

# Tissue-Specific DNA Cleavages in the Globin Chromatin Domain Introduced by DNAase I

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## Summary

Using recombinant chicken DNA clones as probes, we have investigated the DNAase I sensitivity of chromosomal DNA regions bordering the  $\alpha$ - and  $\beta$ -globin structural genes. By both a blot hybridization assay and solution hybridization, we find that regions around these globin genes are preferentially sensitive (relative to the ovalbumin gene) to DNAase I after mild digestion of isolated red cell nuclei. These regions are resistant in cells that do not express globin. The preferential DNAase I sensitivity extends to at least 8 kb on the 3' side of the  $\beta$ -globin gene cluster and to 6 or 7 kb on the 5' side, where relatively resistant DNA fragments have been identified. Using low levels of DNAase I to titrate the sensitivities of coding and adjacent non-coding regions, it was observed that coding regions are organized into a very sensitive structure, while adjacent noncoding regions are organized into a moderately sensitive structure. The blot hybridization assay has also revealed that DNAase I introduces specific, double-stranded cuts into both the  $\alpha$ - and  $\beta$ -globin gene clusters. Many of these cuts are tissue-specific. Several  $\alpha$  gene-specific sites occur toward the 3' side of the  $\alpha$ -coding sequences. The  $\beta$  sites are different in embryonic and adult red cells. In embryonic cells, the cut occurs near the 5' end of an embryonic  $\beta$  gene, while in adult cells there are two cuts, one at approximately 2 kb and the other at approximately 6 kb from the 5' side of an adult gene. Based on the observation that the general region around the origin for replication and promoters for transcription in the SV40 minichromosome is also very sensitive to specific, double-stranded scissions by DNAase I, we speculate that the specific cuts in the globin domain may be structural modifications of the chromatin that are associated with origins for DNA replication or promoters for transcription.

## Introduction

During development of the chick embryo, Hb and Hb mRNA are first detected at approximately 35 hr of incubation in cells from the primitive (or embryonic) lineage of red cells. Steady-state globin mRNA is not detectable in precursor hematocytoblasts (Groudine et al., 1974) present in the area vasculosa before 20–25 hr. As assayed by solution hybridization, the globin chromatin is resistant to DNAase I before 25 hr, but becomes sensitive to DNAase I after overt erythroid differentiation at 35 hr (Weintraub and Groudine, 1976).

The primitive erythroblasts present in the embryonic circulation between 2 and 5 days produce only embryonic  $\beta$ -globin chains, yet both embryonic and adult  $\beta$ -globin genes are sensitive to DNAase I (Stalder et al., 1980) as assayed by blot hybridization. The sensitivity of the adult  $\beta$ -globin gene in embryonic red cells has been interpreted to reflect a "preactivation" chromosome structure associated with this nonexpressed adult globin gene. At day 6 of development a new red cell line, the definitive line, appears in the embryonic circulation. These cells are morphologically very distinct from primitive cells, and they produce adult  $\beta$ -globin chains. The Hb "switching" observed in the chick erythropoietic system at this time is reflected in an apparent conversion of the embryonic  $\beta$ -globin gene from a very DNAase I-sensitive state in primitive cells to a more resistant state in definitive cells. We presume that these events are taking place at the level of a common precursor cell (see Discussion).

The sensitivity of an actively transcribed gene to DNAase I reflects among other characteristics its association with HMG 14 and 17 (Weisbrod and Weintraub, 1978; Weisbrod, Groudine and Weintraub, 1980). When these proteins are gently eluted from individual nucleosomes, the active nucleosomes lose their sensitivity to DNAase I. Upon reconstitution with pure HMG 14 or 17, these nucleosomes regain their sensitivity to DNAase I. Nucleosomes associated with nonexpressed genes do not have a high affinity binding site for these proteins, so the interaction of HMG 14 and 17 with active nucleosomes is very specific.

Here we show that the actively transcribed globin chromatin is very sensitive to DNAase I; however, adjacent noncoding regions are also structurally distinct in that they display a moderate level of sensitivity; thus these regions are cut more readily by DNAase I than, for example, the ovalbumin gene. We define the contiguous chromosomal structure exhibiting high and moderate DNAase sensitivity as a "chromosomal domain." We have also found that DNAase I introduces specific double-strand breaks into both the  $\alpha$ -globin domain and the  $\beta$ -globin domain. Many of these cuts are cell type-specific. The specific  $\beta$ -related cuts were

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found to be different in the primitive and definitive red cell lines.

## Results

### Preferential DNAase Sensitivity of Chromosomal Regions surrounding Globin Genes

Figure 1 shows restriction maps for four recombinant DNA clones containing various chicken globin genes:  $\lambda C\beta G1$  (or  $\beta 1$ ) carries both an embryonic  $\beta$  gene (e) and the adult  $\beta$ -globin gene (a) (Dodgson, Stommer and Engel, 1979);  $\lambda C\beta G2$  (or  $\beta 2$ ) carries both a second embryonic  $\beta$  gene (e) and an adult  $\beta$ -like gene, probably the hatching  $\beta$  gene;  $\lambda C\beta G3$  (or  $\beta 3$ ) is the 5' extension of  $\lambda C\beta G2$  and carries part of the second embryonic  $\beta$  gene (M. Dolan, J. B. Dodgson and J. D. Engel, manuscript in preparation); finally,  $\lambda CaG2$  (or  $\alpha 2$ ) carries the two major  $\alpha$  gene loci coding for the  $\alpha a$  and  $\alpha d$  polypeptide chains. Both of these  $\alpha$  chains are expressed in adult and embryonic red cells (Brown and Ingram, 1974).

To examine the DNAase I sensitivity of regions surrounding the various globin genes, nuclei from mature definitive red cells (containing adult and hatching Hb and obtained from 14 day old chick embryos) were digested with various levels of DNAase I, and the DNA was purified and then restricted with Bam HI. The restricted DNA was separated on 1% agarose gels, blotted to nitrocellulose filters (Southern, 1975) and hybridized to the various nick-translated  $\lambda$ -globin clones or to ovalbumin cDNA.

Figure 2a shows a very low level digest from red cell nuclei treated with 1.5  $\mu$ g/ml of DNAase. Before

restriction, this DNA had an average size of approximately 15 kb. As predicted from the restriction map (Figure 1), DNA which has not been DNAase-treated yields five major bands at roughly molar intensities when digested with Bam HI and then hybridized to  $\lambda CaG2$ . A nonstoichiometric band at approximately 7.2 kb probably represents a junction fragment. The second junction fragment (at 0.8 kb) is very faint, probably because it contains a very low percentage of the probe.

Surprisingly, all the fragments hybridizing to the  $\alpha$  clone are preferentially digested by DNAase I. As controls, there is no preferential digestion of the ovalbumin gene (6.5 kb) in the same DNA sample. Several tubulin-related bands and the RAV-O ev-1 locus (Astrin, 1978) are also not digested. In addition, comparable levels of digestion of MSB nuclei (a lymphoid line of chicken cells that does not synthesize globin RNA) or brain nuclei (not shown) result in no preferential digestion of the  $\alpha$ -globin-related DNA, nor of ovalbumin.

Figure 2b shows that a similar level of sensitivity is displayed by the Bam fragments that hybridize with  $\beta 1$  and  $\beta 2$  probes using the same DNA preparation. As in the case of the  $\alpha$  genes, all the fragments hybridizing to these  $\beta$  clones are preferentially digested by DNAase I, whereas the ovalbumin structural gene displays no preferential sensitivity. The same results are obtained with a variety of restriction enzymes: Hind III, Eco RI, MSP and Kpn. We conclude that for  $\alpha 2$ ,  $\beta 1$  and  $\beta 2$ , both coding regions and adjacent regions are preferentially sensitive to DNAase I, using the blot hybridization assay.

### A Region of Relative Resistance at the 5' Side of the $\beta$ -Globin Domain

With  $\beta 3$  a slightly different story emerges (Figure 2b). The DNA hybridizing to the 8.5 and 4 kb fragments is digested rapidly; however, the DNA hybridizing to the 5 kb and 1.5 kb fragments is relatively resistant. These fragments are located approximately 7 kb from the 5' end of the embryonic  $\beta$ -globin gene cluster (Figure 1).

One technical problem we have encountered using genomic clones as hybridization probes (particularly  $\beta 2$  and  $\beta 3$  and to a lesser extent  $\alpha 2$ ) is that there appears to be a rather high "apparent" background within the lanes. This "apparent" background is very resistant to low salt and high temperature washes and on many occasions resolves into rather discrete bands (see, for example, Figure 3, below). We presume that the globin domains also contain many repetitive sequences which are scattered throughout the genome (Shen and Maniatis, 1980), and that this is the reason for much of the apparent background. Additional evidence for this interpretation comes from the fact that most of the noncoding regions from  $\beta 2$  and  $\beta 3$  clones hybridize to a great number (50–300 per genome) of

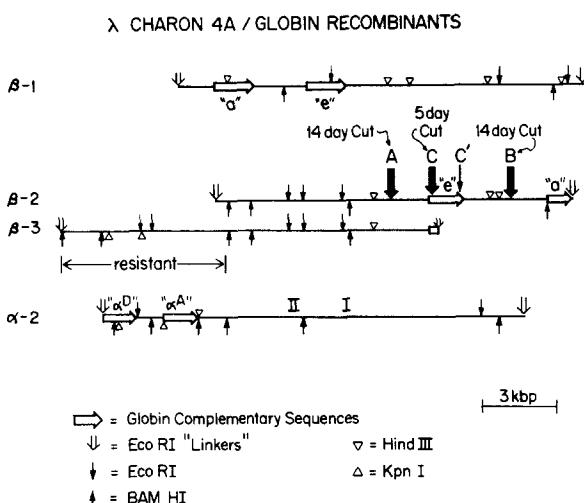


Figure 1. Restriction Maps for Four  $\lambda$ Charon 4a Recombinant Chicken Clones Carrying Globin Genes

"a" and "e" denote adult and embryonic  $\beta$ -globin genes extending over the distance indicated by the thick arrows. The direction of transcription ( $5' \rightarrow 3'$ ) was determined by Dodgson et al. (1978) and M. Dolan, J. B. Dodgson and J. D. Engel (unpublished results). The thick vertical arrows point to sites of preferred cutting by DNAase I as referred to in the text.

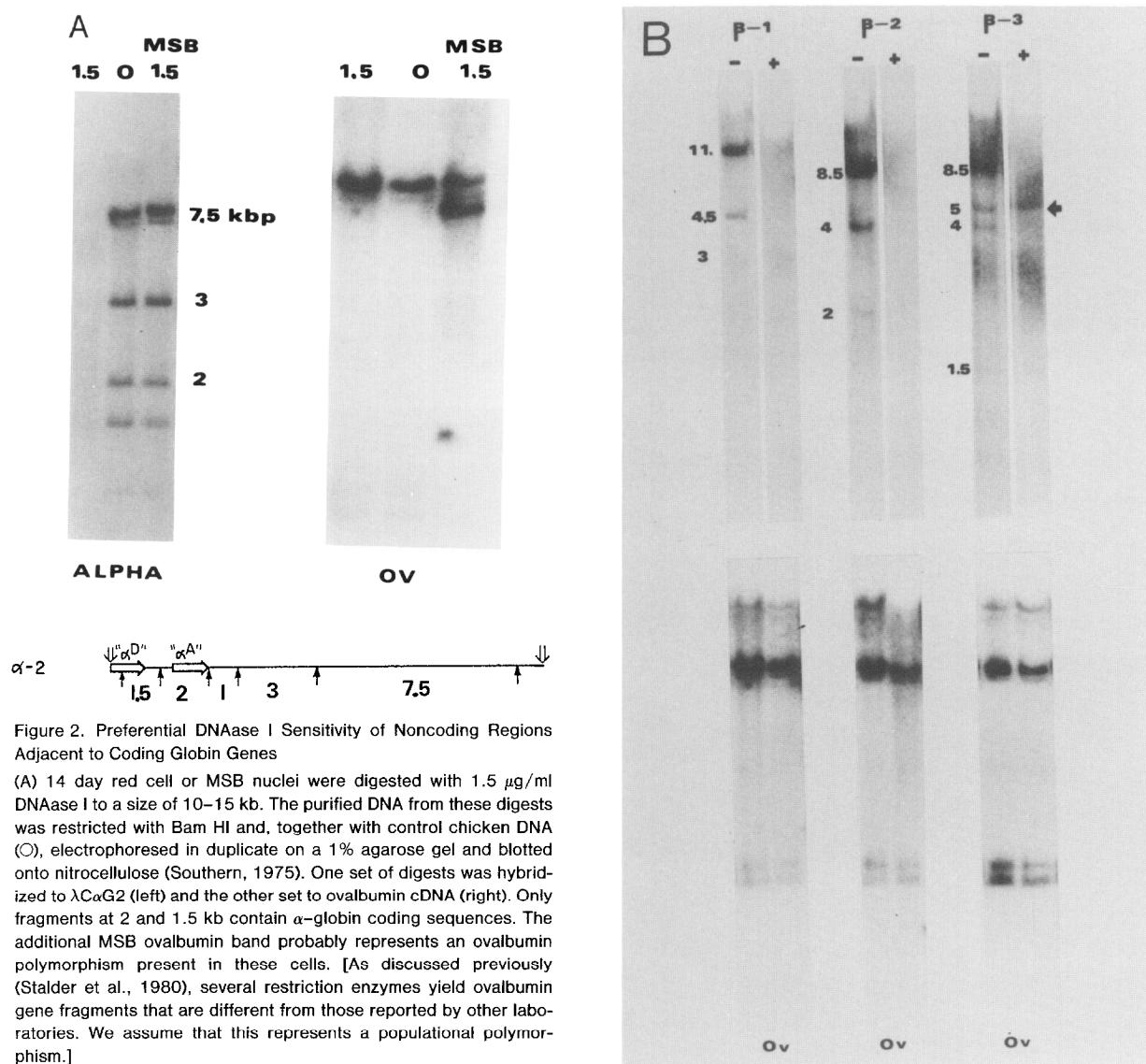


Figure 2. Preferential DNAase I Sensitivity of Noncoding Regions Adjacent to Coding Globin Genes

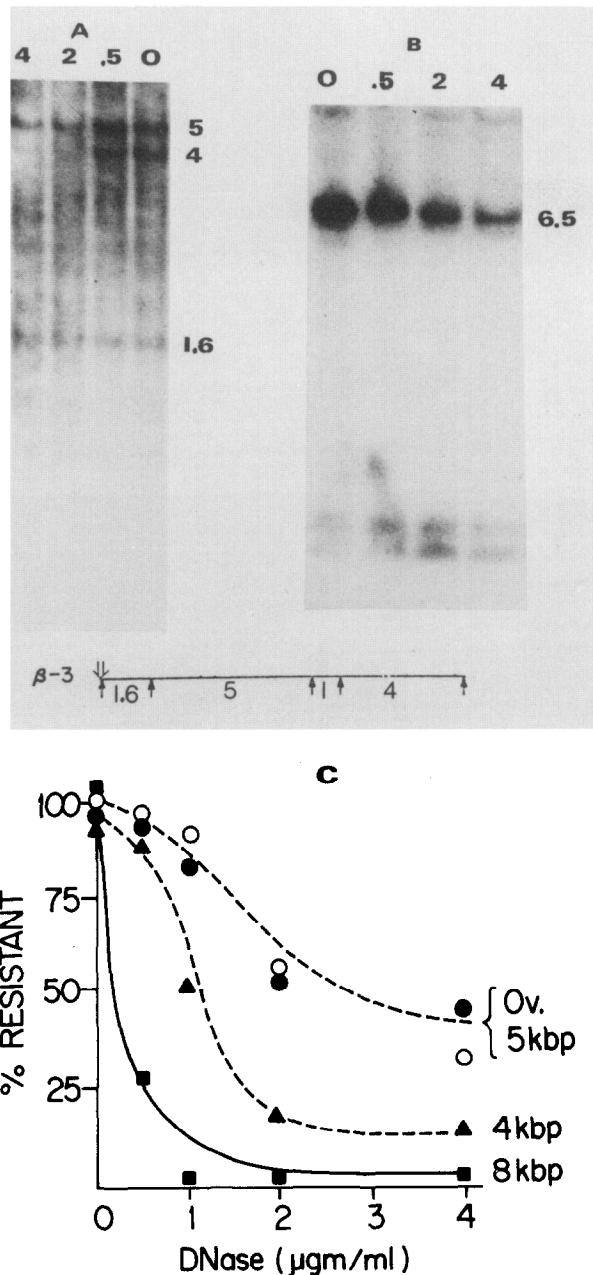
(A) 14 day red cell or MSB nuclei were digested with 1.5  $\mu\text{g}/\text{ml}$  DNAase I to a size of 10–15 kb. The purified DNA from these digests was restricted with Bam HI and, together with control chicken DNA (O), electrophoresed in duplicate on a 1% agarose gel and blotted onto nitrocellulose (Southern, 1975). One set of digests was hybridized to  $\lambda\text{C}\alpha\text{G}2$  (left) and the other set to ovalbumin cDNA (right). Only fragments at 2 and 1.5 kb contain  $\alpha$ -globin coding sequences. The additional MSB ovalbumin band probably represents an ovalbumin polymorphism present in these cells. [As discussed previously (Stalder et al., 1980), several restriction enzymes yield ovalbumin gene fragments that are different from those reported by other laboratories. We assume that this represents a populational polymorphism.]

(B) The same red cell samples as shown in (A) were hybridized to  $\lambda\text{C}\beta\text{G}1$ ,  $\lambda\text{C}\beta\text{G}2$ ,  $\lambda\text{C}\beta\text{G}3$  or ovalbumin cDNA (Ov). (—) and (+) indicate without and with (1.5  $\mu\text{g}/\text{ml}$ ) DNAase I, respectively. The 4 kb fragment in  $\beta 2$ , the 3 kb fragment in  $\beta 1$ , and the 5, 4 and 1.6 kb fragments in  $\beta 3$  are all noncoding. The arrow in the  $\beta 3$  blot points to a relatively DNAase-resistant fragment located at the 5' end of the  $\beta$  domain (see Figures 1 and 3).

recombinant  $\lambda$  clones when the chicken library is probed with these sequences (H. Weintraub, unpublished observations).

To reduce the background so that the kinetics of DNAase I digestion could be examined and the relative resistance of the 5' region of  $\beta 3$  could be established, blots containing DNA digested to rather high levels were hybridized to labeled  $\beta 3$  in the presence of an excess of a cold, competing DNA fragment from  $\beta 2$  (we used the 8.5 kb Bam fragment in  $\beta 2$ ). Under these hybridization conditions, we detect only the 1.6, 5 and 4 kb Bam fragments present in  $\beta 3$  toward the 5' end of the  $\beta$ -globin gene domain (Figure 3A). Many cross-hybridizing fragments are also observed. Figure 3B shows that there is a very gradual decrease in hybrid-

ization to the 6.5 kb ovalbumin fragment at these high DNAase concentrations. A similar decrease is observed with the 5 kb Bam fragment associated with  $\beta 3$ ; however, the 4 kb Bam fragment in  $\beta 3$  is significantly more sensitive [separate experiments show it to have an intermediate level (see below) of sensitivity] and the 1.6 kb fragment slightly more resistant. The resistance of the 1.6 kb band might be related in part to its small target size; however, for  $\beta 2$  and  $\alpha 2$ , correspondingly small Bam fragments are clearly sensitive (Figure 2) to DNAase I. Thus while most of the  $\beta$  gene cluster is sensitive to DNAase I, a region that begins approximately 7 kb from the 5' end of the embryonic  $\beta$  gene in  $\lambda\text{C}\beta\text{G}3$  is relatively resistant to DNase I digestion. Figure 3C shows the results from

Figure 3. Resistance of the 5' Region of  $\beta$ 3 to DNAase I

(A) Red cell nuclei from 5 day red cells were digested with increasing doses of DNAase I, and the digest was analyzed, as described in Figure 2. The samples were again digested with Bam HI, but in this case hybridization was to  $\lambda$ C $\beta$ G3. An excess (20  $\mu$ g) of the 8.5 kb Bam fragment from  $\beta$ 2 was included in the hybridization to decrease the number of CPM hybridizing to repetitive sequences in the chick genome so that the "effective" hybridization probe is that part of  $\beta$ 3 shown at the bottom of the figure.

(B) The same samples as shown in (A) were hybridized to ovalbumin cDNA.

(C) Blots similar to the one shown in (A) and (B), except that whole  $\beta$ 3 was used, were scanned with a soft laser densitometer (Zeineh) and the area under the peaks (above the repetitive sequence hybridization) was determined at each DNAase concentration. The data represent the average from two separate blots. In the top curve, the open circles are from ovalbumin. The closed symbols refer to the Bam fragments present in  $\beta$ 3.

densitometer tracings of related experiments hybridizing ovalbumin and whole  $\beta$ 3 to the same samples of DNAase-treated DNA from embryonic cells. While interpretation of the data is complicated by difficulties associated with differential transfer and binding of the DNA (and also by the background due to repetitive sequences), these effects probably do not change the rather qualitative conclusion that the region 7 kb from the 5' end of the  $\beta$  gene cluster is relatively resistant to DNAase I.

#### A Moderate DNAase I-Sensitive State for Noncoding Adjacent Fragments

Using very low levels of DNAase I, we asked whether the entire domain defined by these probes had the same sensitivity to DNAase I. Figure 4B shows an RI digest (see Figure 1) hybridized to  $\beta$ 1. At low levels of digestion, the 6.1 kb fragment coding for the adult globin gene (arrow) is much more sensitive in adult red cell nuclei than are the adjacent fragments (see also Figure 3). At slightly higher levels of digestion, all these fragments become digested faster than the ovalbumin standard (Figure 4A). In analogous experiments, we have also noted that, using  $\alpha$ 2 and  $\beta$ 2 as probes, coding regions are very sensitive to DNAase I, while adjacent noncoding regions are usually of intermediate sensitivity (data not shown). For conven-

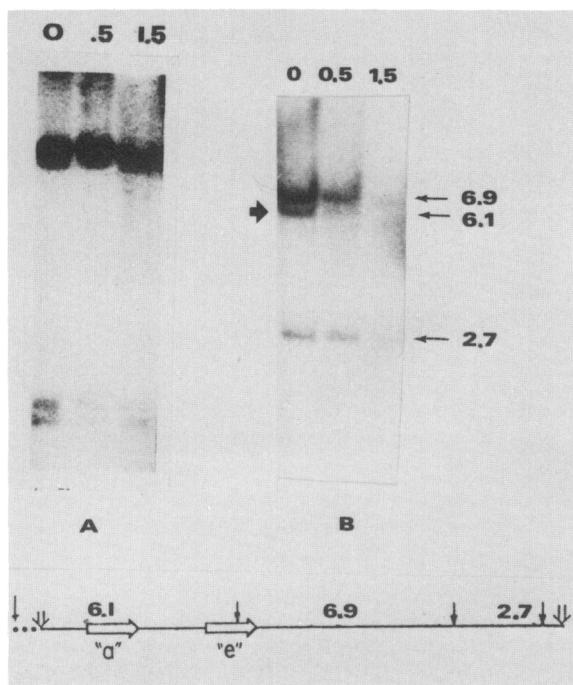


Figure 4. Moderate Sensitivity of Noncoding Adjacent Sequences in the Globin Domain

(A) The same DNA samples as in (B) were digested with Bam HI and hybridized to ovalbumin cDNA. (B) DNAase I-treated samples were digested with RI and hybridized to  $\beta$ 1. The 6.1 kb fragment contains the coding region for both the adult and embryonic genes present in  $\beta$ 1 (Ginder, Wood and Feisenfeld, 1979).

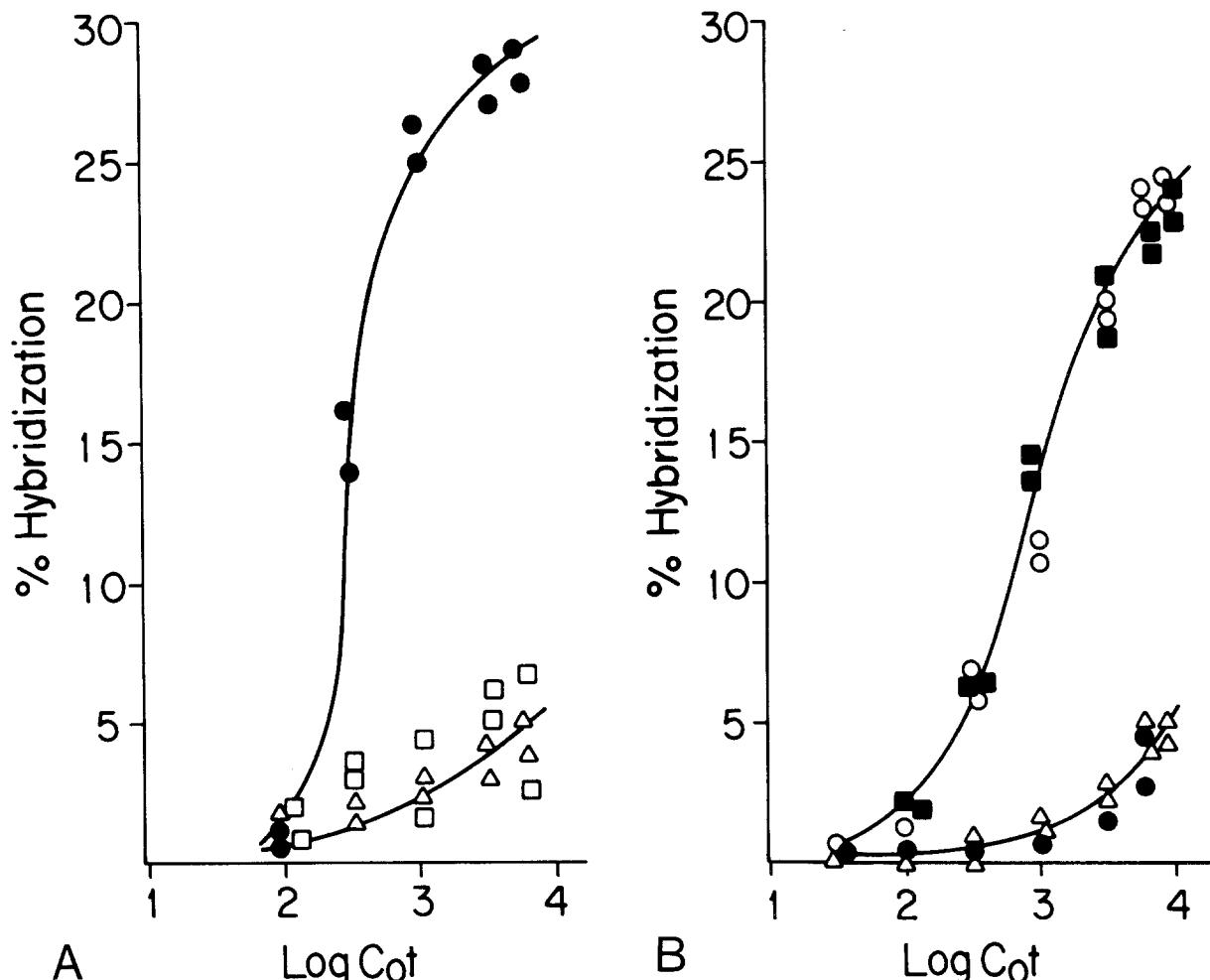


Figure 5. Preferential Sensitivity to DNAase I of Noncoding Regions in the  $\alpha_2$  and  $\beta_1$  Domains As Assayed by Solution Hybridization

14 day nuclei were digested to 15% acid solubility with DNAase I or to 40% acid solubility with staph nuclease. The resistant DNA was then used for solution hybridization.

(A) Hybridization of  $\lambda C\alpha G2$  ( $4 \times 10^8$  cpm/ $\mu g$ , 50,000 CPM input, 12 mg/ml DNA; 20  $\mu l$  volume). (●—●) Hybridization to total DNA; ( $\Delta$ — $\Delta$ ) hybridization to DNAase I-treated DNA; ( $\square$ — $\square$ ) self-hybridization of probe alone. Approximately 33% of  $\lambda C\alpha G2$  is represented by chicken sequences.

(B) Hybridization of  $\lambda C\beta G2$  (conditions as in Figure 5A). (○—○) Hybridization to total DNA; (■—■) hybridization to staph nuclease-treated DNA; ( $\Delta$ — $\Delta$ ) hybridization to DNAase I-digested DNA; (●—●) self-hybridization of probe alone.

(C) Hybridization to ovalbumin cDNA ( $6 \times 10^8$  cpm/ $\mu g$ ; 20,000 cpm input; DNA at 12 mg/ml; 20  $\mu l$  volume). (○—○) Hybridization to staph nuclease-digested DNA; ( $\Delta$ — $\Delta$ ) hybridization to DNAase I-digested DNA. cDNA background was 5–10% and was subtracted from each point as determined from a parallel hybridization.

ience, we refer to the adjacent regions of moderate and high sensitivity as a "chromosomal domain." For the  $\beta$  genes, we have identified the apparent junction of this domain at the 5' side, but not at the 3' side (Figure 3). Although we have not yet found recombinant clones to "hook-up"  $\lambda C\beta G1$  with  $\lambda C\beta G2$  and  $\lambda C\beta G3$ , evidence from cellular DNA blots indicates that only 1.8 kb separates  $\beta 1$  from  $\beta 2$  and that the

direction of transcription of each  $\beta$  gene is in the same orientation; thus it is probable that all the  $\beta$ -related genes are in the same chromosomal domain, as defined by DNAase sensitivity.

#### Digestion of Chromatin Domains by DNAase I As Assayed by Solution Hybridization

To determine whether adjacent, noncoding sequences were also sensitive to DNAase I as assayed by solution hybridization, red cell nuclei were digested to 15% acid solubility with DNAase I. The purified DNA was hybridized in solution to tracer amounts of  $\lambda C\alpha G2$  or  $\lambda C\beta G1$ . Figures 5a and 5b show that this preparation fails to hybridize to these probes; however, hybridization to ovalbumin cDNA was normal (Figure 5c). Since coding regions comprise only 10–20% of the hybridizing component of these probes, we conclude that at these levels of digestion, the entire chromosomal domain defined by these probes is digested to pieces too small to hybridize when the nuclei are digested to 15% acid solubility. Similar results have also been obtained for  $\lambda C\beta G2$ . We do not yet know whether lower levels of digestion also discriminate between coding and noncoding sequences in the globin chromatin domains, as we have observed by blot hybridization.

#### Specific Cutting Sites for DNAase I

At very low levels of DNAase I, specific, discrete subbands appear when the Bam-digested DNA is hybridized to  $\alpha 2$  (Figure 6). The same sub-fragments are

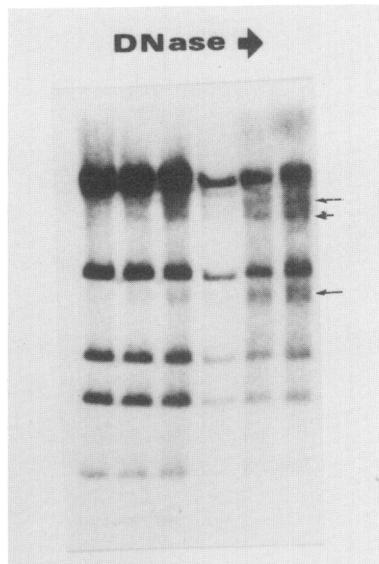


Figure 6. Generation of Specific Subfragments after Low-Level Digestion of Adult (14 Day) Nuclei with DNAase I

14 day red cell nuclei were digested with increasing concentrations of DNAase I (left to right: 0; 0.2; 0.4; 0.6; 0.8; 1.0  $\mu$ g/ml DNAase I) and the digested DNA was purified and restricted with Bam HI and analyzed as described in the legend to Figure 2. Hybridization was to  $\lambda C\alpha G2$ .

observed in adult and embryonic red cells, both of which produce  $\alpha A$  and  $\alpha D$  globin chains. These types of discrete bands are not observed when the DNAase I-treated DNA is not restricted. (We do, however, observe very broad  $\alpha 2$ -related bands without restriction.) Thus we presume that one end of these bands comes from a Bam cleavage, while the other arises from a specific DNAase I cut. Low-level digests of pure DNA with DNAase I fail to generate these bands after Bam restriction. Similarly, digestion of MSB or brain nuclei with DNAase I also does not lead to subbands. While we have not yet mapped the exact location of the  $\alpha$ -related cleavages, it is clear that they are from regions of the  $\alpha$  domain that do not code for  $\alpha$ -globin and, by virtue of their size, must come from the 7.5 or 3 kb Bam fragments, both of which are 3' to the structural genes. More recently, we have also identified a specific cleavage that occurs right at the 5' side of  $\alpha D$ . Treatment of nuclei with increasing concentrations of DNAase I (Figure 6) shows that these sub-fragments appear very early during the course of digestion. They also tend to persist throughout the digestion, and they comprise a very significant fraction of the total radioactivity hybridizing to the 7.5 kb fragment, presumably the fragment from which the larger bands are derived.

#### Specific DNAase I Cutting Sites in the $\beta$ -Globin Domain

Figure 7A shows a control and a low level DNAase I digest of adult (14 day) and embryonic red cell nuclei after restriction with Bam and hybridization to pHb1001, an adult  $\beta$ -globin cDNA clone (a gift from W. Salser). The clone detects two adult  $\beta$ -globin

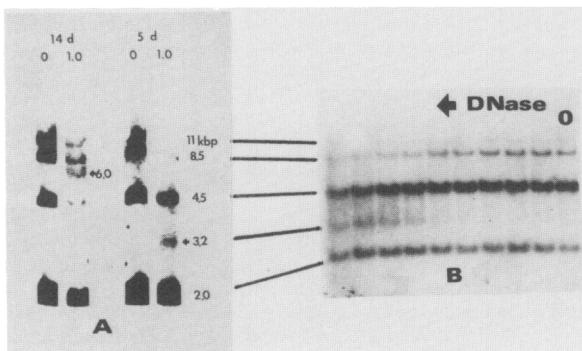
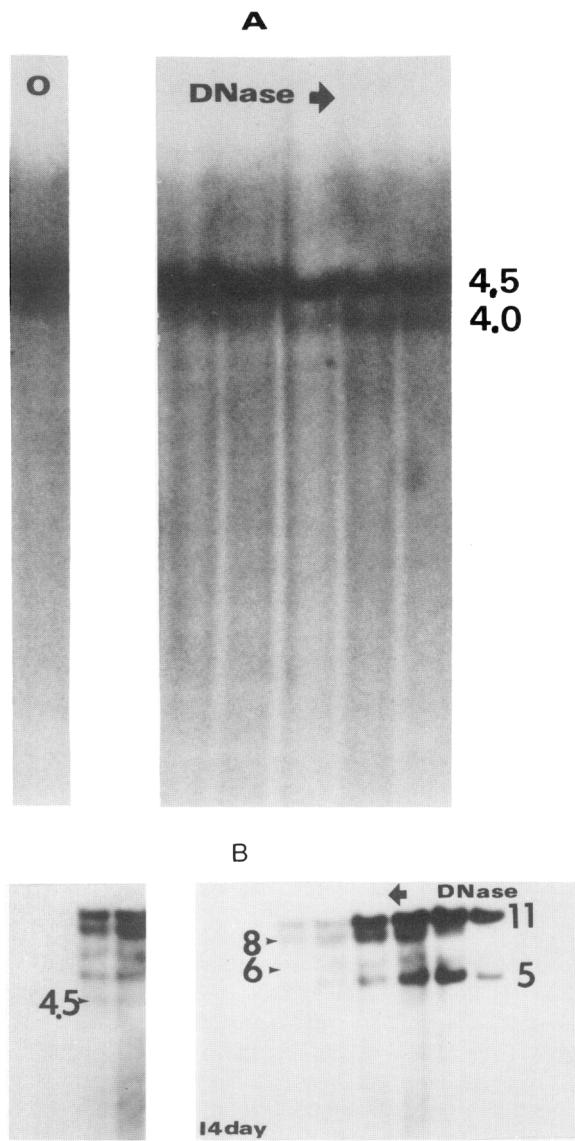


Figure 7. Generation of Specific Subfragments after Low-Level Digestion of Adult (14 Day) and Embryonic (5 Day) Red Cell Nuclei with DNAase I (1  $\mu$ g/ml)

(A) The experimental details were as described in the legend to Figure 6, except that hybridization was to pHb1001. The 11 and 8.5 kb Bam genes are embryonic; the 4.5 and 2.0 kb genes are adult. Most of these genes are detected by cross-hybridization.

(B) 5 day nuclei were digested with increasing concentrations of DNAase I and hybridized to pHb1001 after Bam digestion. In this experiment, hybridization was in 50% formamide at 45°C. Under these conditions pHb1001 hybridizes most intensely with the 4.5 kb Bam gene rather than the 2 kb gene, as observed in (A).



**Figure 8.** Mapping the Specific Cuts in the Adult  $\beta$  Domains  
**(A)** Adult (14 day) nuclei digested with DNAase I (0; 0.2; 0.4; 0.6; 0.8; 1.0  $\mu$ g/ml). All samples were digested with Hind III and hybridized to pB2H2, a subclone of  $\lambda$ C $\beta$ G2 (in pBR322) that contains the embryonic gene flanked by the 4.5 kb Hind III sites (see Figure 1).  
**(B)** Control and DNAase-treated DNA from 14 day nuclei were treated with Eco RI and, after blotting, hybridized to  $\lambda$ C $\beta$ G2. The two lanes on the left are longer exposures of the corresponding lanes on the right.

genes at 2 and 4.5 kb, and two embryonic  $\beta$  genes at 8.5 and 11 kb. Low level DNAase I digestion generates a 6 kb sub-band in adult nuclei and a 3.2 kb sub-band in embryonic nuclei. Thus these  $\beta$  sub-bands are not only tissue-specific (we do not see them in brain or MSB cells), but they are different in adult and embryonic red cell lines. They are also not seen when free DNA is digested with low levels of DNAase I. Because they are detected with a cDNA clone, these sub-bands

must include  $\beta$ -globin coding regions. Figure 7B is a dose response to DNAase that shows that in embryonic red cells, the 3.2 kb band appears very early during digestion, and also that no detectable 6 kb sub-band is observed in embryonic cells. Similarly, the 3.2 kb sub-band is also not detected in adult nuclei over a wide range of DNAase concentrations.

To map the sites of DNAase I cutting, blots were hybridized with  $\lambda$ C $\beta$ G1,  $\lambda$ C $\beta$ G2 and  $\lambda$ C $\beta$ G3.  $\lambda$ C $\beta$ G1 gave no detectable sub-bands;  $\lambda$ C $\beta$ G3 gave weak sub-bands;  $\lambda$ C $\beta$ G2 gave very prominent sub-bands (data not shown). The failure to see sub-bands from  $\lambda$ C $\beta$ G1 (which contains the 11 and 4.5 kb Bam fragments) indicates that neither the 6 kb adult sub-bands nor the 3.2 kb embryonic sub-band arise from the 11 or 4.5 kb  $\beta$  genes. This means that they must arise from regions of the genome defined by  $\lambda$ C $\beta$ G2 and  $\lambda$ C $\beta$ G3, presumably from the region around the 8.5 kb Bam embryonic  $\beta$ -globin gene since, by elimination, this gene is the only one remaining that is large enough to give rise to discrete fragments of 6 and 3.2 kb detectable by hybridization to pHb1001.

In 14 day red cells, Bam digested DNA yields a subfragment of 6 kb, presumably derived from the 8.5 kb gene in  $\lambda$ C $\beta$ G2. Thus the adult cut is 2.3 kb from either or both Bam sites (Figure 1). Digestion of the DNA with Hind III and hybridization with labeled pB2H2 [a subclone of  $\lambda$ C $\beta$ G2 in pBR322 that contains the embryonic  $\beta$ -coding region flanked by the two Hind III (Figure 1) sites 4.5 kb apart] yields the parent fragment at 4.5 kb and a subfragment at 4 kb (Figure 8A). The results with Hind III, together with the Bam digestion data, clearly establish one cut in adult nuclei at the 5' side of the embryonic gene in  $\beta$ 2 (see arrow A in Figure 1). Digestion with R1 and hybridization with  $\lambda$ C $\beta$ G2, however, yields parent molecules of 11 and 5 kb and subfragments of 8 and 2.8 kb (the 2.8 kb fragment is barely visible at this exposure). These fragment sizes are consistent with the previous identification from the Bam and Hind III digests. Unexpectedly, subfragments of 6 and 4.5 kb (Figure 8B) are also generated. This second pair of fragments would arise if there were actually a second site for DNAase I-specific cleavage (see arrow B in Figure 1) approximately 2.3 kb from the other Bam site in  $\lambda$ C $\beta$ G2. Thus we believe that in 14 day red cells there are actually two sites for preferential DNAase cutting, one approximately 2 kb from the 5' end of the adult gene and the second approximately 6 kb from the 5' end of this same gene in  $\lambda$ C $\beta$ G2.

Figure 1 also shows our estimation of the approximate position for the specific DNAase I cut observed in 5 day embryonic red cell nuclei (arrow C). The subfragment obtained after Bam digestion and hybridization to pHb1001 is approximately 3.2 kb. Since we do not see a 3.2 kb subfragment after digestion with DNAase I alone, it follows that one end of the subfrag-

ment is generated by DNAase I and the second end by Bam. This places the DNAase I-sensitive site 3.2 kb from either the left or right Bam sites (arrow C or C'). The two possible cutting sites must therefore be just within either the 3' or 5' side of the coding region for the gene, since the subfragment is detected with the cDNA clone, pHb1001. To determine whether the specific cut occurred at arrow C or C' in Figure 1, DNAase-treated chromosomal DNA was digested with Hind III and the blot was hybridized to pB2H2 (Figure 9). A cut at C' would predict the generation of two subfragments of approximately 3.5 and 1 kb from the parent 4.5 kb fragment; a cut at C would predict the generation of two co-migrating fragments, each about 2.2 kb. When this experiment was performed, a single broad subfragment was obtained (which occasionally resolves into several discrete fragments) at about 2.2 kb, indicating that the specific cut in 5 day primitive erythroblast nuclei occurred near the 5' side (arrow C and not arrow C') of the embryonic  $\beta$ -globin gene in  $\lambda$ C $\beta$ G2. This site was further confirmed in separate experiments, where Bam-digested DNA hybridized to pB2H2 yielded two subfragments, one at approximately 3.2 kb and a second at approximately 4.8 kb. More recently, we have also found that there is a second embryo-specific DNAase I cut at the 5' side of the embryonic gene present in  $\beta$ 1. As with the embryonic gene in  $\beta$ 2, this cleavage is observed only in embryonic red cells and not in adult red cells.

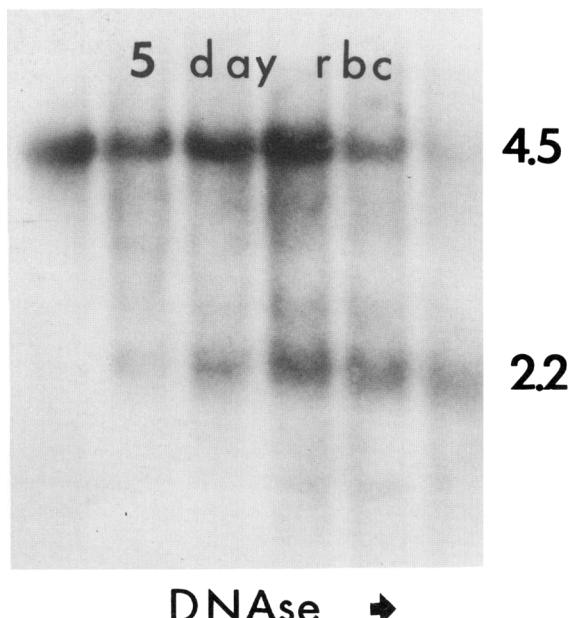


Figure 9. Mapping the Specific DNAase Cleavage in the Embryonic Red Cell  $\beta$  Domain  
5 day red cell nuclei were digested with DNAase I (0; 0.1; 0.2; 0.4; 0.5; 0.8  $\mu$ g/ml). All samples were restricted with Hind III and hybridized to pB2H2 (see Figure 8A). Unlabeled bands probably represent hybridization to repetitive DNA sequences.

## Discussion

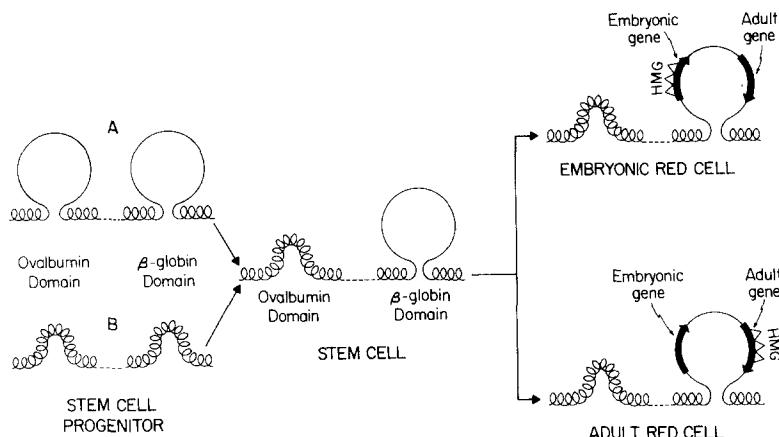
### Structural Domains for Active Genes

Using the blot hybridization assay, we have shown that the chromosome region directly associated with actively transcribed DNA sequences is extremely sensitive to DNAase I. In addition, chromatin regions on either side of these sequences are also preferentially digested; however, these regions display a moderate degree of sensitivity. This type of structure (a chromosomal domain) extends to at least 8 kb on the 3' side of the  $\beta$  and  $\alpha$  domains and to approximately 7 kb to the 5' side of the  $\beta$ -globin gene domain. In the case of the  $\beta$  chromosomal domain the junction with resistant chromatin can be observed, since several restriction fragments beyond this point are relatively insensitive to DNAase I under our conditions.

It has been shown (Flint and Weintraub, 1977; Frlova et al., 1978) that for hamster cells transformed by adenovirus, only those integrated adenovirus DNA sequences that were transcribed were sensitive to DNAase I (after 15% digestion) as assayed by solution hybridization; adjacent adenovirus sequences that were not transcribed were not preferentially digested using this assay. More recently, we have confirmed this observation by blot hybridization; however, because we do not yet have good probes for inactive rat genes, it is difficult to say with certainty whether the nontranscribed adeno sequences fall into the insensitive or moderately sensitive class and, consequently, the exact nature of the integrated adenovirus domain is not certain.

The structural basis for the globin domains is not known. While it is clear that HMG 14 and 17 are responsible, in part, for establishing the DNAase I sensitivity of a large percentage of the very sensitive coding regions (Weisbrod et al., 1980), preliminary results suggest that HMG 14 and 17 are not responsible for the moderately sensitive state, and that treatments that destroy higher-order nuclear structure also eliminate the intermediate level of sensitivity. It is also possible that the adjacent regions actually code for some as yet unidentified red cell specific RNA; however, preliminary evidence suggests that this is not the case, at least for the great majority of the globin domain.

The results presented here can be considered in terms of one very popular model for chromosome organization (Figure 10). The model is based on the organization of lampbrush loops and, to some extent, polytene chromosomes. Most recently, it has its biochemical basis in the experiments first done by Benyajati and Worcel (1976) and Laemmli et al. (1977). [See also Cook and Brazell (1975) and Igo-Kemenes and Zachau (1977)]. When the loop is relaxed, a moderately sensitive DNAase I domain results. This may be the state of the globin domain, but not of the ovalbumin domain in the red cell stem cell (hemato-



ture displaying a marked sensitivity to DNAase I. Until proven otherwise, we postulate that DNAase I sensitivity per se is not necessarily always indicative of HMG association; consequently, in order to prove an association with HMGs, the appropriate reconstitution experiments must be performed (Weisbrod et al., 1980).

cytoblast). With terminal differentiation to an embryonic red cell, HMG 14 and 17 (and possibly other changes as well; see (McGhee and Ginder, 1979) become associated with the newly activated embryonic gene. As the embryo ages, the hematocytoblast (or its descendants) switch to adult red cell production and the adult  $\beta$  gene is now assembled with HMG 14 and 17. Besides focusing one level of control to the base of the loops, the figure also illustrates two extreme states for the progenitor to the hematocytoblast. Ovalbumin and globin domains are both seen to be "open" initially and then differentially "repressed," or repressed initially and then differentially activated. Using very young chick blastoderms, we are currently trying to distinguish between these two possibilities.

#### Specific Cutting by DNAase I

Specific cutting by DNAase I was first observed by Wu et al. (1979) in several Drosophila genes. We have shown here that DNAase I introduces specific double-stranded cuts into regions associated with the  $\alpha$ - and  $\beta$ -globin domains. We have also observed specific cutting in regions near the 5' side of the active, RAV-O, ev-3 locus (but not the inactive RAV-O, ev-1 locus) and regions at the 5' side of the integrated adenovirus genome. We suspect that the specific DNAase cleavages will reflect a number of different types of structural modifications, and that eventually roles for each will have to be defined more precisely.

The most significant observation from our work is that many of the specific cleavages introduced by DNAase I are tissue-specific. This is most dramatically seen in the case of the  $\beta$ -globin genes where the DNAase I sites differ, even between adult and embryonic red cells.

At present, we think that the best clue to the function of these modified chromatin regions comes from analogous observations with the SV40 minichromosome. Here the general region around the origin for replica-

Figure 10. Interpretation of Moderate Sensitivity to DNAase I in Terms of "Lampbrush" Chromosome-Like Loops or Domains

Chromosome domains are either condensed or relaxed. Transcription units become activated in relaxed domains by virtue of their association with HMG 14 and 17 (open triangles). The red cell stem cells (hematocytoblasts) are unique in that they are predicted to be defined by a relaxed globin chromatin domain not yet fully activated by HMGs. The hematocytoblast progenitors can have either relaxed (A) or constrained (B) domains; however, whichever proves to be the case, the state of the domains is seen to be the same for ovalbumin and globin. Finally, we should point out that the association of HMGs with the adult gene in embryonic red cells is not indicated, even though this gene has a struc-

tion and the probable promotor for transcription is preferentially digested by nucleases (Scott and Wigmore, 1978; Waldeck et al., 1978; Varshavsky, Sundin and Bohn, 1979). While many of the specific cellular cutting sites seem to be located at the 5' side of transcribing genes, we would guess that some of these sites are not promotor structures, since several are present on the 3' side of the  $\alpha$ -globin genes where no transcription has yet been detected. If the same type of structure as seen in SV40 is also responsible for some of the specific cutting sites associated with the globin genes, and if some of these structures also act as cellular origins for DNA replication, then the results with the  $\beta$ -globin domain would indicate that the origin is specific (see also Seidman, Levine and Weintraub, 1979), and actually changes during the switch from primitive to definitive (adult) erythropoiesis. [These specific cleavage sites may also contain potential polymerase III transcription units that have homologies to known origins of DNA replication (Jelinek et al., 1980).]

We previously proposed (Weintraub et al., 1977) a mechanism for generating chromosomal diversity (and hence cellular diversity) during the unfolding of a given lineage. The idea was based on the conservative segregation and assembly of nucleosomes (Leffak, Grainger and Weintraub, 1977), and required that cellular origins change in a precise way during development. We are now trying to test whether the sites for DNAase I cutting in the  $\beta$ -globin domain can act as origins in an appropriate recombinant DNA system.

#### Experimental Procedures

##### Cells and Nuclear Digestions

Red blood cells from either 5 day or 14 day White Leghorn chicken embryos were isolated and the nuclei prepared as described (Weintraub and Groudine, 1976). MSB cells were grown as described by Weisbrod and Weintraub (1978).

After the nuclei had been washed several times in reticulocyte standard buffer (RSB) [0.01 M Tris-HCl (pH 7.4), 0.01 M NaCl, 3 mM

$MgCl_2$ ] without NP40, they were digested in RSB with 0.1–1.5  $\mu g/ml$  of pancreatic deoxyribonuclease (DNAase I; Sigma) at a DNA concentration of 1  $mg/ml$  at 37°C for 10 min. The digestion was terminated by adding Na<sub>2</sub>EDTA (pH 7.2) to a final concentration of 2.0 mM, and the DNA was isolated as described by Stalder et al. (1980).

#### Blotting and Restriction Enzyme Digestions

Purified DNA was digested with restriction enzymes (Biolabs) according to the manufacturers recommendations, and the DNA fragments were separated on 1% agarose slab gels. DNA was transferred to nitrocellulose filters (Schleicher & Schuell BA 85) according to the method of Southern (1975) and hybridized to nick-translated DNA probes (spec. act. 2–4  $\times 10^8$  cpm/ $\mu g$ ) (Weinstock et al., 1978) in 1 M NaCl, 2 mM EDTA, 0.05% SDS, 0.05% Na-Sarcosyl, 0.1% Na-pyrophosphate, 50 mM Tris-HCl (pH 8.0), 5  $\times$  Denhardt's solution (Denhardt, 1966), 50  $\mu g/ml$  each of sheared salmon sperm DNA and poly(A), and 5  $\times 10^6$  cpm of <sup>32</sup>P-labeled probe, for 3–5 days at 65°C, after prehybridizing the filters at 65°C overnight in the same solution without the DNA probe. Filters were washed twice at 65°C for 1 hr intervals in hybridization solution, twice in 0.3 M NaCl, and once in 0.15 M NaCl, and then exposed for 1 day to 1 week at –70°C using an intensifying screen (Dupont).

#### Solution Hybridization

For solution hybridization, nuclei were routinely digested with DNAase I to 15% acid solubility as described by Weintraub and Groudine (1976). The DNA was then purified, treated with alkali (0.3 M NaOH) at 37°C overnight, neutralized, and ethanol-precipitated. Hybridization was performed as described previously (Weintraub and Groudine, 1976).

#### Ovalbumin cDNA

Ovalbumin mRNA was a gift from P. Thomas. cDNA was synthesized under conditions similar to those described by Friedman and Rosbash (1977). A 100  $\mu l$  mixture contained 50 mM Tris-HCl (pH 8.1), 10 mM MgCl<sub>2</sub>, 10  $\mu g$  of actinomycin D, 5 mM dithiothreitol, 0.6  $\mu g$  of (dT)<sub>12-18</sub>, 0.5 mM dGTP, 0.5 mM TTP, 5 nM dCTP, 5 nM <sup>32</sup>P-dCTP (11.1  $\times 10^{12}$  becquerels/mmole), 5  $\mu g$  of RNA, and 40  $\mu l$  of avian myeloblastosis virus polymerase. Incubation was for 4 hr at 37°C, and the reaction was stopped by the addition of NaDODSO<sub>4</sub> to 0.1%. The reaction mixture was centrifuged through a 1 ml column of packed Sephadex G-50 layered over sterile sea sand. The excluded cDNA was adjusted to 0.3 M NaOH and incubated at 65°C for 0.5 hr. The mixture was then neutralized, and the cDNA was precipitated with 2.5 vol of ethanol overnight at –20°C. Specific activity of the probe was approximately 2  $\times 10^8$  cpm/ $\mu g$ .

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#### Note Added in Proof

We have found that while the 3.2 kb  $\beta$ -globin sub-band (Figure 7) is not found in digestive brain or MSB cells, it is detectable in digested DNA from cultured fibroblasts. Other sub-bands have thus far only been observed in erythrocytes and not in brain, MSB cells or fibroblasts. While the biological relevance of the 3.2 kb sub-band in fibroblasts is uncertain at the moment, the observation again reinforces the notion that these sub-bands probably reflect a number of different types of chromosomal modifications involved with several different types of chromosomal functions. After this work was submitted, Kuo, Mandel and Chambon (Nucl. Acids Res. 7, 2105–2113) described similar sub-bands from the 3' side of the conalbumin gene and a similar intermediate level of DNAase I sensitivity for flanking regions of the ovabumin gene.