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Transcriptional Regulation of Hemoglobin Switching in Chicken Embryos

MARK GROUDINE,[1,2]* MARY PERETZ,[1] AND HAROLD WEINTRAUB[1]

Division of Genetics, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104,[1] and Department of Radiation Oncology, University of Washington Hospital, Seattle, Washington 98105[2]

We used recombinant chicken deoxyribonucleic acid clones containing embryonic and adult β-globin genes and "runoff" endogenous nuclear transcription to investigate the expression of embryonic and adult β-globin genes during hematopoiesis in the developing chicken embryo. Purified, cloned deoxyribonucleic acids were digested with various restriction enzymes, separated on agarose gels, blotted to nitrocellulose, and annealed with purified nuclear [^{32}P]ribonucleic acid synthesized in vitro from embryonic or adult red cell nuclei. Transcription of the respective globin genes was assayed by hybridization of nuclear [^{32}P]ribonucleic acid to specific restriction fragments containing adult or embryonic coding sequences. Our results indicate that little, if any, transcription from the adult or embryonic β-globin genes is detectable in the heterologous red cell nuclei, even under conditions in which ribonucleic acid processing probably does not occur.

Differential expression of hemoglobin genes during avian development offers a model system for studying gene regulation at both the cellular and molecular levels. In the developing chicken embryo, hemoglobin first appears in first-generation erythroblasts at approximately 30 to 35 h of development. These cells then serve as the progenitors of six subsequent maturational generations of erythroblasts, each of which contains characteristic amounts of hemoglobin and displays unique morphological characteristics. These cells mature rather synchronously and, by day 6 of embryonic development, most have become erythrocytes. Between days 6 and 7, another lineage of hematopoietic cells, the definitive embryonic lineage, enters the circulating erythrocyte population, arising from the endothelial lining of the blood vessels. The relationship between these two lineages and the possible generation of the definitive series from the immediate precursors of the primitive lineage are the focus of current investigation (2, 16, 17).

The definitive series enters the circulating erythroid compartment between days 6 and 7 as fairly mature erythroblasts and by days 11 to 13 matures into inactive erythrocytes. These cells are the major erythroid cells in circulation until hatching, at which time a second definitive line appears. Although minor differences occur, by and large both definitive lines produce similar types of hemoglobin chains (1), and both differ from the primitive series in the synthesis of specific β chains.

In this and former studies, we have focused on the switch in β-chain synthesis between the primitive and first definitive series. Given the similarities in β-chain synthesis between the two definitive lines, we have not distinguished between the two types of definitive cells, and, for convenience, refer to both as adult red cells since they both produce adult β chains. In contrast, given the distinctive embryonic β chains synthesized in the primitive series, we refer to these cells as embryonic red cells.

In examining the control of embryonic and adult globin gene expressions, we previously reported that adult-specific globin ribonucleic acid (RNA) is not detectable in steady-state cytoplasmic or nuclear RNA (nRNA) from embryonic red blood cells (11). The experiments reported here also focus on the question of whether transcription of the adult and embryonic β-globin genes is restricted to their respective hematopoietic lineages. By employing adult and embryonic β-globin gene-containing recombinants from a library of random chicken deoxyribonucleic acid (DNA) fragments (4, 5) and runoff endogenous nuclear transcription (12, 21) we demonstrate that little, if any, transcription from the embryonic or adult β-globin genes is detectable in the heterologous red cell nuclei, even under conditions in which RNA processing is most likely not occurring.

MATERIALS AND METHODS

Cell and nucleus isolation. Red blood cells from either 5- or 12-day White Leghorn chicken embryos were isolated by vein puncture as described previously

(11, 20) and washed twice with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). Nuclei were isolated by lysis of cells in reticulocyte standard buffer [0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4; 0.01 M NaCl; 3 mM $MgCl_2$] containing 0.5% Nonidet P-40 (British Drug House). The nuclei were washed several times in reticulocyte standard buffer containing Nonidet P-40, suspended at a DNA concentration of 1 to 2 mg/ml in a solution of 40% glycerol, 50 mM Tris-hydrochloride (pH 8.3), 5 mM $MgCl_2$, and 0.1 mM ethylenediaminetetraacetate (EDTA), and used immediately or stored at −70°C.

Nuclear transcription and RNA isolation. Transcription of nuclei and subsequent RNA isolation were performed with modifications of procedures described by McKnight and Palmiter (12). Reaction volumes (200 μl) contained nuclei with 160 to 200 μg of DNA, 30% glycerol, 2.5 mM dithiothreitol, 1 mM $MgCl_2$, 70 mM KCl, 0.25 mM guanosine triphosphate and cytosine triphosphate, 0.5 mM adenosine triphosphate, and 200 μCi of uridine [α-^{32}P]-triphosphate (450 Ci/mmol, ICN). Some reactions were conducted in the presence of 0.5% Sarkosyl (Sigma Chemical Co.) or 1 mg of heparin per ml (Sigma). Nuclei were incubated for 2.5 to 15 min at 26°C, and the reactions were terminated by the addition of deoxyribonuclease I (DNase I) (iodoacetate treated) to 20 μg/ml and further incubation at 26°C for 5 min. The reactions were then deproteinized by incubation at 42°C for 30 min in a solution of 1% sodium dodecyl sulfate, 5 mM EDTA, and 10 mM Tris-hydrochloride (pH 7.4), with 100 μg of proteinase K per ml and subsequent phenol-chloroform extraction. Yeast transfer RNA was added to the resultant aqueous phase to a final concentration of 100 μg/ml, and the aqueous phase was then precipitated at 4°C with cold 5% trichloroacetic acid in the presence of 30 mM $Na_4P_2O_7$. After 30 min, the precipitate was collected on a 25-mm (0.45-μm pore size) nitrocellulose filter disk (Schleicher & Schuell Co., no. BA85) by suction and washed three times with 10 ml of 3% trichloroacetic acid and 30 mM $Na_4P_2O_7$. The disks were transferred to glass scintillation vials, and 0.9 ml of DNase buffer (20 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES] [pH 7.5], 5 mM $MgCl_2$, 1 mM $CaCl_2$) and 25 μg of DNase I (iodoacetate treated) were added. The disks were then incubated for 30 min at 37°C. DNase digestions were terminated by the addition of EDTA to 15 mM and sodium dodecyl sulfate to 1%. RNA was then eluted from the disks by incubation of the filters in the above solution and vial at 65°C for 10 min. After removal of the solution, the filters were incubated in 0.5 ml of 1% sodium dodecyl sulfate–10 mM Tris-hydrochloride (pH 7.5)–5 mM EDTA for an additional 10 min. The two solutions were combined, incubated at 37°C in the presence of 25 μg of proteinase K per ml for 30 min, and extracted twice with equal volumes of phenol-chloroform and twice with chloroform alone. The final aqueous phase was precipitated at −20°C with 0.1 M NaCl and 2.5 volumes of ethanol. The ethanol precipitate was recovered by centrifugation for 30 min at 11,000 rpm in the HB-4 rotor of a Sorval RC-5 centrifuge and suspended in 50 μl of 10 mM Tris-hydrochloride (pH 7.5) and 2 mM EDTA. Omission of the trichloroacetic acid precipitation step resulted in high backgrounds upon subsequent hybridization to Southern blots of restriction enzyme digests of various clones (see below).

Restriction enzyme digestion, blotting, and hybridization. Purified β-globin clone DNAs (provided by D. Engel and J. Dodgson) were digested with restriction enzymes (BRL, BioLabs) according to the manufacturer's recommendations. DNA fragments were separated on 1 or 1.4% agarose slab gels by overnight electrophoresis, and the DNA was transferred to nitrocellulose filters (Schleicher & Schuell Co., no. BA85) by the method of Southern (15). After drying under a heat lamp for 10 min and baking at 80°C under vacuum for 2 to 3 h, the blots were prehybridized at 42°C for 4 to 16 h essentially as described by Wahl et al. (18) in a solution of 50% formamide, 5× SSC, 50 mM phosphate (pH 6.5), 0.02% (wt/vol) each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone, and 250 μg/ml of sonicated, denatured salmon sperm DNA per ml. Hybridization with [^{32}P]RNA was performed at 42°C in four parts of the same buffer and one part of 50% (wt/vol) dextran sulfate. The RNA was denatured at 65°C for 5 min, cooled on ice, and added to the hybridization buffer before addition to the blot. Hybridizations were conducted for 4 days, and the blots were washed with four changes of 2× SSC–0.1% sodium dodecyl sulfate for 5 min each at room temperature and then washed with two changes for 15 min each of 0.3× SSC–0.1% sodium dodecyl sulfate or 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C. Blots were wrapped in Saran Wrap while still damp and exposed for various times to Kodak X-O-Mat film at −70°C with Dupont Cronex Hi-Plus intensifying screens.

In attempting to optimize the conditions of hybridization of in vitro elongated [^{32}P]RNA and immobilized, transferred clone DNA restriction fragments, we have observed no difference in the intensity of the signal when using a 10-fold range of DNA (0.1 to 1.0 μg of coding sequence) and, therefore, conclude that, for the purpose of these studies, hybridization was essentially under the conditions of DNA excess. In addition, no increase in signal was observed after 4 days of hybridization, indicating that saturation was achieved under these conditions.

RESULTS

Preferential transcription of embryonic and adult β-globin genes in their respective lineages. In the following experiments, we employed two recombinant DNA clones containing chicken β-globin genes (provided by D. Engel and J. Dodgson). λCβG-1 (or β1) contains both an embryonic β gene and an adult β-globin gene, and λCβG-2 (or β2) contains a second embryonic β gene as well as an adult β gene, probably the hatching β gene (16, 17). As an assay for the transcription of these various genes in embryonic and adult red blood cells, purified cloned DNAs were digested with various restriction enzymes, separated on 1% agarose gels, blotted to nitrocellulose filters (15), and hybridized

to purified [^{32}P]RNA synthesized in vitro from embryonic or adult red cell nuclei as described above. In this system, RNA chain initiation probably does not occur, so we were measuring the steady-state distribution of elongating endogenous RNA polymerase molecules along each transcription unit. Under these in vitro conditions, the elongation rate is approximately 100 bases per min (12), and, in all of the experiments to be described below, the same results were obtained whether the nuclei were incubated for 2.5 or 15 min. In addition, more than 90% of uridine [^{32}P]triphosphate incorporation was inhibited by α-amanitin (2 μg/ml), and, under such conditions, no globin-specific transcripts were detectable within normal exposure times.

Figure 1a shows the ethidium bromide staining patterns and resultant hybridization to specific regions of β1 after digestion of the clone with *Bam*HI and hybridization with [^{32}P]RNA from either adult or embryonic red cell nuclei. As indicated, digestion of β1 with *Bam*HI resulted in the generation of three chicken sequence-containing fragments: (i) 15 kilobase pairs (kbp), containing λ and noncoding chicken DNA from the 3′ end of the β-globin chromosomal clone; (ii) 10.9 kbp, containing the embryonic gene and sequences flanking this gene on both the 3′ and 5′ ends; and (iii) 9.3 kbp, containing the adult gene and λ DNA. Annealing of blots from *Bam*HI-digested β1 with [^{32}P]nRNA isolated from 5-day embryonic red blood cells resulted in almost exclusive hybridization to the 10.9-kbp embryonic fragment, although a very small signal (<1% of that observed from the 10.9-kbp fragment) could be detected from the 9.3-kbp adult β1 fragment. Conversely, hybridization with [^{32}P]nRNA from 12-day adult red cell nuclei resulted in the almost exclusive labeling of the 9.3-kbp adult fragment, although, again, a small amount of hybridization was observed with the 10.9-kbp embryonic fragment with very long exposures. Given our previous report of the cross-hybridization of the embryonic and adult β-globin genes in β1 (16, 17), we suspect that the small signal observed from the heterologous fragments most likely represents cross-hybridization. Additional evidence for this comes from the observation that low-salt washes preferentially reduce hybridization to the heterologous β-globin gene fragments (data not shown).

Additional data supporting the lineage-dependent transcription of the adult β1 globin gene are shown in Fig. 1b. In this experiment, we hybridized [^{32}P]nRNA from either embryonic or adult red cell nuclei to the complementary DNA

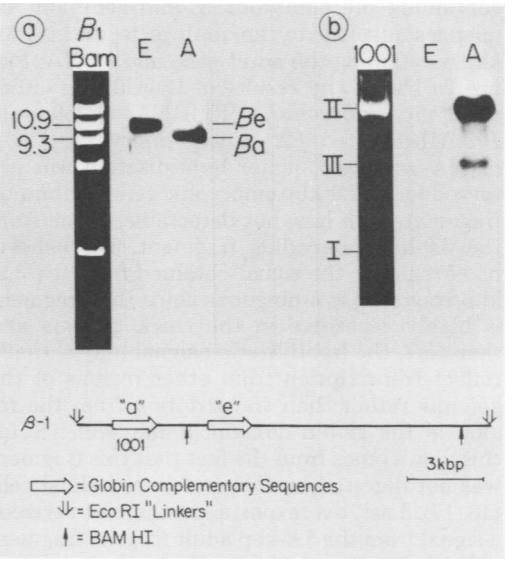

FIG. 1. *Hybridization of in vitro-synthesized [^{32}P]nRNA of 5-day embryonic (lane E) and 12-day adult (lane A) red blood cells to (a) BamHI restriction fragments of β1 and (b) complementary DNA plasmid Hb1001. (a) a and e denote adult and embryonic β-globin genes, respectively, extending over the distance indicated by the thick arrows. The direction of transcription (5′ →3′) was determined by Dodgson et al. (4, 5) and M. Dolan, J. Dodgson, and J. Engel (unpublished data). Restriction sites and kilobase pairs of globin sequence-containing fragments are as indicated. The β1 BamHI lane shows the ethidium bromide stain of β1 fragments after restriction and electrophoresis in a 1% agarose gel: βe and βa indicate those fragments containing embryonic globin sequences and adult globin genes, respectively. (b) Complementary DNA plasmid Hb1001 (14) is homologous to the adult β1-globin gene, as indicated in the diagram of β1. I, II, and III refer to the expected forms of unrestricted plasmid after electrophoresis in a 1% agarose gel.*

plasmid Hb1001 (provided by W. Salser) which contains the coding sequences for the adult β1-globin gene. As shown in Fig. 2b, significant hybridization to this complementary DNA clone was observed only with [^{32}P]nRNA from the 12-day adult red cells, and not that from embryonic cells.

We next analyzed transcription of the embryonic and adult globin genes located on β2. These sequences lie to the immediate 5′ side of β1. Digestion of β2 with *Bam*HI resulted in the generation of five fragments containing chicken sequences which, from the 5′ → 3′ direction, were as follows: (i) 14.8 kbp, containing λ arm and the noncoding sequence; (ii) 0.9 kbp, noncoding; (iii) 3.9 kbp, noncoding; (iv) 7.9 kbp,

containing the embryonic globin gene and sequences adjacent to the adult gene; and (v) 5.8 kbp, containing the adult gene and λDNA. Figure 2a shows the results of hybridizing either adult or embryonic [^{32}P]nRNA to blots of *Bam*HI-digested β2. When embryonic [^{32}P]-nRNA was used, major hybridization was observed to the 7.9-kbp embryonic gene-containing fragment, with less, but detectable, signal from the 3.9-kbp noncoding fragment. We believe, however, that the signal obtained from the 3.9-kbp fragment is ambiguous since this fragment is highly repetitive in the chick genome and therefore, the hybridization signal may actually reflect transcription from other regions of the genome rather than transcription from the region in the globin domain. Some evidence for this view comes from the fact that this fragment was not digested by DNase I in red blood cells (16, 17). Last, overexposure of the blot revealed a signal from the 5.8-kbp adult β2 gene fragment equal to less than 1% of the signal observed from the embryonic β2 fragment. Again, this may reflect cross-hybridization.

When [^{32}P]nRNA from 12-day adult red blood cell nuclei was hybridized with the *Bam*HI-digested β2 blot, almost equal signals were observed from the 5.8-kbp (adult) and 7.9-kbp (embryonic) fragments. In addition, as with embryonic [^{32}P]nRNA, minor signals from the 3.9-kbp noncoding (and repetitive sequence-containing) fragment were also observed with the adult [^{32}P]nRNA. Given the large size of the embryonic 7.9-kbp β2 *Bam*HI fragment, it is possible that hybridization between this region and adult red blood cell [^{32}P]nRNA could result from transcription from sequences outside of the embryonic gene. To test this possibility, we hybridized [^{32}P]nRNA from embryonic adult red blood cells to a blot containing a pBR322 subclone of β2, β2H2, which contains the embryonic β2 gene and its 5′ and 3′ flanking sequences defined by the adjacent *Hin*dIII sites. Significant hybridization was observed between β2H2 and [^{32}P]-nRNA from 5-day embryonic nuclei, but essentially no hybridization was detected between 12-day adult [^{32}P]nRNA and β2H2 at a similar exposure (Fig. 2b). Thus, we feel that these results indicate that hybridization between [^{32}P]nRNA from adult red blood cells and the 7.9-kbp *Bam*HI embryonic β2 gene-containing fragment is a consequence of transcription from a region outside the embryonic β2 coding sequences, possibly just to the left of the *Bam*HI site at the 5′ side of the adult gene in β2 or to the right of the *Bam*HI site of 2.5 kbp on the 5′ side of the embryonic β gene in β2. Also, since these bordering regions contain repetitive DNA

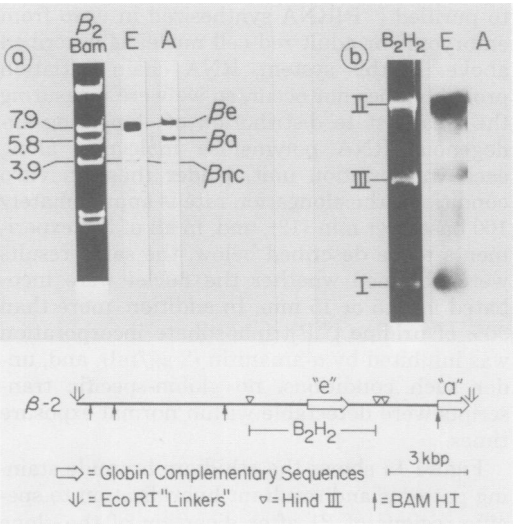

FIG. 2. *Hybridization of in vitro-synthesized [^{32}P]nRNA of 5-day embryonic (lane E) and 12-day adult (lane A) red blood cells to (a) BamHI restriction fragments of β2 and (b) plasmid β2H2. (a) Details are similar to those described in the legend to Fig. 1, except that β2 rather than β1 was used in this analysis. (b) Plasmid β2H2, a subclone of β2 (in pBR322), contains the embryonic β2 gene flanked by the 5′ and 3′ sequences defined by the adjacent HindIII sites. I, II, and III refer to the unrestricted forms of this plasmid.*

sequences, again, it is possible that the transcription signal actually comes from another region of the genome besides the globin domain. DNase I sensitivity experiments support this possibility (16, 17). Thus, we conclude that as our probes approach the size of the known coding regions for adult and embryonic β-globin genes, our ability to detect lineage-specific runoff transcription in β2 is greatly enhanced. In general, both embryonic genes were transcribed in embryonic cells, whereas both adult genes were transcribed in adult cells. Heterologous transcription was at least 100-fold lower in the respective red cell lineages and may even be zero if the weak signal observed proves to come from cross-hybridization.

Transcriptional domain of β1. Given the large sizes of the β1 *Bam*HI fragments containing coding regions of the embryonic and adult β genes contained in β1, little information regarding the transcription of regions around these genes can be derived from the above experiments. Clearly, no transcription can be seen from the 15.5-kbp fragment containing the 3′ region of the chromosomal domain defined by β1 and the λ arm on the right (Fig. 1a).

Given the limitations of our current mapping of the embryonic β gene-containing region of the β1 clone, analysis of the extent of the embryonic β1-globin gene transcription domain by runoff synthesis and hybridization to *Bam*HI-*Hin*dIII, *Msp*I, or *Sac*I digests of β1 (Fig. 3a to c) revealed that major hybridization was observed only in the embryonic coding sequence-containing fragments. Based upon these various experiments, actively transcribed regions of the embryonic gene appeared to be limited to the coding sequences, but could extend as far as 0.9 kbp to the 5′ side of the gene (*Bam*HI site) and 1.8 kbp on the 3′ side (*Hin*dIII site).

The adult β1 gene transcription domain can be mapped with greater precision due to the presence of *Msp*I and *Sac*I sites 0.9 kbp to the 3′ side of the coding sequence and a *Sac*I site approximately 0.4 kbp to the 5′ side of the adult gene (9). As shown in Fig. 3b, the only *Msp*I β1 fragments demonstrating significant hybridization to [^{32}P]nRNA from the adult red cell nuclei were the 1.4- and 1.2-kbp fragments, both of which contain adult coding sequences. The 1.4-kbp fragment is entirely coding, and the 1.2-kbp fragment contains approximately 0.3 kbp of coding sequences and 0.9 kbp of 3′ flanker. In addition, hybridization of [^{32}P]nRNA from 12-day red blood cells to a blot of *Sac*I-digested β1 (Fig. 3c) revealed hybridization to the 1.3- and 1.5-kbp fragments containing adult coding sequences. The 1.3-kbp fragment contains approximately 0.9 kbp of 3′ flanker and 0.4 kbp of coding sequence, whereas the 1.5-kbp fragment contains approximately 0.4 kbp of 5′ flanker and 1.1 kbp of coding sequence. Of significance, we observed no hybridization to a larger *Sac*I fragment containing additional 5′ noncoding chicken sequences and λ DNA. Thus, using this assay, we place initiated RNA polymerases within 0.4 kbp on the 5′ side and 0.9 kbp on the 3′ side of the most stable globin nRNA precursor. It is important to emphasize that the size of the transcription unit as measured by runoff transcription represents a different and independent method from measuring the size of the most stable, labeled nuclear transcript. In the case of the chicken α genes, these two measurements do not, in fact, correspond (Weintraub and Groudine, Cell, in press).

Effect of Sarkosyl and heparin on transcription of globin genes. Although the experiments described in the preceding section indicate that transcription of adult and embryonic β-globin genes is restricted to their respective developmental compartments, two alternative possibilities could also explain our results. (i) Both embryonic and adult globin genes are transcribed in both adult and embryonic red cells, but the heterologous transcripts are degraded so rapidly as to avoid detection, even in our 2.5-min pulses. (ii) Polymerases are present on both embryonic and adult globin genes in red cells of both developmental compartments, but are unable to elongate in the heterologous red cells due to inhibitory chromosomal proteins. Some evidence against preferential degradation comes from the observation that the adult-to-embryonic signal (in either adult or embryonic nuclei) was unchanged during either a 2.5- or 15-min incubation. Nevertheless, we have attempted to test these possibilities by performing the elongation assay in the presence of Sarkosyl or heparin. These compounds remove histones and most other chromosomal proteins, but leave initiated RNA polymerases still bound to DNA and capable of elongation, but not new initiation (6–8, 10); thus, blocked RNA polymerases would most likely elongate if histones are usually preventing such elongation in the intact nuclei. In addition, since heparin is a known inhibitor of ribonuclease (3, 13) and since either heparin and Sarkosyl would be expected to strip proteins from heterogeneous nuclear ribonucleoprotein (hnRNP) and decrease the specificity of sequence-specific RNA nucleases, degradation of specific RNA sequences in the presence of these compounds is less likely.

When either adult or embryonic red blood cell nuclei are incubated in the presence of Sarkosyl or heparin, an approximate 50% decrease in incorporation of uridine [^{32}P]triphosphate is observed, presumably due to a decrease in elongation rate (12). However, no change in the ratio of transcripts from the adult or embryonic β-globin genes was observed in the presence of these compounds (Fig. 3b and c). If either sequence-specific posttranscriptional degradation or blocked RNA polymerases were major regulatory mechanisms in globin gene switching, we would have expected a significant decrease in the ratio of adult to embryonic or embryonic to adult globin gene transcription in the adult and embryonic red cell nuclei, respectively. Similarly, no change in ratio between coding and noncoding regions was observed under these conditions, which utilize a very short (2.5 min) labeling period (Fig. 3b and c). Thus, our results indicate that a major regulatory mechanism in governing hemoglobin switching in the chicken embryo resides at the level of transcription of the respective β-globin genes by RNA polymerase.

To confirm this conclusion, adult and embryonic nuclei were mixed, and transcription was allowed to take place for 2.5 min, either in the

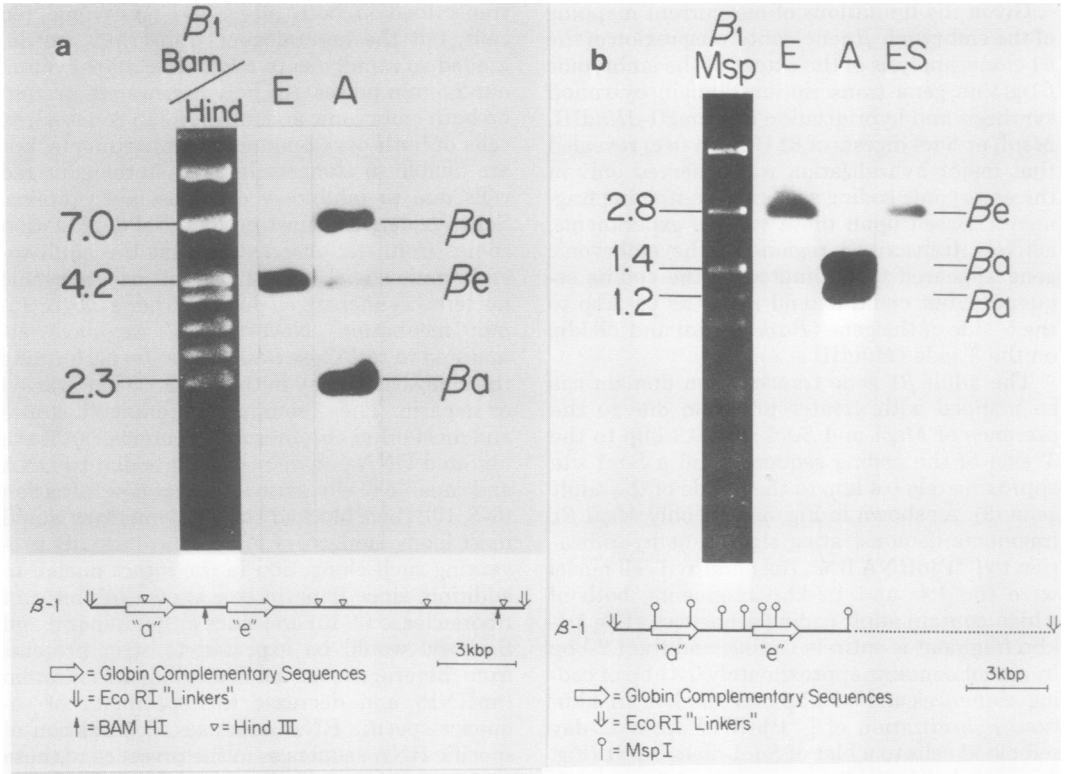

FIG. 3. *Hybridization of in vitro-synthesized [^{32}P]nRNA of 5-day embryonic (lane E) and 12-day adult (lane A) red blood cells to clone β1 DNA fragments generated by digestion with (a) BamHI–HindIII, (b) MspI, and (c) SacI. In addition, [^{32}P]-nRNA was also synthesized in 5-day embryonic red blood cells pretreated and incubated in the presence of Sarkosyl (lane ES) (identical results were obtained when the embryonic nuclei were treated with heparin; similarly, [^{32}P]nRNA from adult nuclei incubated in the presence of Sarkosyl or heparin gave an identical pattern of hybridization to that obtained in lane A [not shown]). Several additional MspI sites are located 5′ to the adult gene (a) in β1 (the embryonic gene is indicated as "e"). However, these are not shown in (b); the size of the fragments generated by digestion at these sites was insufficient for our analysis.*

absence or presence of Sarkosyl. For both conditions, normal amounts of transcription and normal hybridization to both adult and embryonic β-globin genes were observed. This mixing experiment suggests that if the observed differential transcription results from differential degradation then such a process would not only have to be resistant to heparin and Sarkosyl (see above), but would also have to function in *cis*, since, if freely soluble factors were involved, we would have expected that such factors from adult nuclei would degrade the embryonic transcript in the embryonic nuclei and vice versa. As a result, no hybridization to either adult or embryonic β genes would be expected when transcription occurs in the mixed nuclear system. We therefore think that the most likely explanation for the differential expression of β-globin genes in the adult versus the embryonic lineage in chickens is that the genes are differentially controlled at the level of transcription.

In attempting to determine the resolution of our assay, we have used an avian endogenous retrovirus genomic clone as a standard (data not shown). All embryos used in these experiments were virus-free, and over 90% contained a steady-state maximum of one copy per cell of virus-related RNA. About 10% of the embryos, however, contained approximately 50 copies of viral RNA per cell. Thus, on average, each cell contained about five copies of virus-related RNA sequences in steady state. In all of the experiments reported here, we have exposed the hybridized blots for periods of time beyond that needed to yield a clear signal from the endogenous retrovirus DNA fragments. By using such exposures as standards, we estimate that fewer than five copies per cell from the heterologous β-globin genes or from regions outside the coding sequence (in β1) were transcribed in our system. Clearly, this estimate does not control for different rates of RNA processing but nevertheless serves as a rough approximation of the sensitivity of the assay.

DISCUSSION

By employing in vitro runoff transcription of nuclei and subsequent hybridization of the synthesized [^{32}P]nRNA to DNA fragments generated from β-globin clones, we have demonstrated that in embryonic red cells, initiated RNA polymerases are present on the embryonic, but not on the adult β-globin genes, and in adult red cells, nascent RNA polymerases are present on the adult but not the embryonic β-globin genes. Our attempts to determine whether post-transcriptional controls such as differential processing or degradation are important in determining this switch have revealed that such mechanisms most likely do not play a significant role in this phenomenon.

We previously reported that the switch from primitive to definitive erythropoiesis is accompanied by a decrease in DNase I sensitivity of the embryonic β-globin genes as well as the loss of specific DNase I cutting sites at the 5′ side of the embryonic β-globin genes (16, 17, 20). Thus, our current demonstration that expression of the embryonic β-globin genes is limited to the primitive red blood cells is consistent with the notion that a specific chromosomal conformation is coupled to transcription of this gene. In contrast, as assayed by blot hybridization, both the embryonic and adult β-globin genes are digested at approximately the same rate in primitive erythroid cells (16). As assayed by solution hybridization, however, the embryonic β-globin genes are more sensitive than the adult β genes in embryonic cells (20). Thus, it was proposed (17) that the blot sensitivity of the adult gene in embryonic cells reflects a preactivation chromosomal state. The experiments reported here support the conclusion that the adult genes are in a preactivation structure since they show that transcription of the adult genes is limited to the adult cells and is barely, if at all, detectable in embryonic cells. Thus, although an altered chromosomal state is present before overt expression and may, in fact, be required for subsequent expression, that altered conformation of chromatin is insufficient to permit transcription per se.

In our system, heterologous transcription is, at the most, 1% that of homologous transcription. This allows us to estimate whether any polymerases are bound and initiated at heterologous β-globin promoters. Possibly, these polymerases would fail to elongate because the chromatin structure of these genes is not permissive for elongation, either in vivo or in vitro. The globin gene is about 1.5 kbp or about 510 nm. Assuming an RNA polymerase diameter of 30 nm, there is enough room for perhaps 15 tightly packed polymerases along the extended gene. Thus, if a polymerase was initiated in vivo on the heterologous genes, but not elongating, one would expect a signal of about 6% that of the homologous β-globin gene when the supposed block to elongation is removed by in vitro transcription in the presence of Sarkosyl or heparin. Since the signal that we obtained (particularly in the Sarkosyl experiments) was more than sixfold below this level, we think it likely that polymerases are not initiated at promoters associated with the inactive β genes. However, in the appropriate conditions, RNA polymerase II can initiate in vitro on cloned DNA fragments (19); thus, we assume that there are definite mechanisms preventing such initiation in vivo.

It is likely that the folding of DNA into chromatin prevents RNA polymerase binding and initiation at promoter sites.

Our attempts to map the transcriptional domain of the β-globin genes have centered on the adult β gene due to the availability of a detailed restriction map of the regions around this gene (9). Our experiments indicate that transcription is most likely limited to a maximum of 0.4 kbp to the 5′ side of the gene and 0.9 kbp to the 3′ side. Since these findings are reproducible when transcription occurs in the presence of Sarkosyl or heparin, polymerases do not appear bound (and initiated) to sufficiently long regions of the adult β1-globin domain to account for a postulated large "premessenger" RNA molecule containing this particular β-globin gene. Clearly, the data cannot rule out a small number of very long β-globin transcripts. It is also possible that elongation in vivo is so fast over noncoding regions bordering these genes that in our in vitro assay, the steady-state distribution of nascent polymerases along these regions was very low, and, hence, these regions were poorly represented in the transcription assay.

We have also observed that in the in vitro elongation assay, the 3′ sides of the β1 globin gene coding sequence consistently produce more ^{32}P-transcripts than the 5′ regions of the gene (see β1 *Sac*I and embryonic β1 *Msp*I and *Sac*I blots). A small contribution to this effect could come from the fact that while the system elongates, initiation probably does not occur; hence one might expect a small degree of bias toward the 3′ end. However, since the system only elongates about 0.25 kbp in our typical reaction, this effect is probably very small. Rather it may be that the rate of RNA chain elongation is not constant across the length of the gene and that there is a relative pause toward the 3′ end.

Finally, it will be important to extend this type of assay to non-globin-producing cells and, most important, to precursor erythroblasts present in the area opaca of the 20-h chicken blastoderm. Our preliminary results show that not only are the globin genes DNase I resistant in these precursors, but they are also not being transcribed.

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