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Chicken Globin Gene Transcription Is Cell Lineage Specific during the Time of the Switch†

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ABSTRACT: Posttranscriptional silencing of embryonic globin gene expression occurs during hemoglobin switching in chickens [Landes, G. M., Villeponteau, B., Pribyl, T. M., & Martinson, H. G. (1982) *J. Biol. Chem. 257*, 11008–11014]. Here we use Percoll density gradients to fractionate the red blood cells of 5–9-day embryos in order to determine the cellular source and the timing of this posttranscriptional process. By means of nuclear "run-on" transcription in vitro we show that it is within mature primitive cells that production of embryonic globin mRNA is terminated posttranscriptionally. In contrast, young definitive cells produce little (or no) embryonic globin mRNA because of regulation at the transcriptional level. Thus the lineage specificity of embryonic and adult globin gene expression is determined transcriptionally, and the post-transcriptional process described by Landes et al. is a property of the senescing primitive cells, not a mechanism operative in the hemoglobin switch. This conclusion is supported by [^{3}H]leucine incorporation experiments on Percoll-fractionated cells which reveal no posttranscriptional silencing of the embryonic genes during the early stages of the switch. In the course of our studies we have noticed a strong transcriptional pause near the second exon of the globin genes which is induced by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and which resembles a natural pause near that position.

During avian development there are, as in mammals, switches in the types of hemoglobins expressed. Prior to 6 days of incubation circulating erythroid cells in chicken embryos express exclusively embryonic hemoglobins while after the 12th day of development only adult hemoglobins are expressed (Bruns & Ingram, 1973). The molecular and cellular mechanisms governing these switches are difficult to address experimentally because a complete cellular change in erythroid lineage takes place simultaneously with the molecular hemoglobin switch. Erythroid cells of the primitive lineage arising from the blood islands of the area vasculosa are the first to enter circulation during the second day of incubation. These cells mature as a cohort and are the only recognizable erythroid cells in the embryo until the 5th day when cells of the definitive lineage begin entering circulation. As the circulation expands, the definitive erythroid cells rapidly outnumber the senescing primitive cells which become virtually undetectable by 16 days of development (Bruns & Ingram, 1973). The switch in hemoglobin expression and the switch in erythroid lineage are well correlated. Prior to 5 days of incubation only embryonic hemoglobins are expressed at a time when only primitive cells are found in circulation. Adult hemoglobins begin to appear during the 6th day of incubation simultaneously with the first appearance of definitive erythroid cells.

In mature primitive and definitive erythroid cells taken from embryos prior to (i.e., at 5 days) or following completion of (12–14 days) the hemoglobin switch, the choice among globins is made at the transcriptional level (Groudine et al., 1981; Landes et al., 1982). For such embryos, either before or after the switch, transcriptional specificity, translational output, and cell lineage are all correlated. However, during the actual period of switching (6–9 days), when both primitive and definitive cells are present simultaneously in the circulation, the rate of transcription of the embryonic genes remains disproportionately high, indicating the contribution of a posttranscriptional process that silences the embryonic genes (Landes et al., 1982). These and related data led to the suggestion that the embryonic globin genes are active in im-

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In-Register Homodimers of Smooth Muscle Tropomyosin†

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ABSTRACT: Gizzard smooth muscle tropomyosin dimer molecules were dissociated by guanidinium chloride and reassociated by dialysis against 1 M NaCl. Several properties of the protein were changed by this treatment. There was a large decrease in tropomyosin's low-salt viscosity, owing to reduced end-to-end polymerization, the helix unfolding profile changed from a one-step to a two-step process, and the ability to form intramolecular, interchain, disulfide-cross-linked homodimers increased dramatically. Thus, the native molecule, thought to exist predominantly as the $\beta\gamma$ heterodimer which cannot form disulfide cross-links [Sanders, C., Burtnick, L. D., & Smillie, L. B. (1986) *J. Biol. Chem. 261*, 12774–12778], reassembles, after dissociation, to form predominantly parallel, in-register $\beta\beta$ and $\gamma\gamma$ homodimers able to form disulfide cross-links. This suggests that the physical properties, including the end-to-end interaction, of gizzard tropomyosin homodimers differ considerably from those of the heterodimer. This is a first step toward a molecular understanding of the end-to-end interaction of smooth muscle tropomyosin.

Muscle contraction involves the interaction of the myosin-containing thick filaments with the actin-containing thin filaments, and the hydrolysis of ATP by this actomyosin complex provides energy for the contractile process. Rod-shaped tropomyosin binds end-to-end along the actin thin filament and together with troponin in striated skeletal muscle [see Leavis and Gergely (1984) for a review] or with caldesmon in smooth gizzard muscle [see Marston and Smith (1985) for a review] takes part in the Ca^{2+} regulation of contraction. Tropomyosin's end-to-end interaction is thought to play a crucial role in the cooperative features of this regulation in skeletal muscle [see Leavis and Gergely (1984) for a review]. It has been observed that gizzard tropomyosin regulates with a greater cooperativity than does skeletal muscle tropomyosin (Lehrer & Morris, 1984). The fact that gizzard tropomyosin has a greater end-to-end interaction than skeletal tropomyosin (Tsao et al., 1956; Dabrowska et al., 1980; Sanders & Smillie, 1984; Nowak & Dabrowska, 1985) may, in part, account for this observation.

The end-to-end polymerization of tropomyosin increases with decreasing salt concentration as inferred from the increase in viscosity with decreasing salt concentration for both skeletal (Bailey, 1948; Kay & Bailey, 1960; Ooi et al., 1962) and gizzard (Tsao et al., 1956; Dabrowska et al., 1980; Sanders & Smillie, 1984; Nowak & Dabrowska, 1985) tropomyosins. The end-to-end interaction of skeletal tropomyosin involves the overlap of eight to nine terminal residues (Johnson & Smillie, 1975, 1977; McLachlan & Stewart, 1975; Phillips et al., 1979). However, the molecular basis of gizzard tropomyosin's greater end-to-end interaction is unknown.

Gizzard tropomyosin, like skeletal tropomyosin, is composed of two coiled-coil, α-helical polypeptide chains with an α-helical content close to 100%, giving rise to a high ellipticity at 222 nm in the circular dichroism spectrum (Woods, 1969a, 1976). Skeletal tropomyosin contains 2 types of chains (Cummins & Perry, 1973), α and β, both containing 284 residues with a cysteine residue at position 190 (Mak et al., 1980). Gizzard tropomyosin also contains 2 types of chains, β and γ [also referred to as α and β, respectively; see Lau et al. (1985)], of roughly equal concentration (Cummins & Perry, 1974) and each containing 284 residues (Helfman et al., 1984; Sanders & Smillie, 1985; Lau et al., 1985). The β chain contains one cysteine residue at position 36 (Helfman et al., 1984; Sanders & Smillie, 1985), and the single cysteine of the γ chain is located at position 190 (Lau et al., 1985). The two chains of a skeletal tropomyosin molecule are in-register and parallel since they can easily and quantitatively form an intramolecular, interchain disulfide bond at cysteine-190 by oxidation (Johnson & Smillie, 1975; Stewart, 1975) or disulfide exchange (Lehrer, 1975). In contrast to skeletal tropomyosin, gizzard tropomyosin forms only a fraction of disulfide cross-links with difficulty by oxidation (Strasburg & Greaser, 1976; Lehrer et al., 1984), and none are formed by disulfide exchange (Lehrer et al., 1984). Cross-linking of gizzard tropomyosin with succinimide cross-linkers results in small amounts of cross-linked dimers, predominantly heterodimers, suggesting that gizzard tropomyosin is composed predominantly of heterodimers, which cannot form disulfide cross-links since the cysteines on the two chains are widely separated (Sanders et al., 1986).

As part of a study to understand the role of tropomyosin in smooth muscle regulation, we observed that there was a large drop in the low-salt viscosity of gizzard tropomyosin after the protein was dissociated with guanidinium chloride and reassociated by dialysis. Concomitant with this change there was a change in gizzard tropomyosin's helix unfolding profile and a dramatic increase in the ability to form disulfide-cross-linked $\beta\beta$ and $\gamma\gamma$ homodimers. These results, together with the view that native gizzard tropomyosin is composed primarily of heterodimers (Sanders et al., 1986), suggest that the physical properties, including the end-to-end interaction, of the heterodimer and homodimers of gizzard tropomyosin differ considerably.

Experimental Procedures

Chicken gizzard tropomyosin was prepared at 4 °C as described previously (Graceffa, 1987) and will be referred to as native gizzard tropomyosin (N-TM).[1] In some cases, N-TM

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[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; N-TM, native gizzard tropomyosin; DR-TM, N-TM chains dissociated and then reassociated; DRDR-TM, DR-TM chains dissociated and then reassociated.

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Table I: Conditions for Separation of Red Blood Cells from Different Age Embryos[a]

embryo age (days)	vol of cells (mL) added to 30 mL of Percoll	spin time (min)
5	12.0	15
6	4.5	30
7	4.5	30
8	4.2	30
9	3.9	30
10	3.6	30
11	3.3	30
12	3.0	30

[a] The parameters varied to achieve optimal separation were the initial density of the Percoll–red blood cell mixture and the time of centrifugation at 27000*g*. The initial density was varied by changing the volume of cells (in PBS) added to a stock solution of Percoll (made isotonic with PBS) having a density of 1.132 g/mL.

Southerns. The methods are essentially those used by Landes et al. (1982). After the specific clones were digested with the appropriate enzymes, 1.5 μg of DNA was electrophoresed in a 2% agarose gel (Tris–acetate buffer) and blotted onto nitrocellulose by using 20 × SSC. The blots were prehybridized overnight.

Dot Blots. DNA samples were depurinated by incubation in 0.2 M sodium acetate, pH 4, at 55 °C for 15 min, and then NaOH was added to 0.3 M. The mixture was incubated at room temperature for 20 min and neutralized, and then an equal volume of 20 × SSC was added. Each sample was applied to a miniblot apparatus (BRL), filtered through nitrocellulose (0.45 μm from Millipore Corp.) with minimal suction, and baked for 2 h at 80 °C under vacuum.

Hybridization. This was carried out essentially as described in Landes and Martinson (1982), using 50% formamide at 43 °C for 72 h with washing at high stringency (65 °C, 0.1 × SSC).

Clones. All the globin clones used as well as their map positions in the globin region are described in Villeponteau et al. (1982). However, the nomenclature has been revised slightly.

Results

Separation of Chicken Embyro Erythroid Cells in Percoll Density Gradients. The circulating blood in chicken embryos consists of a heterogeneous mixture of erythroid cells of different lineages and at different stages of maturation. In order to study the globin gene switch in cells of known lineage and degree of maturation, we have adapted the use of Percoll for the separation of large quantities of red blood cells into fairly homogeneous populations using self-generated density gradients. Percoll density gradients offer several advantages over sedimentation velocity methods of cell separation. Such advantages include speed, simplicity, capacity, minimal interference by cell aggregation, and improved resolution for density-related cellular parameters.

As erythroid cells mature, their density increases. Figure 1A illustrates that the increasingly mature cells from embryos of increasing age between 5 and 9 days of development band at higher densities. It can be seen that the cells at 6 days of development are highly heterogeneous owing to the simultaneous presence of large numbers of both immature definitive and mature primitive cells.

Figure 1B illustrates that the differences in banding position reflect genuine differences in cell density. Cells from a 7-day embryo were fractionated in a shallow gradient, and the top and bottom banding cells were then respun in fresh gradients. Figure 1B shows that the cells reband true. Moreover, no contaminating cells were observed in the rest of the gradient upon examination in a hemacytometer. The same result was obtained when a 2-h incubation at 0 °C prior to the second centrifugation was included, indicating that cell density is a stable parameter which can be used reliably for separation of the different red blood cells of the chicken embryo. Optimal conditions for cell separation for different age embryos are presented in Table I.

Table II: [^{3}H]Leu Incorporation by Percoll-Separated and Nonseparated Red Blood Cells[a]

Percoll fraction	cpm incorporated in 20 min	40 min	60 min
5 (top)	6228	11754	15901
4	5260	7360	8603
3	4516	6773	8735
2	2511	4325	6246
1 (bottom)	1625	2571	3206
average	3748	6609	8801
unseparated	4223	5327	7435

[a] Unseparated or Percoll-separated red blood cells from 7-day embryos were incubated in the presence of [^{3}H]Leu at 37 °C. Aliquots were taken at 20, 40, and 60 min, trichloroacetic acid precipitated, and counted in scintillation fluid. The numbers are presented as cpm incorporated per 10^6 cells. The average incorporation of counts for the separated cells was calculated as the weighted average of the counts from all five fractions. The value for unseparated cells was obtained directly from an aliquot of cells that had not been fractionated.

Table III: In Vitro Transcription of Nuclei from Percoll-Separated and Nonseparated RBC[a]

Percoll fraction	cpm per 10^4 nuclei
5 (top)	796
4	675
3	944
2	517
1 (bottom)	825
average	740
unseparated	680

[a] Red blood cells from 9-day embryos were fractionated and washed free of Percoll. Nuclei were then isolated and allowed to transcribe in vitro. The average transcriptional activity of 9-day cells after Percoll separation was calculated as the weighted average of the transcriptional activities for all five fractions. The value for unseparated cells was obtained separately for nuclei from an aliquot of unfractionated cells.

Representative photomicrographs of cell spreads from the different fractions of Percoll-separated 6-day cells are shown in Figure 1C. The most dense fraction at the bottom contains almost exclusively large and lightly stained mature primitive cells while the least dense fraction at the top contains primarily the smaller and darker, less mature definitive cells. Upon examination of several micrographs we determined that cross-contamination amounted to about 3% and 7%, respectively. Moreover, the few dense definitive cells that can be observed near the bottom of the gradient (not shown) have a decreased cytoplasmic/nuclear ratio, characteristic of more mature cells, compared to those at the top. Thus there is a good separation of cells according to maturity both within and between lineages.

In order to determine whether centrifugation in Percoll has any affect on the metabolic state of the cells, we analyzed the in vivo protein-synthesizing capacity (Table II) and the in vitro transcriptional activity (Table III) for 9-day separated red blood cells and compared these values to those for unseparated red blood cells. It is evident that the average values of radioactivity incorporated into either protein or nascent nuclear transcripts for the separated cells are close to the values for unseparated blood, suggesting that the manipulations involved

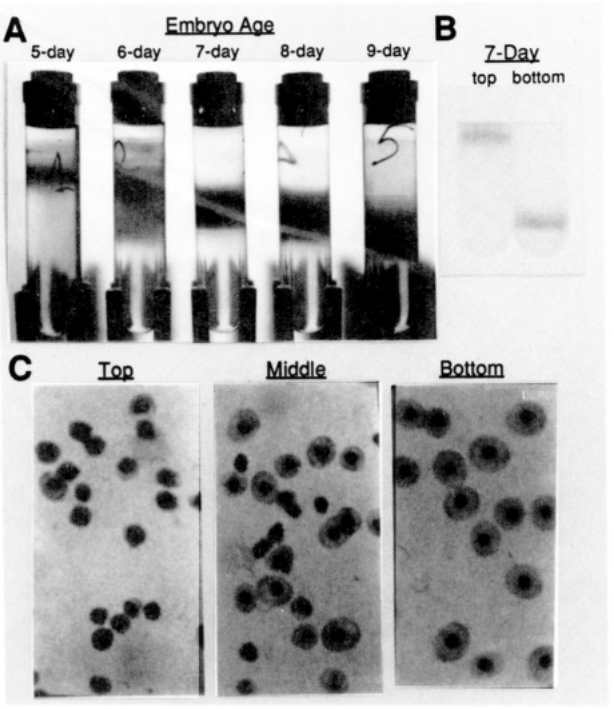

FIGURE 1: Fractionation of red blood cells in Percoll gradients. (A) Cell density increases with embryo age. Erythroid cells in PBS (1.5 mL) were mixed with 10 mL of Percoll/PBS and then centrifuged in a Beckman Ti-75 rotor at 17 000 rpm for 30 min to generate the gradient and band the cells. (B) Percoll-separated cells reband true. Red blood cells in PBS (4.5 mL) were mixed with 30 mL of Percoll/PBS and centrifuged in a Sorvall SS-34 rotor at 15 000 rpm for 15 min. Cells from the top and bottom of the gradient were washed free of Percoll, resuspended in 4.5 mL of PBS, and then mixed with fresh Percoll/PBS and recentrifuged. (C) Percoll-separated cells from 6-day embryos were stained and then photographed under a Zeiss microscope. Fraction 5 of the gradient (top) contains primarily small, blue definitive cells. Fraction 1 (bottom) contains primarily large, pink primitive cells. The definitive cells (i.e., the smaller, darker cells) in each fraction show a decrease in the cytoplasmic/nuclear ratio as the fractions get denser.

in this type of separation do not affect the metabolic integrity of the cells.

Posttranscriptional Silencing of ρ during the Switch: The Young Definitive Cells. To investigate the basis of the posttranscriptional silencing of the ρ gene during switching, we have analyzed both transcription itself and the levels of accumulated transcripts for cells separated on Percoll gradients. If, as originally suggested (Landes et al., 1982), the least mature definitive cells present at 6 and 7 days of development transcribe all the globin genes but express only the adult globin proteins owing to differential processing of the transcribed RNA, then we would expect the cells from the least dense Percoll fractions (i.e., the immature definitive cells) to exhibit active embryonic gene transcription but to contain much lower levels of stable embryonic gene transcripts. This would be reflected in a ratio of embryonic to adult transcripts that would be much higher for the nascent RNA than for steady-state RNA. We have used "run-on" transcription of isolated nuclei followed by hybridization to Southern blots of cloned DNAs to estimate nascent RNA levels. Steady-state transcript levels were measured by hybridizing total cellular RNA (fragmented and end labeled) to similar blots.

Table IV shows the relative levels of nascent and steady-state RNA for the embryonic ε and ρ genes, compared to that of the adult β gene for fractionated cells. The ε/β and ρ/β ratios are essentially the same for both nascent and steady-state RNA for the top-banding, least mature cells at 6 and 7 days

Table IV: Comparison of Transcription Rate and Transcript Accumulation for ε and ρ RNA in 6- and 7-Day Percoll-Separated Red Blood Cells[a]

	ε/β		ρ/β	
Percoll fraction	nascent	steady state	nascent	steady state
6 day				
5 (top)	0.27	0.16	0.38	0.33
4	0.44	0.45	0.47	1.6
3	0.92	2.2	0.76	3.4
2	1.1	9.2	1.4	12
1 (bottom)	2.1	8.4	3.4	8.0
7 day				
5 (top)			0.60	0.55
4			1.3	2.3
3			1.4	3.0
2			2.6	5.0
1 (bottom)				

[a] Nuclei from 6- and 7-day Percoll-separated cells were allowed to transcribe in vitro. The level of transcription (nascent) of the embryonic genes, ε and ρ, was quantitated by densitometry of the resulting autoradiograms and normalized with respect to the β gene. The same treatment was applied to autoradiograms of hybridized end-labeled whole cell RNA (steady state) from the same cell fractions.

of development, indicating little or no preferential degradation of the embryonic globin RNA sequences in these cells. Therefore, in the young definitive red blood cells entering the circulation at 6 and 7 days of embryonic development no significant amount of posttranscriptional silencing of the embryonic genes occurs, at least at the level of RNA processing. Indeed, even the small amount of embryonic gene transcription itself in the top-banding cells (evidenced by the nonzero ε/β and ρ/β ratios) can be accounted for by the small numbers of contaminating primitive cells present in the top Percoll fractions.

Inspection of the remaining ratios in Table IV confirms the validity of the Percoll fractionation procedure. Thus, as the proportion of primitive cells increases going down each gradient, so does the proportion of embryonic globin sequences. Moreover, as expected, the increase is greater for the steady state than for the nascent RNA, reflecting the accumulated stable mRNA in the more mature primitive cells lower in the gradient.

The data of Table IV have ruled out posttranscriptional silencing of the embryonic genes (especially for ρ which is the most active and gives the best signal) at the level of RNA processing for 6- and 7-day cells but leave open the possibility of such an effect at the level of translation. To evaluate this possibility, we compared hemoglobin protein synthesis with the levels of mRNA present in Percoll-fractionated cells. Red blood cells from 5-, 6-, and 7-day embryos were isolated and fractionated, and each fraction was divided into two, one part for RNA isolation and the other for [^{3}H]Leu incorporation into hemoglobin. The isolated RNA was end-labeled and hybridized to a dot blot of embryonic and adult β-type globin clones and an α-globin control (Figure 2A). The ratios of embryonic (ε or ρ) vs adult (β) globin transcripts obtained from densitometric scans of the resulting autoradiograms are shown as ε/β and ρ/β in Table V. The pulse-labeled hemoglobins were separated in isoelectric focusing gels and fluorographed (Figure 2B). The ratios of the rates of synthesis of embryonic (E or P) vs adult (A) hemoglobins were obtained from densitometric scans and are shown in Table V as E/A or P/A (E, P, and A hemoglobins contain ε, ρ, and β as their β-like chains, respectively).

Table V reveals no significant translational control between 5 and 7 days of development. In all cases the ε/β and ρ/β mRNA ratios adequately account for the pattern of hemo-

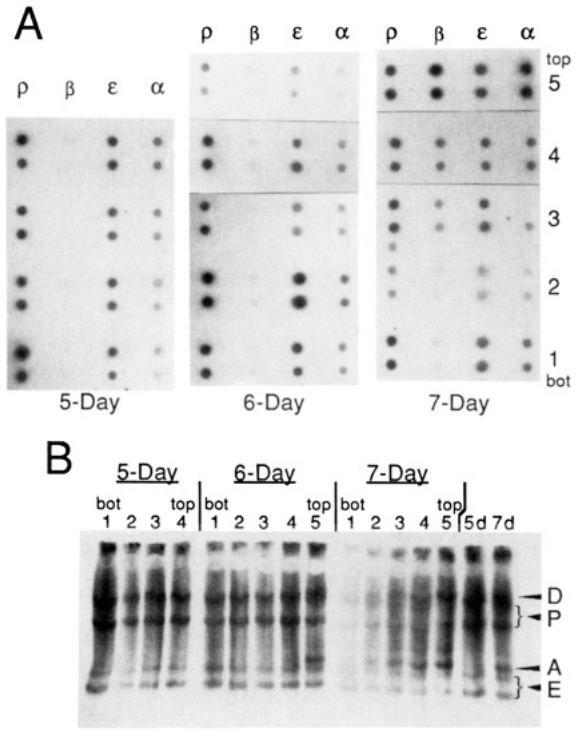

FIGURE 2: (A) Globin mRNA steady-state levels and (B) hemoglobin synthesis in Percoll-separated 5-, 6-, and 7-day red blood cells. Total cell RNA was end-labeled and hybridized to duplicate dots of cloned DNA containing the indicated globin gene sequences. Hemoglobin was pulse-labeled by incubation of the Percoll-separated cells for 1 h at 37 °C in the presence of [^{3}H]leucine. The hemoglobins were separated in an isoelectric focusing gel (pH 7–9) and fluorographed with PPO/Me_2SO. The lanes labeled 5d and 7d contain hemoglobins from 5- and 7-day blood not separated in Percoll. The positions of the different hemoglobins (D, P, A, and E) are indicated.

globin synthesis as shown by the E/A and P/A ratios.

Therefore, these data, which show no translational control, together with the previous data showing no differential RNA processing, demonstrate that globin gene switching in chickens is regulated at the level of transcription between 5 and 7 days of development.

Late Transcription by Old Primitive Cells. During switching the principal red blood cell types present in the embryo are the young definitives and the old primitives. Since the embryonic globin genes are not being transcribed, or silenced, to any significant extent in the young definitive cells at 6 or 7 days of development, we focused next on the old primitives present at 9 days. It was for the 9th day of development that Landes et al. (1982) obtained the strongest evidence for the posttranscriptional silencing of the ρ-globin gene.

The histograms in Figure 3A show the levels of transcription of the ρ and β genes in nuclei from immature and mature cells from 9-day embryos. The data show that, if anything, more ρ gene transcription fractionates with the mature (bottom) than with the immature (top) cells (despite a low level of $\beta \rightarrow \rho$ cross-hybridization which augments ρ hybridization preferentially for the immature cells). The bottom cells consist of mature definitive cells mixed with small numbers of senescent primitive cells. Since the definitive cells, specialized for β gene expression, cannot be presumed to decrease β and increase ρ gene transcription during maturation, we conclude that it is the senescent primitive cell population that transcribes the ρ gene at 9 days.

Senescing primitive cells, however, are thought to be transcriptionally inactive. For example, by 9 days, primitive cells

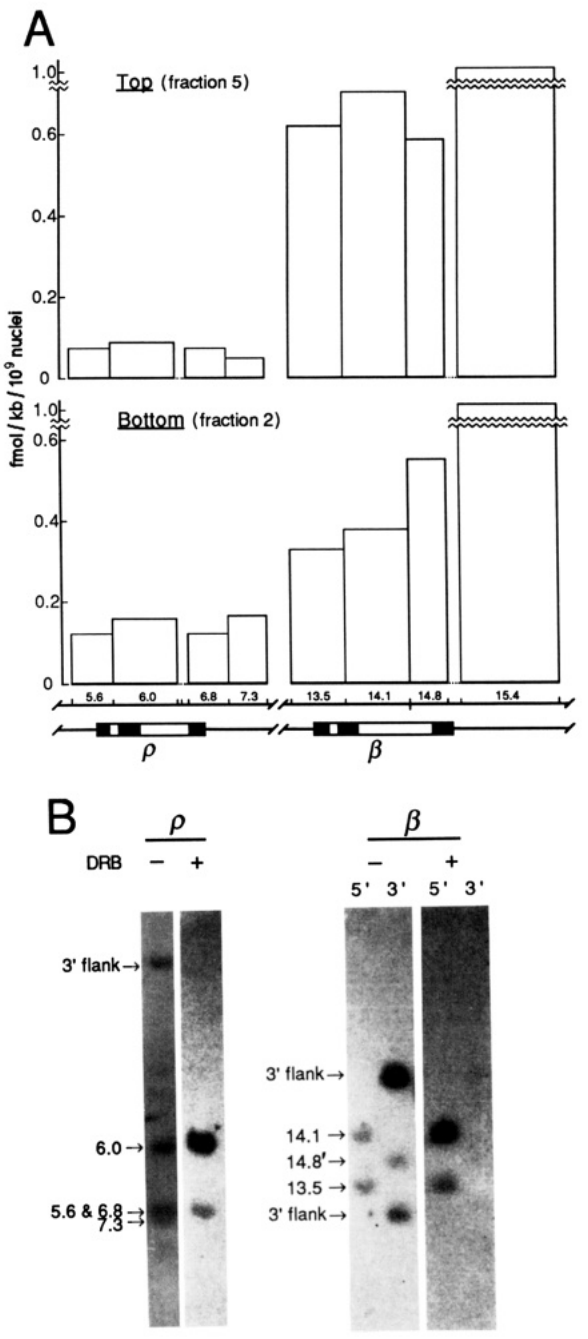

FIGURE 3: Transcription of the ρ and β genes in 9-day red blood cell nuclei. (A) Nuclei from mature primitive cells transcribe ρ in vitro. Run-on transcripts from nuclei of Percoll-separated cells were hybridized to a Southern blot of restriction enzyme digested clones containing the ρ and β-globin genes. Fraction 5, from the top of the gradient, contains primarily the least mature definitive cells while fraction 2, from near the bottom, contains a mixture of mature primitive and definitive cells. The extent of hybridization was quantitated by densitometry and plotted as a histogram as previously described (Landes et al., 1982). Absolute hybridization values were determined following excision and scintillation counting of selected bands from the Southern blots. However, for comparison to previous data (Landes et al., 1982; Villeponteau et al., 1982) it is necessary to adjust for a calculation eror which rendered those values too large by 100-fold. (B) Mature primitive cells transcribe ρ in vivo. Intact erythroid cells from 9-day embryos were incubated in the presence (+) or absence (−) of DRB for 1 h. Nuclei were then isolated and allowed to transcribe in vitro in the absence of DRB. Hybridization was as above. The ρ gene is contained in clone pCBG4 which was cut with *Pst*I. The β gene is contained in clones pCBG13 (5′ part) and 15 (3′ part) which were cut with *Hha*I. The fragments map as shown in part A except for pCBG15 which was cut with *Hpa*II for part A. However, *Hha*I fragment 14.8′ (part B) is very similar in size and position to *Hpa*II 14.8 (part A).

Table V: Comparison between Levels of Steady-State RNA and Rate of Protein Synthesis for the Embryonic Hemoglobins[a]

	RNA		protein	
Percoll fraction	ϵ/β	ρ/β	E/A	P/A
5 day				
4 (top)	10.8	12.4	>10	>10
3	10.4	13.3	>10	>10
2	9.3	10.3	>10	>10
1 (bottom)	8.3	16.1	>10	>10
6 day				
5 (top)	3.0	4.3	1.2	4.4
4	5.4	11.6	~7	>10.0
3	13.6	18.1	>10.0	>10.0
2	14.0	12.6	>10.0	>10.0
1 (bottom)	11.8	13.2	>10.0	>10.0
7 day				
5 (top)	0.5	0.6	0.1	0.8
4	1.3	2.1	0.3	2.2
3	2.1	2.9	0.6	1.9
2	3.1	3.1	0.9	2.7
1 (bottom)	6.3	8.4	7.5	5.7

[a] The steady-state levels of the embryonic ϵ and ρ mRNAs were quantitated by densitometry of the dot blot in Figure 2A and then normalized to the level of the adult β-globin message. The rates of embryonic hemoglobin (E and P) synthesis were quantitated from the fluorogram in Figure 2B and normalized to the rate of synthesis of the adult hemoglobin A. Where hemoglobin A was too low for accurate quantitation, we show ">10" for the ratio.

show only traces of cytoplasmic reticulum (Bruns & Ingram, 1973), have pycnotic nuclei (Lucas & Jamroz, 1961), and have essentially finished hemoglobin synthesis (Campbell et al., 1971). Therefore, we considered the possibility that the run-on transcription assay may be artifactual in showing ρ gene transcription in the senescent primitive cells. This transcription assay is based on the in vitro extension of nascent RNA by RNA polymerases which in vivo were tightly complexed with the DNA as active transcription complexes. However, it is possible that the transcription complexes are not actively transcribing in vivo but are immobilized during nuclear pycnosis [e.g., see Gariglio et al. (1981)] and are then reactivated during the manipulations in vitro.

In order to determine whether the ρ transcription observed at 9 days is artifactual, we used the transcription inhibitor DRB. DRB has been reported to induce premature termination (Tamm et al., 1980; Skolnik-David et al., 1982), to cause increased pausing of polymerases at specific gene sequences (Maderious & Chen-Kiang, 1984), and to act as an inhibitor of initiation (Zandomeni et al., 1986; Mukherjee & Molloy, 1987). The effects of DRB are reversible (Maderious & Chen-Kiang, 1984; Zandomeni et al., 1986), and whatever its mechanism of action, the net result of DRB treatment is a change in the steady-state distribution of polymerases engaged in transcription in vivo.

In our experimental design *intact cells* are treated with DRB. The nuclei are subsequently isolated by using buffers lacking DRB and are allowed to transcribe in vitro in the absence of DRB. Thus any observed effect of DRB on nuclear transcription must have been exerted in vivo. Moreover, any DRB-induced change in the distribution of polymerases obviously can only occur if the polymerases can move, i.e., if they are actively transcribing in vivo. Therefore, we treated 9-day red blood cells with DRB and assayed, by nuclear run-on transcription, the RNA polymerase distribution across the ρ gene for nuclei from cells that were previously treated or untreated with DRB. If there is a difference, the polymerases are transcribing in vivo.

The autoradiograms of Figure 3B show the hybridization of run-on transcripts from the nuclei of 9-day red blood cells incubated in the absence (−) or presence (+) of DRB. Since the vast majority of 9-day cells are definitive cells known to be engaged in active transcription of the adult β-globin gene, we use this gene as the control. The β panel of Figure 3B shows that DRB treatment of cells leads to reduced run-on transcription of the β 3′ flanking sequences as well as of fragment 14.8′, which contains the third exon, but increased transcription of fragment 14.1, which contains the second exon (see map in Figure 3A). Thus DRB appears to induce pausing within or near the second exon of the β gene in vivo such that the third exon becomes depleted of polymerases.

Figure 3B shows that the effects of DRB on the ρ gene are similar to its effects on the β gene. Polymerases are depleted from the 3′ flanking region, including fragment 7.3, but are increased in density within the second exon contained in fragment 6.0 (exons 1 and 3 are not resolved in this blot). Thus the polymerase distribution over the ρ gene at 9 days is, like that of the β gene, sensitive to DRB, indicating that the ρ gene is indeed active in vivo. This shows that primitive cells as late as in the 9th day of development are still engaged in transcription of the ρ gene in vivo despite being fully mature and displaying many senescent characteristics including the loss of protein-synthesizing ability. Therefore, it is within these cells that ρ is silenced posttranscriptionally.

Discussion

The above experiments have established that during the initial stages of hemoglobin switching in chick embryos (6–7 days of development) the switch is regulated principally if not solely at the level of transcription. Moreover, by corroborating further the linkage between transcriptional specificity and erythroid cell lineage, these experiments (Table IV and Figure 3A) favor, for chick embryogenesis, the cellular model for hemoglobin switching (Ingram, 1972; Godet, 1974; Papayannopoulou et al., 1977; Beaupain, 1985). According to this model one type of hemoglobin is replaced by another in the embryo as a consequence of substituting one specifically committed cell lineage for another rather than by altering the regulation of specific genes within existing, more flexibly committed cells. Although Chapman and Tobin (1979) have previously reported that both embryonic and adult globins are present simultaneously in immature definitive cells, Beaupain (1985) has reported immunofluorescent data at variance with those of Chapman and Tobin (1979).

The posttranscriptional silencing of the embryonic globin genes previously described by Landes et al. (1982) apparently operates toward the end of the switching period (ca. 9 days) and involves only the primitive cells which, because of senescence and dilution by the definitive cells, are already inconsequential to the embryo. Thus the posttranscriptional turnoff of the embryonic genes is more appropriately viewed as a concomitant of the inevitable senescence and death of the primitive cells rather than as a significant contributor to the molecular mechanism of the hemoglobin switch itself. Interestingly, the machinery for processing and translation of mRNA is dismantled in the 9-day primitive cells even as active transcription continues. Nine-day primitive cells have been postmitotic for several days, they have nuclei that are pycnotic, and most of them have a cytoplasm in which hemoglobin synthesis itself is essentially finished (Bruns & Ingram, 1973; Lucas & Jamroz, 1961; Campbell et al., 1971). A similar situation has recently been shown to exist for the definitive cells of the adult (Affolter et al., 1987).

However, the continued transcription of the embryonic genes in the isolated nuclei of senescing primitive cells is surprising, less for the simple fact that it occurs, than because it is dy-

namically responsive to treatment with DRB in vivo. This rules out for these cells the conventional explanation for the existence of active run-on transcription (Gariglio et al., 1981), namely, that RNA polymerases have become trapped within the condensed, inactive chromatin structure of the nucleus in vivo and are activated upon incubation in vitro. Moreover, it is interesting that DRB induces pausing near the second exon not only of ρ and β (Figure 3B) but also of ϵ and β^H (data not shown). Gariglio et al. (1984) have reported that the RNA polymerases of mature erythrocytes of the adult chicken are paused, naturally, at a position on the β gene which is similar to that of the DRB-induced pause site that we report here. Perhaps, for the longer circulating erythrocytes of adult chickens, transcription is not permitted to continue indefinitely and attenuation at this position is the mechanism used to abort transcription. At least two previous examples in which DRB accentuates natural pause or termination sites have been reported (Maderious & Chen-Kiang, 1984; Skolnik-David et al., 1982).

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