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Biochemistry

Hemoglobin switching in sheep: Only the γ gene is in the active conformation in fetal liver but all the β and γ genes are in the active conformation in bone marrow

(pancreatic DNase I/complementary DNA/recombinant plasmids/messenger RNA/erythroid cells)

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ABSTRACT **Differential expression of the closely linked γ, β^A (or β^B), and β^C globin genes in sheep results in the production of fetal hemoglobin (Hb F, $\alpha_2\gamma_2$) during gestation and the adult hemoglobins (Hb A, $\alpha_2\beta^A_2$, and Hb B, $\alpha_2\beta^B_2$) after birth. Erythropoietic stress in certain animals leads to production of Hb C ($\alpha_2\beta^C_2$). The molecular mechanism of differential expression of these genes in nuclei of fetal and adult erythroid cells has been investigated by analysis of their susceptibility to digestion by DNase I (genes that are in the conformation associated with active transcription are sensitive to this nuclease). The concentration of globin gene sequences in DNA from control and DNase I-digested nuclei was determined by annealing to synthetic DNAs and analogous cDNA probes derived from recombinant plasmids containing one of the sheep globin genes. In nuclei from sheep fetal liver erythroid cells, the γ genes but not the β genes were digested by DNase I; the γ locus was open but the β^A or β^C loci was closed, consistent with synthesis of only Hb F by these cells. DNase I digestion of nuclei from bone marrow of anemic sheep making only Hb C or Hb B resulted in equivalent digestion of the β and γ gene sequences, although γ mRNA was not detected in these cells. Digestion by DNase I did not decrease the globin gene sequence concentration in residual DNA of spleen nuclei. As a further control, DNA from digested bone marrow and spleen nuclei were shown to anneal equally well to a cDNA prepared from liver polysomal mRNA. Differential expression of the γ and β globin genes in sheep fetal erythroid cell appears to be based on differences in chromatin structure. The γ globin gene remains in the active conformation in adult erythroid cells; failure of γ mRNA to accumulate in these cells probably reflects transcriptional or post-transcriptional regulation.**

At about the time of birth, many animals replace fetal hemoglobin with adult hemoglobin. Although Hb F synthesis in man may persist into adult life for genetic reasons, it also reappears in the presence of certain hemotologic disorders (1, 2). The possibility of deliberately enhancing Hb F synthesis in adults might offer an opportunity for the alleviation of human diseases such as sickle cell anemia. The sheep offers a convenient model for the study of the regulation of hemoglobin synthesis (1). In the sheep, several closely linked genes encode for globins which combine with α globin to form particular hemoglobin tetramers. In fetal life, the γ gene is expressed, resulting in synthesis of Hb F ($\alpha_2\gamma_2$), whereas, in adult life, sheep that are homozygous for the β^A globin gene make Hb A ($\alpha_2\beta^A_2$). The β^B gene is an allele of the β^A gene; animals homozygous for the β^B gene synthesize only Hb B ($\alpha_2\beta^B_2$). In addition, sheep that produce Hb A have a third gene, β^C, which is expressed briefly during neonatal life and in adults under anemic stress, resulting in synthesis of Hb C($\alpha_2\beta^C_2$).

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There appears to be a good correlation between the quantities of the individual globin mRNAs present in cytoplasm and the pattern of globin synthesis (3–5), suggesting that the differential expression of the globin genes is regulated in the nucleus. The globin genes in erythroid cells are a part of the small fraction of the total nuclear DNA available to act as a template for RNA transcription. The active fraction of chromatin is often referred to as euchromatin, in contrast to condensed and inactive heterochromatin. Both the active and inactive portions of chromatin share a basic structural organization into ν bodies composed of 180–240 nucleotide base pairs complexed to histone octamers (6–12). Nonetheless, structural differences almost certainly exist between active and inactive chromatin and are reflected by the accessibility of particular genes to transcription by RNA polymerase. Transcriptionally active genes in nuclei appear also to be more susceptible to digestion by pancreatic DNase (DNase I) (11–12). This enzyme has served as a useful probe of gene structure.

In the present study, the conformation of the various genes in nuclei from erythropoietic cells of sheep have been examined by using DNase I as a probe. In sheep fetal erythroid cells, only the γ gene is in the active conformation, whereas in nuclei from adult sheep bone marrow the γ and β globin genes are sensitive to DNase I.

METHODS

Preparation of nuclei, DNase I digestion, and recovery of DNA

Cells obtained from the hematopoietic liver of middle-trimester fetuses and from the bone marrow and spleen of young lambs made anemic by phlebotomy were washed twice in phosphate-buffered saline (GIBCO). More than 85% of the bone marrow cells of the anemic animals were erythroblasts. All subsequent manipulations were performed at 4°C. Nuclei were obtained by lysing the cells with gentle homogenization in 0.1 M sucrose/3 mM $MgCl_2$/3 mM $CaCl_2$/10 mM Tris·HCl, pH 8/0.1% Triton X-100 (Research Products International)/0.5 mM dithiothreitol. The nuclei were centrifuged (2200 rpm, 10 min) through a cushion of a similar solution but 0.3 M in sucrose and lacking Triton X-100. The nuclear pellet was washed twice with gentle homogenization in 10 mM Tris·HCl, pH 8/10 mM NaCl/3 mM $MgCl_2$ and suspended in the same buffer at a DNA concentration of 1 mg/ml. DNase I was added to a concentration of 10 μg/ml and the mixture was incubated for 30–90 sec, releasing 5–25% of the total nucleic acids as determined by solubility in cold 7% perchloric acid. The suspension was cooled

Abbreviation: Hb F, fetal hemoglobin ($\alpha_2\gamma_2$).

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to 4°C and centrifuged; the nuclei were resuspended in 5 mM EDTA/10 mM NaCl/containing proteinase K (Boehringer) at 100 μg/ml and incubated at 37°C for 2 hr. NaCl was added to a final concentration of 0.5 M and the incubation was continued overnight. Sodium dodecyl sulfate was then added to a concentration of 0.5% and the incubation was continued for an additional 2 hr. Further steps in purification of DNA included phenol/chloroform extraction, ethanol precipitation, and desalting through Sephedex G-25 as described (11, 13). The yield of DNA was between 50 and 80% and the fragment size ranged from 150 to 250 nucleotides as assessed by centrifugation on an alkaline sucrose gradient. Control DNA, recovered from nuclei that were not exposed to DNase I, was sonicated before hybridization.

Preparation of specific cDNAs

Synthetic cDNA. Probes specific for the β^A, β^C, or γ sequences were prepared by using mRNA from reticulocytes of fetal and neonatal animals that contained sequences specific for either Hb F, Hb A, Hb B, or Hb C (3). Polysomes were also prepared from adult liver, and the poly(A)-containing mRNA species were recovered on oligo(dT)-cellulose. Each mRNA was used as a template for RNA-directed DNA polymerase, yielding, in the case of the globin cDNAs, mixtures of an α and non-α species. [^{32}P]dCTP (specific activity, 200–250 Ci/mmol; New England Nuclear or Amersham/Searle) was used to label the cDNAs, yielding probes with specific activities of approximately 300,000–365,000 cpm/ng. To obtain pure γ or β cDNA, α cDNA sequences were removed by annealing the mixed cDNA to a nonhomologous mRNA—e.g., Hb B cDNA ($\alpha + \beta^B$ cDNA) to Hb F mRNA ($\alpha + \gamma$ mRNA). Selected thermal denaturation of the partially mismatched β^B cDNA·γ mRNA duplexes allowed removal of the α cDNA·α mRNA duplexes by chromatography on hydroxyapatite (3).

Plasmid-Derived cDNA. Double-stranded synthetic DNA containing the sequences of the β^B, β^C, or γ genes were prepared and inserted into the *Eco*RI site of pMB-9 by the A·T tailing method (14, 15) and cloned into *Escherichia coli* χ 1776 under P-3 containment conditions (as specified by the National Institutes of Health Guidelines for Recombinant DNA Research) (details to be published elsewhere). The recombinant plasmids used for preparation of hybridization probes contained 550–600 base pair inserts of one of the three genes. Plasmid DNA (100–150 μg) was digested with *Hha* I and the resulting DNA fragments were fractionated on a sucrose gradient (5–20%) in 100 mM NaCl/10 mM Tris·HCl, pH 7.5/1 mM EDTA. The largest *Hha* I fragment which contains the globin gene insert was recovered by ethanol precipitation. Nick-translation (16, 17) in the presence of [^{32}P]dCTP resulted in a specific activity of approximately 10^8 cpm/μg. Each plasmid fragment was annealed in 50% formamide/0.5 M NaCl (3) to homologous mRNA [e.g., the fragment containing the β^B globin gene was annealed to Hb B mRNA (α and β^B)] at 66.5°C. At this temperature, DNA will not reanneal but a duplex between mRNA and the "cDNA" strand of the insert is formed. Digestion with nuclease S1 resulted in destruction of all DNA except that in duplex with mRNA. The single-stranded cDNA fragment was then recovered by alkaline sucrose gradient centrifugation.

Extraction and analysis of total cellular RNA

Total cellular RNA was extracted by a modification of the method of Strohman *et al.* (18) except that the cells were lysed directly in 6 M guanidine HCl and the RNA was recovered by precipitation with 0.5 vol of ethanol before it was purified by cesium chloride centrifugation according to the procedure of Glisin *et al.* (19). Annealing to cDNA under stringent hybridization conditions was as described (3, 4).

DNA·cDNA hybridization

Both synthetic and plasmid-derived cDNA were used in measuring the globin gene sequence concentration in control DNA and DNA from nuclei exposed to DNase I. In a total reaction mixture of 150 μl, 1.5 or 2.0 mg of DNA was mixed with approximately 10 pg of cDNA; individual capillary tubes containing 20 μl of this mixture were incubated at 58.5°C for times ranging from 10 min to 48 hr. Use of this temperature, only 3–4° below the melting temperature of homologous globin DNA duplexes, prevents heterologous crossreaction between globin gene sequences. Reaction conditions and analysis of duplex formation by nuclease S1 resistance were as described (20).

RESULTS

Conformation of the individual globin genes

In Fetal Liver. In sheep during the middle trimester of fetal development, the liver is the major site of erythropoiesis, and Hb F ($\alpha_2\gamma_2$) accounts for more than 95% of total hemoglobin synthesis (21). Animals homozygous for the β^A globin gene were selected for study; their genome contains the β^A, β^C, and γ globin genes. As shown in Fig. 1, there was no difference in the rate of hybridization of synthetic β^C cDNA to control DNA or to DNA from nuclei from which 15% of the DNA had been released by DNase I. However, there was a significant reduction in the concentration of γ globin gene sequences in DNA from the digested nuclei compared to the control. On analysis of DNA from fetal erythroid nuclei exposed to DNase I with the totally pure plasmid-derived γ, β^B, or β^C cDNAs, the concentration of γ globin gene sequences was markedly less than the concentration of β^C or β^A gene sequences (Fig. 2). (Because of the close homology between the β^A and β^B globin genes, the β^B probe was used to measure the concentration of β^A gene sequences). The rate of annealing of the plasmid-derived β^C and β^B cDNAs to control DNA was identical to the results shown in Fig. 2 (data not shown), whereas the concentration of γ globin gene sequences in control DNA was 5-fold greater than in DNA from nuclei exposed to DNase I. In both experiments, the γ globin gene was sensitive to DNase I and thus in the open or active conformation, consistent with synthesis of γ mRNA and production of γ globin by fetal erythroid cells, whereas the

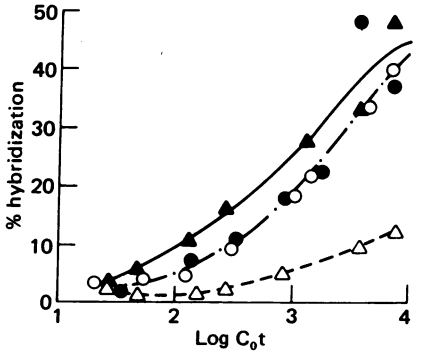

FIG. 1. Annealing of synthetic γ (▲, △) or β^C (●, ○) cDNAs to DNA extracted from erythroid cell nuclei of a fetus that was homozygous for the β^A globin gene. The genome of this animal contained genes for γ, β^A, and β^C globins. DNA was prepared from control nuclei (▲, ●) and from nuclei that had been digested (△, ○) with DNase I leading to release of 15% of the total nucleic acids. The slow rate of reaction and failure of the probes to be completely protected from nuclease S1 by a vast excess of genomic globin DNA sequences are thought to be related to the high temperature at which the annealing reaction was conducted (58.5°C) and the small size of the DNA fragments.

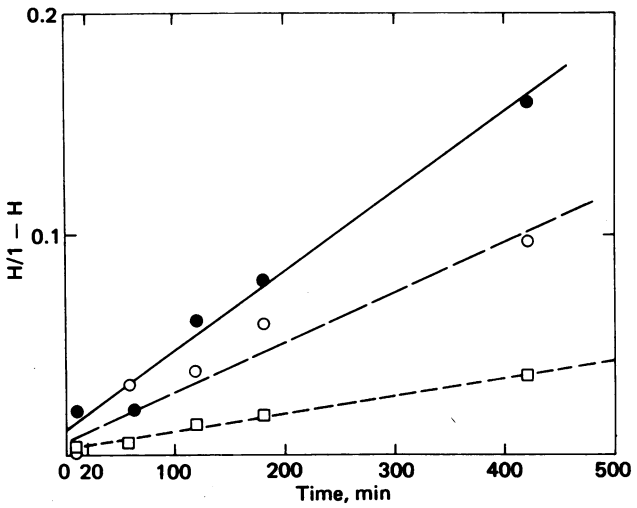

FIG. 2. Annealing of DNA from fetal erythroid nuclei from which 15–20% of the DNA had been released by DNase I to plasmid-derived cDNA specific for β^C (●), β^B (○), or γ (□) globin gene sequences. The genome of this animal contains the genes for β^C, β^A, and γ globins; the β^B probe crossreacts nearly completely with β^A gene sequences. H is the fraction of the cDNA counts resistant to nuclease S1 digestion. For the initial portion of the annealing reaction, H/1 − H is linearly related to the time of annealing and directly proportional to the reactive sequences in the DNA sample (12, 20).

β globin genes were resistant to DNase I and therefore in an inactive conformation in chromatin.

In Bone Marrow. Anemic stress in animals homozygous for the β^A gene results in a switch from Hb A ($\alpha_2\beta_2^A$) to Hb C ($\alpha_2\beta_2^C$) production. Nuclei obtained from the bone marrow and spleen of anemic young animals were digested with DNase I; the residual DNA was recovered and annealed to synthetic β^A, β^C, and γ cDNA probes (Fig. 3). The spleen is not an erythropoietic organ in the sheep, and DNase I digestion of nuclei from spleen does not affect the concentration of the globin gene sequences in comparison to DNA from control spleen or bone marrow nuclei (data not shown). Therefore, DNA from digested spleen nuclei was used as a control. The concentrations of γ, β^A, and β^C globin gene sequences were all markedly decreased by DNase I-mediated release of 20% of DNA from bone marrow nuclei.

As a further control, a cDNA probe complementary to the poly(A)-containing polysomal mRNA of adult sheep liver was prepared. The quantitatively preponderant mRNA species would be expected to encode for the synthesis of albumin, globulin, and other proteins synthesized by the liver. The genes corresponding to most of the polysomal liver mRNA molecules should be in the inactive conformation in both spleen and bone marrow nuclei and therefore resistant to DNase I. Results of annealing this cDNA to DNA extracted from nuclei of bone marrow and spleen that had been exposed to DNase I as well as to DNA from control bone marrow nuclei are shown in Fig. 4. The rate of annealing of all three DNA samples was identical; thus, DNase I did not alter the concentration of sequences of genes that are not expressed.

Similar experiments were performed in sheep that were homozygous for the β^B globin gene; these animals lack the β^C gene (22) and therefore anemia does not produce a switch in hemoglobin phenotype. Nuclei obtained from the bone marrow and spleen of such an animal were digested with DNase I and annealed to plasmid-derived β^B and γ cDNAs (Fig. 5). The β^B and γ sequences were equally and markedly decreased after DNase I digestion in comparison to the globin gene sequences in DNA from similarly treated spleen nuclei. Thus, in anemic sheep all of the globin genes are equally susceptible to DNase I, suggesting that they are all in an open or active conformation.

Globin mRNA sequences in fetal and adult erythroid cells

Total cellular RNA was recovered from liver erythroid cells of a fetus homozygous for the β^A globin gene and from bone marrow cells of an anemic lamb homozygous for the β^B globin gene. In the fetal erythroid cells the preponderant mRNA was γ although there was a small quantity of β^C mRNA sequences present (Fig. 6). Possibly a few fetal erythroid cells were synthesizing β^C mRNA; a difference in the structure of the chromatin in 10% or less of the total cells would not be detected in the DNase I experiments. β^A mRNA is also absent or is found in very low levels in the fetal erythroid cells (3, 4, 21). In bone marrow cells, only β^B mRNA was present and no γ mRNA sequences were detected (Fig. 6), despite the fact that the γ globin gene is in the active conformation in nuclear chromatin (Fig. 5).

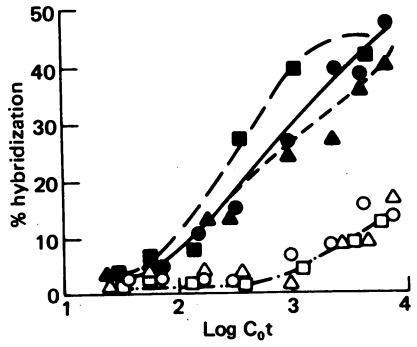

FIG. 3. Analysis of DNA extracted from bone marrow and spleen nuclei of a severely anemic lamb; the nuclei has been digested with DNase I to release 20% of the total nucleic acids. The lamb was homozygous for the β^A globin gene and therefore its genome included the β^C globin gene as well as β^A and γ globin genes. Only the β^C globin gene is expressed during acute and severe anemia (1, 3–5). DNA was annealed to synthetic β^A, β^C, or γ cDNA. Spleen DNA: ▲, γ cDNA; ■, β^A cDNA; ●, β^C cDNA. Bone marrow DNA: △, γ cDNA; □, β^A cDNA; ○, β^C cDNA.

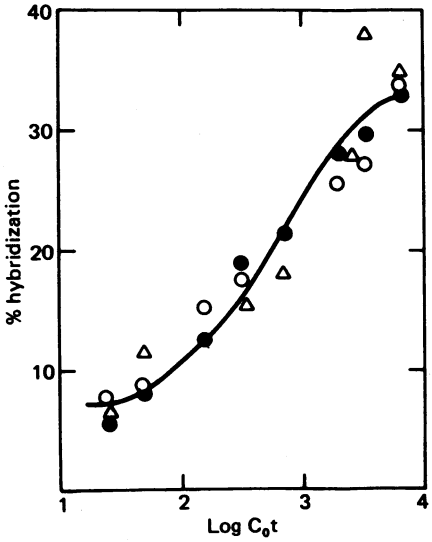

FIG. 4. Annealing of synthetic cDNA prepared from polysomal mRNA of adult sheep liver to control DNA (△) or to DNA from spleen (●) or bone marrow (○) nuclei that had been exposed to DNase I. The nuclease-digested DNAs were the same samples used to obtain the data displayed in Fig. 3.

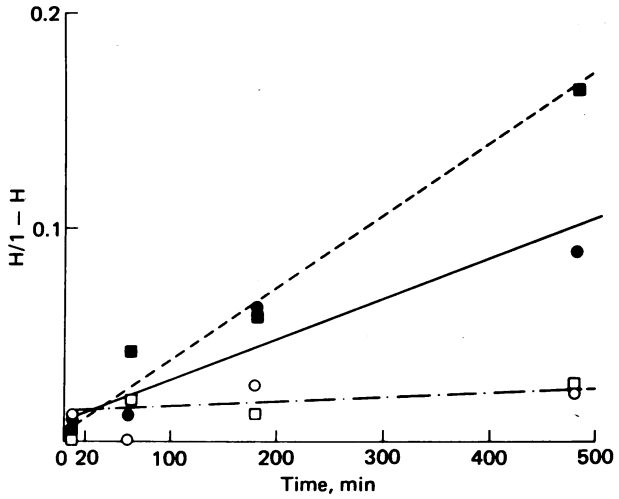

FIG. 5. Annealing of β^B (●, ○) or γ (■, □) plasmid-derived cDNA to DNA extracted from spleen (●, ■) or bone marrow (○, □) nuclei from which 15–20% of the DNA has been released by DNase I. This animal had been made anemic by repeated phlebotomy; it was homozygous for the β^B globin gene and therefore continued to make Hb B during anemia.

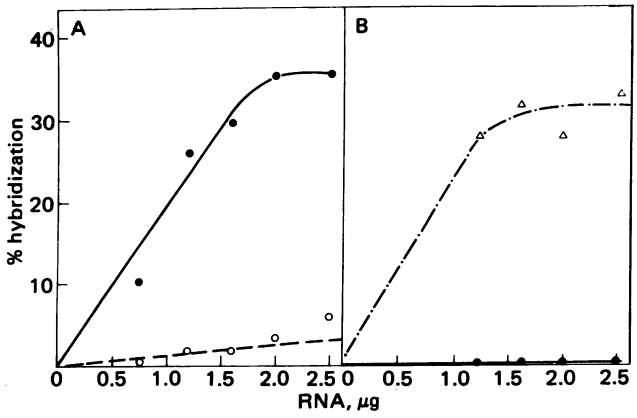

FIG. 6. Annealing of plasmid-derived cDNA to total cellular RNA. Incubation was at 68.5°C for 48 hr, and each 10-μl reaction contained 15 pg of cDNA as well as the specified amount of RNA. The high temperature at which hybridization was performed accounts for the fact that the cDNA was not completely protected at high RNA inputs because incubation at 50°C results in incorporation of 90–100% of the probe into nuclease S1-resistant duplex (data not shown). (*A*) RNA extracted from fetal erythroid cells of midgestation lamb homozygous for the β^A globin gene. ●, γ cDNA; ○, β^C cDNA. (*B*) RNA extracted from bone marrow erythroid cells of an anemic lamb that was homozygous from the β^B globin gene. △, β^B cDNA; ●, γ cDNA.

DISCUSSION

Our results show that in nuclei from fetal erythroid cells the γ globin genes but not the β globin genes are susceptible to DNase I, but in nuclei from bone marrow cells of anemic lambs the β and γ globin genes are equally sensitive to the nuclease. The specificity of DNase I for genes whose conformation in chromatin renders them susceptible to active transcription has been demonstrated in various tissues (11–13, 23, 24). In our experiments, the DNase I reaction also appears to be specific for several reasons. First, exposure of erythroid nuclei to DNase I leading to release of only 15–20% of the total nuclei acid results in a decrease in the concentration of globin gene sequences to 20% or less. Second, the globin genes are susceptible to DNase I only in nuclei from erythropoietic tissues such as fetal liver and neonatal erythroid bone marrow and not in nuclei from adult spleen or liver. Third, DNase I does not result in a significant decrease in gene sequences that are unexpressed in erythroid tissue as revealed by annealing DNA from digested bone marrow nuclei to a cDNA probe prepared from the polysomal mRNA of adult liver.

What is the significance of DNase I sensitivity? Billing and Bonner (25) first demonstrated that mild digestion of chromatin with DNase I resulted in specific degradation of DNA that was associated with radioactive nascent RNA molecules. Weintraub and Groudine (11) demonstrated that DNase I specifically digested the adult globin genes in nuclei from chick erythrocytes, leaving the embryonic globin genes intact. The globin genes in nuclei from fibroblasts and brain cells were not susceptible to DNase I. Similarly, DNase I digested the ovalbumin gene in nuclei from chicken oviduct but not in nuclei from chicken liver (12). The integrated viral DNA sequences in Swiss mouse cells infected with Moloney murine leukemia virus were more sensitive to DNase I in nuclei from cells actively producing virus than in nuclei from cells not shedding viral particles (24). In hamster cells, only that portion of the integrated adenovirus genome that was represented by cytoplasmic mRNA sequences was susceptible to DNase I; nonexpressed portions of the viral genome were not sensitive (23). The unique structural features of transcriptionally active chromatin that render the included DNA sequences susceptible to DNase I are not known. DNase I releases a single protein from trout testes nuclei (26) and a small subset of nonhistone proteins from chicken erythrocyte nuclei (27); perhaps these proteins are important in maintaining the active conformation of certain portions of the genome. Modification of the histones associated with active genes might also be a factor because acetylation of chromatin renders the associated DNA sequences more sensitive to DNase I (28).

The pattern of sensitivity of the individual globin genes in fetal erythroid cell nuclei is consistent with the results reviewed above. γ globin is produced and β globins are not. Correspondingly, the γ globin genes are in the transcriptionally active or DNase I-sensitive conformation whereas the β globin genes in the transcriptionally inactive portion of nuclear chromatin are resistant to DNase I. Differential expression of the individual globin genes in fetal erythroid cells appears to be correlated with their conformation in chromatin.

However, the regulation of the individual globin genes in adult erythroid cells may occur by a different mechanism. Despite the DNase I-sensitive or active conformation of the γ globin genes in bone marrow erythroid cell nuclei, γ globin mRNA sequences were not detected in total cellular RNA. It should be noted that DNase I sensitivity does not appear to be related to the rate or frequency of transcription of individual genes. Genes in chicken oviduct which are transcribed rarely are as sensitive to DNase I as are the frequently transcribed gene for ovalbumin (29). Similarly, the marked acceleration in the rate of transcription of the globin genes after dimethyl sulfoxide-mediated induction of mouse erythroleukemia cells occurs without a change in the conformation of the globin genes; the genes are highly sensitive to DNase I even in nuclei from uninduced cells (13). Despite its active conformation in adult erythroid cells, the γ globin gene may be infrequently transcribed. Alternatively, the rates of transcription of the γ and β genes might be equivalent in adult erythroid cells, with differential accumulation of cytoplasmic mRNA being related to modulation of the various processing steps necessary for production of mature mRNA. Little evidence exists for primary regulation at this level, however, suggesting that differential expression of the β and γ genes in adult erythroid cells is based on different rates of transcription.

Current evidence suggests that fetal and adult erythrocytes are derived from common erythroid stem cells (1, 21). Our

study suggests that there may be sequential activation of the individual globin genes in the nuclear chromatin of cells that make up the erythroid lineage, with progressive opening of the γ followed by the β genes. The γ globin genes remain in the open or DNase I-sensitive conformation in chromatin in adult erythroid cells despite the lack of accumulation of γ globin mRNA. The adult β globin genes are not expressed until just before birth and are in the inactive conformation in nuclei of midgestation fetal erythroid cells. The differences in the pattern of DNase I sensitivity between fetal and adult erythroid cells may indicate two levels of regulations of globin gene expression. In the fetus, gene expression may reflect globin gene conformation whereas in the adult the absence of major structural differences among the globin genes supports regulation of a transcriptional or a post-transcriptional stage of mRNA metabolism.

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