$-, or β-globin genes; human actin gene sequence; or pBR322 alone was loaded onto nitrocellulose and hybridized to ^{32}P-labeled nuclear runoff-transcription products. No hybridization, including that to the actin sequence, was observed for transcripts produced when nuclei were incubated with α-amanitin at 2 μg/ml, a concentration that specifically inhibits polymerase II transcription (data not shown).

transcription in either fetal liver or adult bone marrow cells, our failure to observe the −200 site is presumably due to low or nonexistent ε gene expression in our erythroid populations. In the published reports of hypersensitivity in the ε promoter in cell lines, the yield of DNase I-produced subbands is low, perhaps reflecting the number of cells within the total population that are actively expressing ε. Our studies did reveal some minor fetal-specific DNase I-hypersensitive sites (Fig. 2) in the ε-globin gene region, which are absent in adult erythroid cells. These may be part of a chromatin structure that persists after the ε-globin gene has been turned off. It is possible that the cells that have expressed the ε gene are gradually replaced by cells expressing the fetal and adult β-type globins and that these minor hypersensitive sites reflect the diminishing numbers of the primitive erythroid cells. Alternatively, this could reflect the transition from a transcriptionally active region to an inactive one as a result of the gradual switch to predominantly fetal globin-gene expression during differentiation and maturation of erythroid precursors (22).

The −6.1-kb Hypersensitive Site in Development. An important feature of cellular differentiation is that specific determinative events occur in precursor cells whose effects are subsequently passed on to progeny cells. The major hypersensitive site located 6.1 kb upstream of the ε-globin gene is unusual in that it is erythroid-specific and stable throughout development, yet not directly associated with transcription. Perhaps these features distinguish this site, on a functional level, from other DNase I-hypersensitive sites, previously described (6), that correlate with gene activity (for review, see ref. 23). Thus, this −6.1-kb site may be funtionally similar to an erythroid-specific site located upstream of the chicken β^{A}-globin gene, which also represents a stable structure (7). This β^{A} hypersensitive site was induced in chicken embryo fibroblasts, either by salt shock or by infection with a temperature-sensitive Rous sarcoma virus (RSV) at nonrestrictive temperature, and remained present for at least 20 cell doublings after shifting to the RSV-restrictive temperature or returning the cells to normal medium conditions, despite the absence of any globin transcription. It has therefore been proposed that structural features of chromatin can be propogated in the absence of the original stimulus and may serve as molecular analogues of deterministic events in embryogenesis.

The function that such a site might serve in globin gene expression is described in a model proposed by Allan *et al.* (24): Initially, in primitive erythropoietic precursor cells, no transcription of the ε-globin gene occurs. Subsequently, the chromatin adjacent to the ε gene is reorganized, allowing the region that extends ≈4.5 kb 5′ of the major ε cap site to become transcriptionally permissive, resulting in a popula-

tion of ε mRNAs with heterogenous 5′ ends. Regulatory signals, perhaps supplied by newly synthesized *trans*-acting factors in embryonic erythroid cells, would then redirect the upstream initiations down to the major ε cap site. For a detailed discussion, as well as experimental evidence supporting this model, see ref. 25. Although the major −6.1-kb site is further upstream than the −4.5-kb transcriptional boundary described, it may be involved in the initial establishment of a transcriptionally active domain. In support of this, the thalassemia syndromes that involve deletions which remove this 5′ ε region all give rise to the null [$(\gamma\delta\beta)^0$] phenotype (2, 26).

The presence of this site in adult as well as fetal erythroid cells also raises the possibility that an erythroid-specific transcription unit is located near this site. When RNAs from K562 cells, which express the ε-globin gene, and from fetal livers of various ages were hybridized with a probe 5′ of the −6.1-kb site, no steady-state transcript was detected (data not shown). Another possibility is that regulatory events occurring at this site are involved with the repression of ε transcription. However, although we were unable to analyze a population of normal erythroid cells that express ε, this site is present in the erythroleukemia cell line, K562, which does express the ε gene (refs. 20 and 21 and data not shown). It should be noted, however, that this does not exclude the repression model, since ε expression in K562 cells may only occur in a subpopulation of the cells.

Experiments addressing the (overall) conformation of the β-globin locus indicate that all of the globin genes are contained within a DNase I-sensitive domain in erythroid tissue and in an insensitive domain in nonerythroid tissue. Thus, an important regulatory event would be the transition of this domain from an inactive or closed state to one in which the structure was altered so as to allow secondary, gene-specific regulatory events to occur. In this model, the −6.1-kb region may play a role in regulating this transition and therefore be necessary for the expression of any of the β-globin genes. Other gene-specific factors would be required for the preferential expression of different genes in the β-globin gene cluster during development.

Note Added in Proof. While this manuscript was in press, Tuan *et al.* (27) reported the presence of the −6.1-kb ε hypersensitive site in several erythroid cell lines and adult bone marrow.

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