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Human fetal to adult hemoglobin switching: Changes in chromatin structure of the β-globin gene locus

(DNase I-hypersensitive sites)

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ABSTRACT **We have investigated the chromatin structure of the chromosomal DNA regions containing the human $^{G}\gamma$-, $^{A}\gamma$-, δ-, and β-globin structural genes in both fetal and adult erythropoietic tissues and in two human erythroleukemia cells lines before and after induction. Our results indicate that DNase I introduces specific cuts into the β-globin gene cluster in erythroid cells but not in leukocytes. The predominant sites are located at the 5′ sides of the $^{G}\gamma$-, $^{A}\gamma$-, δ-, and β-globin genes, within 200 base pairs of the respective cap sites. Examination of fetal liver cells has revealed the presence of hypersensitive sites at the 5′ side of all four genes, whereas analysis of adult bone marrow has revealed the characteristic sites near the δ- and β-globin genes but no hypersensitive sites at the 5′ termini of the $^{G}\gamma$- or $^{A}\gamma$-globin genes. The presence of δ and β hypersensitive sites in fetal cells suggests that the increment in expression of the δ and β genes during development most likely involves the modulation of another pathway to gene expression. Using isolated nuclei from HEL and K562 cells, we have found that the $^{G}\gamma$, $^{A}\gamma$, δ, and β genes are preferentially sensitive [relative to the pro-α_2(I) collagen gene] to mild digestion with DNase I, whereas these genes are as resistant as collagen genes in cells that do not express globin. These findings are discussed within the context of chromatin structural correlates of hemoglobin switching.**

During the perinatal period of human ontogeny there is a switch from fetal ($^{G}\gamma$ and $^{A}\gamma$) to adult (δ and β) globin synthesis (reviewed in ref. 1). Although the molecular organization of the human globin genes has been characterized extensively (1), the molecular control of this switch remains unknown. Presumably, at least part of this control operates through conformational changes in chromatin of the β-globin genomic region, changes that lead to conformational states that permit transcription of only certain genes of the locus at any given developmental time. Here we examine whether changes at the level of β-globin chromatin are in fact associated with the switch of human fetal hemoglobin (Hb F) to adult hemoglobin (Hb A).

Our initial strategy has been to define the location of DNase I-hypersensitive sites around the $^{G}\gamma$-, $^{A}\gamma$-, δ-, and β-globin genes in cells that express these genes differentially. These regions are detected by the ability of DNase I to introduce double-strand cuts into chromosomal DNA that are manifest as subbands on Southern blots (2, 3). Our results indicate that fetal liver erythroid cells, which synthesize predominately $^{G}\gamma$ and $^{A}\gamma$ chains, but which also contain small amounts of δ chains in all cells, display hypersensitive sites around all four genes. These sites map 75–150 base pairs (bp) upstream from their respective cap sites. In contrast, an erythroid-enriched bone marrow fraction of cells from an adult displays hypersensitive sites only around the δ and β genes; the $^{G}\gamma$ and $^{A}\gamma$ sites are not detectable. Using two human cell lines of hematopoietic origin, K562 and HEL (4, 5) we find that all four genes are located in a domain that is more sensitive to DNase I than is bulk DNA. Our results indicate that Hb F to Hb A switching appears to be associated with changes in chromatin structure that lead to the loss of hypersensitive sites of the two fetal genes. Since no differences in the presence or location of δ and β hypersensitive sites are detectable between fetal and adult cells, other correlates of gene expression must underlie the increase in expression of these genes in adult cells.

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MATERIALS AND METHODS

Adult erythroblasts were isolated by centrifugation of bone marrow cells from a patient with sickle cell anemia who synthesized less than 2% Hb F. Smears prepared from the interphase cells used in our experiments revealed the presence of approximately 50% nucleated erythroid cells. Fetal liver specimens (54–120 days of gestation) were finely minced with scissors and flushed repeatedly through a syringe fitted with an 18 gauge needle to obtain a single-cell suspension. Smears prepared from these cells showed them to consist of approximately 65% nucleated erythroid cells. K562 cells and HEL cells were maintained in suspension cultures as described (4, 5). For globin induction, hemin (50 μM) was added to the cultures at logarithmic phase growth and the cells were harvested 4 days later. Cytocentrifuge smears from a small sample of these cells were used for fluorescent labeling with anti-γ chain monoclonal antibodies to assess the level of hemoglobin induction by hemin.

Isolation of nuclei, DNase I digestions, DNA purification, blot hybridization, and nuclear runoff transcription were as described (6–8). The globin-specific runoff transcription products were analyzed by hybridization of the ^{32}P-labeled nuclear RNA to recombinant DNA molecules containing intervening sequences (IVSs) of $^{G}\gamma$, δ, or β human globin genes dot-blotted to nitrocellulose filters as described (9).

RESULTS

Loss of γ-Region Hypersensitive Sites in Adult Erythroid Cells. Nuclei isolated from fetal liver cells, from an erythroid-enriched cell fraction from adult bone marrow, and from peripheral leukocytes were digested with various concentrations of DNase I. The purified DNA was redigested with several restriction endonucleases and electrophoresed on agarose gels. Fig. 1 shows the results of blot-hybridization of these various samples with a probe containing sequences from the second IVS of the $^{G}\gamma$ gene. *Eco*RI digestion of these DNase I-digested

Abbreviations: bp, base pair(s); kbp (or kb), kilobase pair(s); IVS, intervening sequence.

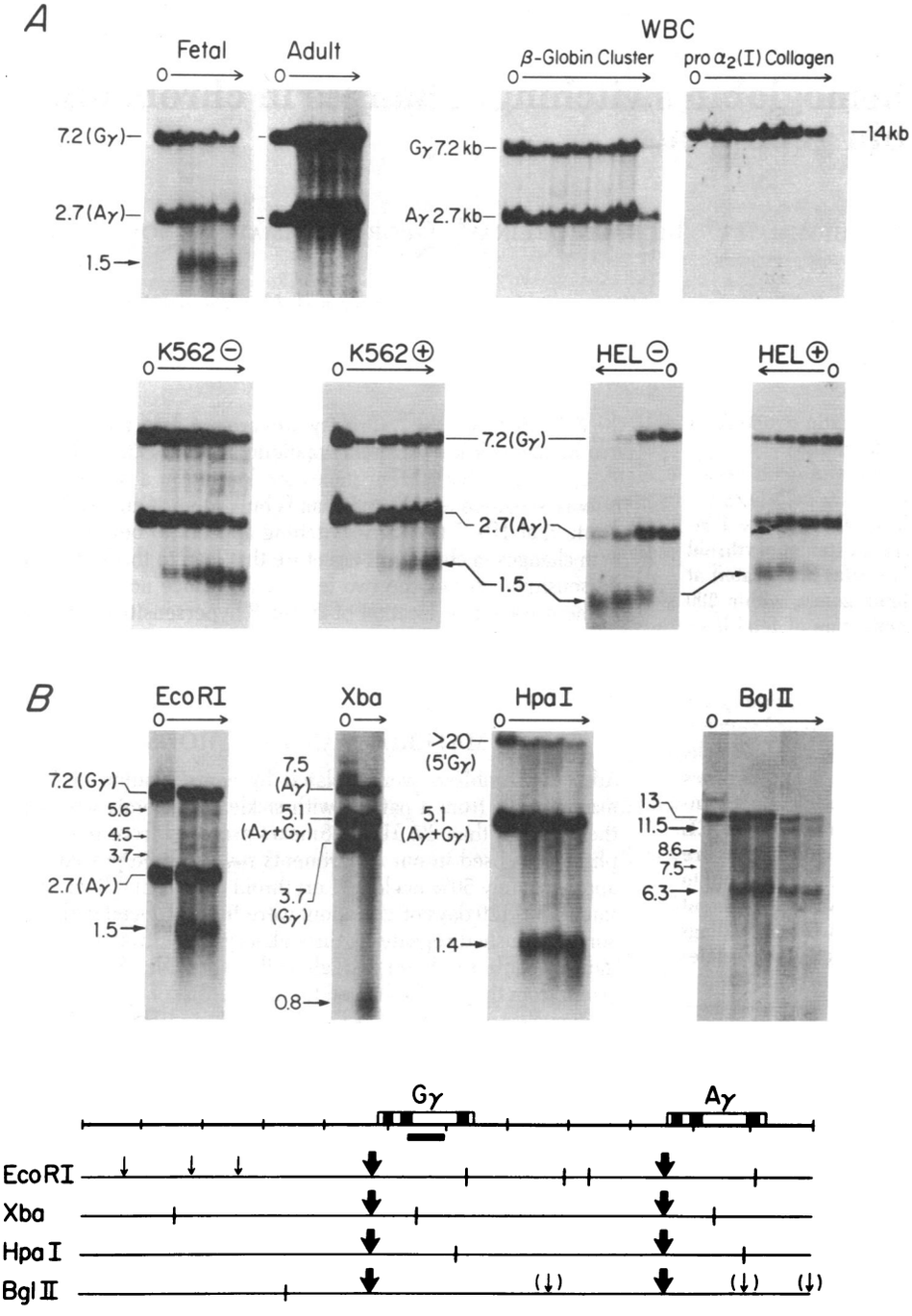

FIG. 1. Presence and location of DNase I-hypersensitive sites in the $^{G}\gamma$ and $^{A}\gamma$ regions of the human β-globin gene cluster. (*A*) After digestion of nuclei with DNase I at increasing concentrations (indicated by direction of the arrow above each blot), the DNA was purified, digested with *Eco*RI, and blot-hybridized to a $^{G}\gamma$ IVS probe consisting of a 457-bp fragment extending from the *Bam*HI site in the second exon to a *Pvu* II site in the second intron of this gene. The DNA sequences of the $^{G}\gamma$ and $^{A}\gamma$ genes are identical in this region (10). The leukocyte (WBC) blot was rehybridized to the pro-α_2(I) collagen probe described in the legend to Fig. 7. kb = kbp. (*B*) DNA samples prepared from DNase I-digested fetal liver nuclei were digested with *Eco*RI, *Xba*, *Hpa* I, and *Bgl* II and blot-hybridized to the $^{G}\gamma$ IVS probe described for A. The probe is derived from a region of the $^{G}\gamma$ gene indicated by the heavy black line on the map at the bottom of the figure (marked at 1-kbp intervals). The bold arrows on both the blots and the restriction map reveal the presence of subbands and the locations of the major hypersensitive sites, whereas the thin arrows indicate the minor sites. The vertical lines on the various restriction endonuclease maps indicate the location of cutting sites of these enzymes in this region of the cluster of β-globin-like genes. The same results are obtained upon analysis of HEL and K562 samples. The ⊕ refers to induction of globin synthesis in these cells.

samples reveals the presence of a 1.5-kbp subband in fetal cells (Fig. 1*A*). In contrast, no such subband is detected in the adult cells, even with overloading of the DNase I-digested samples on the blot. As a control for the generation of subbands in the bone marrow sample, these samples were hybridized to either the δ- or the β-specific probe; such fragments were readily detectable with these probes (see below). These results indicate that the developmental inactivation of γ-globin expression correlates with a specific change at the chromosomal level, namely the loss of a DNase I-hypersensitive site in the γ region of the β-globin cluster.

Locations of γ, δ, and β Gene Hypersensitive Sites in Fetal and Adult Erythroid Cells. The specific locations of the hypersensitive sites around the γ genes were determined by digesting the various DNase I-digested fetal liver samples with several restriction endonucleases and hybridizing the resultant blots to the $^{G}\gamma$ probe. The results of this analysis are shown in Fig. 1*B*. Since *Bgl* II digestion results in the appearance of 11.5- and 6.3-kbp subbands, we conclude that hypersensitive sites are located at the 5′ ends of both the $^{G}\gamma$ and $^{A}\gamma$ genes. In addition, since the sequences of these genes have been determined and the regional restriction map is known, the subbands generated by *Eco*RI, *Xba* I, and *Hpa* I allow us to map fairly accurately the location of these sites. For example, the predominant subband generated by *Xba* I is 800 bp long; by sequence the *Xba* I restriction site is 682 bp 3′ to the cap site. Thus, the hypersensitive site must be located approximately 120 bp upstream from the cap site. (The other predicted subband with *Xba* I is approximately 4.2 kbp, generated from the *Xba* I site within the second IVS of the $^{G}\gamma$ gene and extending to the 5′ hypersensitive site of the $^{A}\gamma$ gene; with these particular gels, this subband is most likely hidden by the 3.7 kbp pa-

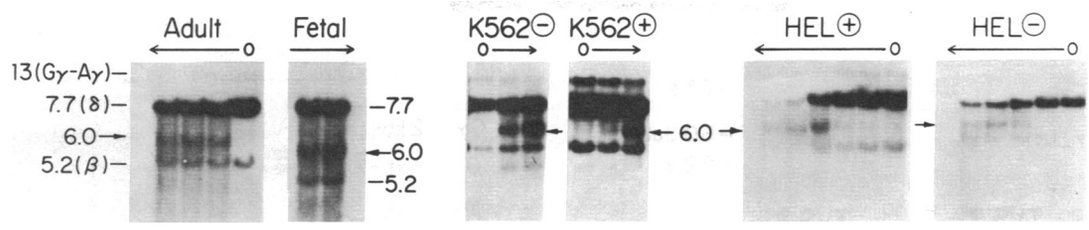

FIG. 2. Hypersensitive sites in the region of δ-globin chromatin of various erythroid cells. DNAs from the same DNase I "series" described in the legend to Fig. 1 were digested with *Bgl* II and blot-hybridized to an IVSδ probe prepared by subcloning the 960-bp fragment that extends from the *Bam*HI site in the second exon to the *Eco*RI site in the third exon (11).

rental $^{G}\gamma$ fragment.) Similar analyses of the subbands generated after *Eco*RI and *Hpa* I digestions reveal that these sites are approximately 50 to 150 bp 5′ to the cap site.

In addition to the major hypersensitive sites at the 5′ end of the γ-globin genes, several minor sites are also observed. These are indicated on the map in Fig. 1*B* with thin arrows; the sites between 2 and 4 kbp 5′ to the $^{G}\gamma$ gene are observed reproducibly, whereas the sites indicated by arrows in parentheses are not always observed. Neither the major nor the minor sites are observed in DNase I-digested nuclei from peripheral leukocytes (Fig. 1*A*).

Figs. 2 and 3 illustrate the results of our analysis of hypersensitive sites in the δ region of the β-globin gene cluster. Whereas *Bgl* II digestion of DNase I-digested nuclei from adult and fetal cells reveals the presence of a 6.0-kbp subband, no such subband is observed in similarly analyzed peripheral leukocytes (not shown). Extensive mapping of this site (Fig. 3) and knowledge of the sequence of the gene indicate that this site is located in a region approximately 75–175 bp 5′ to the cap site. *Bam*HI digestions did not reveal any subbands (not shown); thus, because *Bam*HI defines the 5′ end of the probe, subbands generated with the *Eco*RI, *Pst* I, and *Hpa* I must be located near the 5′ end of the gene, as indicated in Fig. 3.

Our analysis of various DNase I-digested samples with probe specific for the β gene revealed at least three major hypersensitive sites around and within this gene in fetal liver cells (Fig. 4), in adult bone marrow cells, and in HEL cells (data not shown). *Eco*RI digestion results in the appearance of a strong 1.5-kbp subband that maps 100–150 bp 5′ to the cap site. *Bam*HI, which defines the 5′ end of the probe, reveals the presence of two major sites and one minor (and variable) site. The major sites are located approximately 200 bp 5′ and 800 bp 3′ to the poly(A) addition site. The presence of these latter two sites is confirmed by digestion with *Hpa* I, which in addition reveals the presence of a minor site within the second IVS of this gene. *Hpa* I digestion does not reveal the 5′ site due to the large size of the predicted subband; however, a suggestion of the corresponding subband can be observed directly below the parental 9.4-kbp band (Fig. 4). Similarly, our failure to observe the site in the second IVS with *Bam*HI and *Eco*RI could be due to the sizes of the predicted subbands and their obfuscation by various parental bands.

Location of β-Globin Gene Cluster Hypersensitive Sites in Erythroleukemic Cell Lines. The two human erythroleukemia lines, K562 (4) and HEL (5), have normal globin genomic maps and synthesize ε, $^{G}\gamma$, and $^{A}\gamma$ globin chains (as well as α and ζ), the amount of which can be augmented by hemin or other "inducers" (5, 13); however, neither cell line synthesizes β globin chains regardless of inducers. K562 cells contain low levels of δ transcripts after stimulation but no detectable levels of δ transcripts under either condition (14, 15). An analysis of DNase I-hypersensitive sites in the δ-globin gene-containing region of these lines is illustrated in Figs. 1*A* and 2. K562 and HEL cells contain characteristic γ and δ hypersensitive sites both prior to (−) and after (+) induction with hemin, and these sites are identical in location to those described for fetal cells (see Figs. 1*B* and 3). The multiple β-gene hypersensitive sites observed

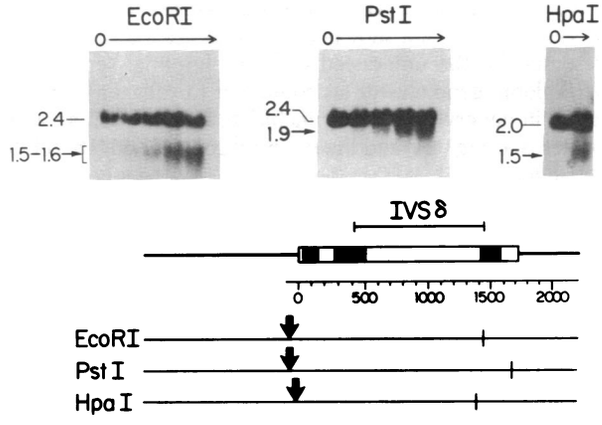

FIG. 3. Location of the hypersensitive site as determined by digestion of the DNase I-generated samples with *Eco*RI, *Pst* I, and *Hpa* I and hybridizing with a δ probe. The location of the IVSδ probe (described in the legend to Fig. 2) is indicated by the line over the δ gene represented on the map.

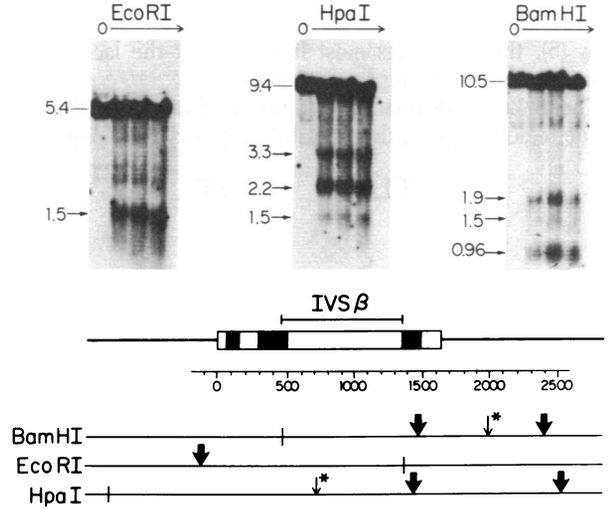

FIG. 4. Mapping of the β-globin hypersensitive sites in fetal liver cells. Samples were digested with *Eco*RI, *Hpa* I, or *Bam*HI and blot-hybridized to the IVSβ probe illustrated by the thin line drawn above this gene and the map. This probe extended from the *Bam*HI site at the end of the second exon to the *Eco*RI site at the start of the third exon and includes 923 bp of the β-globin gene (12). The asterisks near arrows on the map indicate that these sites are not always detected. Similar results were obtained with adult bone marrow cells and HEL cells (data not shown).

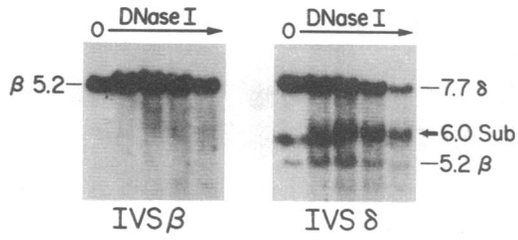

FIG. 5. Absence of β-globin hypersensitive sites from K562 cells. DNA samples from DNase I-digested, hemin-stimulated K562 cells were digested with *Bgl* II and hybridized to the IVSβ probe; the same blot was then rehybridized to the IVSδ probe. No significant hypersensitive sites are observed with the β probe, whereas the δ probe reveals the presence of the characteristic subband indicative of a hypersensitive site at the 5′ end of the δ gene. The signal observed with the δ probe in the "0" sample above the 5.2-kbp β fragment extends beyond the boundaries of the "0" lane and is artifactual.

in fetal and adult cells (Fig. 4) are also present in pre- and post-induction HEL cells (blots not shown). In contrast, no β-related sites are observed in the DNase I digests of K562 nuclei, either before or after (Fig. 5) hemin stimulation. As a control, rehybridization of the same K562 blot with a probe specific for the δ gene reveals the presence of the diagnostic δ subband (Fig. 5).

The presence of δ-globin cluster hypersensitive sites in K562 cells appears to be correlated with the expression of specific genes. In HEL cells, however, there appears to be a dissociation between the presence of the nuclease-sensitive sites around the δ and β genes and δ and β expression. To determine if the presence of 5′ hypersensitive sites around the δ and β genes in HEL cells correlates with overt transcription of these genes, we investigated the steady-state distribution of elongating endogenous polymerase molecules in the β domain by runoff endogenous nuclear transcription. As revealed in Fig. 6, no δ or β transcription products are detectable in these cells. These same results were obtained when Sarkosyl or heparin was included in the reactions (not shown), making it unlikely that these observations are based upon specific differences in γ, δ, and β RNA processing, or due to initiated but blocked polymerases on the δ and β genes (8). Given the sensitivity of the runoff assay (8), these findings most likely reflect the lack of polymerase molecules along these genes.

The β Locus Domain. Definition of domains of the β genomic region requires the availability of very pure populations

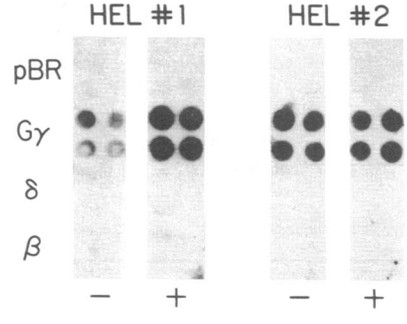

FIG. 6. Absence of RNA polymerases along the β-globin gene in HEL cells. Samples (0.5 μg) of DNA from recombinant clones (described in the preceding figure legends) were immobilized in quadruplicate on nitrocellulose. Nuclear runoff transcription was performed with nuclei isolated from two different HEL cell populations, both before (−) and after (+) stimulation with hemin. In HEL population no. 1, the observed hemin-induced increase in γ chain synthesis (5) is clearly associated with an increase in the transcriptional activity of these genes, whereas, in population no. 2, the hemin-related control of γ-globin synthesis appears to be post-transcriptional. pBR, plasmid pBR322 DNA.

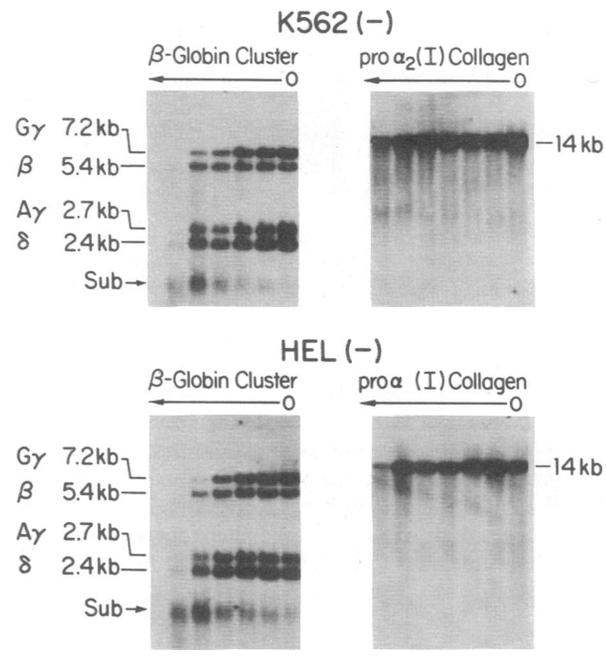

FIG. 7. DNase I sensitivity of the β-globin gene cluster is independent of the transcriptional activity of each of these genes. DNA samples from DNase I digests of nuclei isolated from unstimulated (−) cultures of HEL and K562 cells were digested with *Eco*RI and blot-hybridized to a combination of the $^{G}\gamma$, δ, and β probes previously described. These probes were then removed and the blot was rehybridized to a pro-α_2(I) collagen probe provided by F. Ramirez. In both HEL and K562 cells, all of the β-globin genes disappear at lower levels of DNase I digestion than does the collagen gene.

of normal erythroid cells. Since such highly pure populations of normal erythroid cells are not currently available for analysis, we attempted to gain some insight into the chromatin structure of the β-globin locus by analyzing the nuclease sensitivity of this region in the HEL and K562 cell lines.

Fig. 7 shows *Eco*RI digests of unstimulated HEL and K562 genomic DNA, previously digested in nuclei with DNase I and blot-hybridized to a mixture of $^{G}\gamma$-, δ-, and β-specific probes and then, after removal of the globin probes by melting, rehybridized to a pro-α_2(I) collagen genomic DNA subclone. All of the globin genes in this cluster are more sensitive to DNase I than is the collagen gene, even though the collagen gene is a larger target (14 kbp) than any of the globin genes. When digests from stimulated HEL and K562 cultures are subjected to the same analysis (not shown), the results are identical to those presented for the uninduced cells. Preferential sensitivity of the β-globin gene cluster (as compared to collagen) is not observed in nonerythroid cells, including human peripheral leukocytes (see Fig. 1A) and the nonerythroid HL-60 (16) cell line (not shown).

DISCUSSION

Changes in Chromatin Structure Related to the Normal Hb F to Hb A Switch. In the developmental history of human erythroid cells, adult globin genes are expressed along with the fetal globin genes in the fetal stage. Thus, in essence, the γ to β switch in man involves a turning off of the fetal genes and the increase in expression of the already activated δ and β genes. Our experiments have revealed that the presence of active γ-, δ-, and β-globin genes in human fetal cells is associated with the presence of hypersensitive sites at the 5′ sides of all of these genes, and the turning off of the γ-globin genes in adult cells is accompanied by the disappearance of hypersensitive sites 5′

to the γ-globin genes. Thus, while the disappearance of hypersensitive site 5′ to the γ-globin genes is associated with the transcriptional inactivation of the fetal genes, the change in the level of transcriptional activity of the β-globin genes during human development is not associated with a change in this aspect of chromatin structure. One possible explanation for this observed change in the transcriptional competence of the β-globin gene in these different developmental compartments is the differential methylation of this gene in fetal and adult erythroid cells (17). For example, the differences in δ- and β-globin gene transcription observed in fetal and adult erythroid cells might be based on changes in the kinetics of promoter binding or the rate of RNA elongation by RNA polymerases along these genes, phenomena that may possibly be influenced by the degree of DNA methylation of the gene (18).

In the β-globin gene, hypersensitive sites are also detected in the second IVS as well as 200 bp 5′ and approximately 800 bp 3′ to the poly(A) addition site. At present, the significance of these non-5′ sites is unclear, although their location within or near regions important in RNA processing suggests that they may serve as indicators of regulatory events. Particularly intriguing is the presence of a major site at 800 bp 3′ to the poly(A) addition site of the β gene, given the observation regarding the presence of nascent RNA polymerases throughout a region approximately 1 kbp 3′ to the mouse β (19) and chicken α^D genes (20). One testable hypothesis is that this 3′-most site represents a structure important in the termination of elongation catalyzed by RNA polymerase molecules.

Comparisons Between the Human and Avian Systems. Of particular interest in attempting to correlate chromatin structural changes with the cellular aspects of hemoglobin switching is that in the case of a hypersensitive site 5′ to the chicken β^A-globin gene, such a structure seems to be rather stable, remaining and being propagated to progeny cells in the absence of the original events leading to its initial formation and in the absence of continued transcription of the gene (6). This observation led us to suggest that the appearance of 5′ sites might be the molecular equivalent of deterministic events during development. Within the context of the deterministic model of hypersensitive structures, the absence of $^G\gamma$ and $^A\gamma$ sites from human adult erythroid cells would suggest that if a common progenitor stem cell with the capacity to give rise to stem cells of both fetal and adult lineage exists, it would contain no γ hypersensitive sites. Thus the bifurcation in commitment to the fetal and adult lineages would occur at a very early stage in the hematopoietic lineage. Alternatively, the stability of these sites may not be a general phenomenon, and the bifurcation in commitment might occur during the course of differentiation of the adult progenitors. In this model, the adult progenitors would arise from cells already containing γ hypersensitive sites, and these sites would be lost during subsequent differentiation to the adult lineage. Both of these concepts are under much current investigation at the cellular level.

Chromatin Structure in Erythroleukemic Cell Lines. Our studies of K562 and HEL cells have provided some insights on the chromatin structure of the β-globin domain. In these lines, all four of the β-globin-like genes are in a conformation in chromatin that renders them more sensitive to DNase I digestion than "bulk" DNA. Since no differences in these sensitivities are observed in the stimulated and unstimulated cells, the increased amount of the γ gene products in hemin-induced K562 cells does not appear to be regulated at the level of formation of an active chromosomal "domain" or "loop." The observations that the δ and β genes are as sensitive as the $^G\gamma$ and $^A\gamma$ genes in HEL cells and that β is as sensitive as the $^G\gamma$, $^A\gamma$, and δ genes in K562 cells are of interest, given the reports that neither cell line contains detectable β-globin RNA (14, 15). One possible explanation for these results is that under these particular conditions of digestion, we are failing to distinguish between the high mobility group protein-mediated "very" DNase I-sensitive structure characteristic of transcribed sequences and the "intermediate" DNase I sensitivity associated with nontranscribed regions within an active domain (e.g., see ref. 20).

In K562, the transcribed $^G\gamma$, $^A\gamma$, and δ genes contain 5′ hypersensitive sites, whereas no such structure is found around the nontranscribed β gene. If this cell line bears any relevance to normal human hemoglobin switching, these results could indicate the presence of a stage during erythroid cell development in which δ and β expression may be normally dissociated. Alternatively, a structural lesion (e.g., a mutation) 5′ to the β-globin gene could account for the loss of hypersensitive sites and β-globin gene expression. In HEL cells, the presence of DNase I-hypersensitive sites in the 5′ regions of the nontranscribed δ and β genes might reflect a preactivation state of these genes in cells that may be arrested in a specific developmental compartment.

The discrepancies between normal cells and erythroleukemic lines illustrate both the limits and potential usefulness of the latter. Thus, while the human erythroleukemic cell lines permit the recovery of large numbers of pure populations of cells, their main use in analyzing the molecular correlates of hemoglobin switching may be in providing sufficient material to rigorously dissect various aspects of chromatin structure originally detectable in the small numbers of normal erythroid cells available for analysis (e.g., accurate mapping of hypersensitive site) or to permit the generation of initial observations (e.g., domain concepts), which can subsequently be tested by examination of normal developmentally regulated erythroid cells.

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