Haemoglobin and globin synthesis in the isolated primitive and definitive erythroid cells of chicken embryos. Evidence for a non-clonal mechanism at the haemoglobin switch

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SUMMARY

Primitive and definitive erythroid cells of chicken embryos aged 4–8 days, were separated by unit gravity sedimentation and pulse labelled with [3H]- and [14C]leucine. The haemoglobin and globin synthesis in the cell populations was analysed by chromatofocussing, isoelectric focussing, urea starch gel electrophoresis, and immunofluorescence or radioimmunoassay, using globin specific antibodies.

We found that both embryonic and adult α-globins are present in primitive erythroid cells, but relatively more of the adult α-type globins are synthesized in the late primitive erythroid cells. In young definitive erythroid cells exclusively adult α-type globins are synthesized. From these findings we conclude that a command to synthesize adult α-globin is perceived in both cell types at the time of the switch. This supports an environmental model rather than a clonal model of haemoglobin switching.

INTRODUCTION

The existence of four embryo-specific haemoglobins in chicken embryos aged 2 to 6 days has been recognized for some time (Schalekamp et al. 1972). Two minor haemoglobins were shown to comprise the two adult α-globins besides a common embryo-specific β-globin. The two major embryonic haemoglobins were shown to contain two different embryo-specific α-globins, together with a common β-globin, which we took for an adult β-globin at that time.

At day 6, coinciding with a shift from the primitive (PE) to definitive (DE) erythroid lineages, a sudden transition occurs, in that the two major embryonic haemoglobins are replaced by two more adult haemoglobin types, whereas the two minor embryonic haemoglobins remain. These findings and more recent work (Schalekamp et al. 1976) are in overall agreement with the independent findings of the group of Ingram (Bruns & Ingram, 1973; Brown & Ingram, 1974; Keane et al. 1974; Keane & Abbott, 1980) and Cirotto (Cirotto et al. 1975). Since the nomenclature used by Ingram's group has now been generally
Table 1. *Haemoglobin and globin nomenclature*

| Haemoglobin | (1) A<br>D | A_2<br>A | E_1<br>D | E_2<br>M | E_3<br>P' | E_4<br>P | P''<br>E | E_5<br>H | E_6
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<td>α globin</td>
<td>γA_1E_1E_2&lt;br&gt;α^D</td>
<td>αA_2E_5E_6&lt;br&gt;α^A</td>
<td>γA_1E_1E_2&lt;br&gt;α^D</td>
<td>γA_1E_1E_2&lt;br&gt;α^D</td>
<td>δE_3&lt;br&gt;α^A</td>
<td>δ(γ)E_4&lt;br&gt;α^A</td>
<td>—&lt;br&gt;α^A</td>
<td>αA_2E_5E_6&lt;br&gt;α^A</td>
<td>αA_2E_5E_6&lt;br&gt;α^A</td>
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<tr>
<td>β globin</td>
<td>βA_1E_6&lt;br&gt;β^A</td>
<td>βA_2E_1&lt;br&gt;β^A</td>
<td>βA_2E_1&lt;br&gt;β^A</td>
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<td>—&lt;br&gt;β^A</td>
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<td>βA_1E_6&lt;br&gt;β^A</td>
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(1) As used by our group previously (Schalekamp *et al.* 1972, 1976).
(2) As we plan to use from now on. This nomenclature is based on that of the group of Ingram (Brown & Ingram, 1974; Bruns & Ingram, 1973) and further modified by the group of Weintraub (Groudine & Weintraub, 1982). The main discrepancies concern the identity of the β^A and β^P in Hb A, Hb D, Hb P and Hb P' and the occurrence of an embryonic-type Hb D (E_1) in our previous studies. The presence of an α^E or α^S in Hb E and/or Hb H is sometimes suggested; the identity of β^H is still uncertain.
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It is fascinating that in chickens, most – if not all – of the globins found in either embryonic or adult red cells are already expressed in the yolk-sac-derived PE cells, which are present from day 2 of incubation onward, and that the later DE cells, which develop intra-embryonic from day 6 of incubation, differ qualitatively only by the absence of certain embryo-specific globins. Indeed although the expression of the adult globins changes quantitatively during development, only one adult globin at most, is restricted to the definitive lineage. The fact that changes in globin content during development are mainly quantitative as opposed to qualitative suggests that such a control is more susceptible to environmental stimuli than has been considered. However to substantiate this hypothesis, it is necessary to measure the actual rates of synthesis of all the globins in individual PE and DE cells at different stages of ontogenesis.

To clarify this issue, we now report further studies analysing the haemoglobin content of purified erythroid cell populations by more refined methods, i.e. chromatofocussing, isoelectric focussing, immunofluorescence and radioimmunoassay. Antibodies specific for the three \( \alpha \) globins were prepared for use in the immunotechniques. In addition, measurements of the ratios of globin synthesis in isolated PE and DE cells around the time of the switch are used to assess how the pattern of globin gene expression relates to the developmental stage of erythropoiesis per se.

**MATERIALS AND METHODS**

White Leghorn adult and embryonic chickens were bled from the wing and vitelline vein respectively (Schalekamp *et al.* 1972). The blood cells were washed three times in a tenfold volume of phosphate-buffered saline (PBS, pH 7.0) with in-between centrifugation (1000 g, 10 min, 15°C).

**Cell separation**

PE and DE cells of 7- and 8-day embryos were separated on a velocity sedimentation gradient of 0-7–2% (w/v) bovine serum albumin (BSA) in 300 ml PBS, at unit gravity. About \( 4 \times 10^8 \) cells in 30 ml 0.3% BSA in PBS were applied per analysis. The separation was complete in 3 h. Cell fractions were judged microscopically. Only pure fractions were pooled. The in-between overlapping cell fractions were discarded. Giemsa-coloured cytocentrifuge preparations are shown in Fig. 1.

**Preparation of haemolysate**

PBS washed cells were lysed in distilled water (10^8 cells/ml) for 15 min at 0°C. Lysis was completed by addition of the non-ionic tween Serdox NP10 (Non-idet tegentype, BV v. Delden, Netherlands), to a final concentration of
Fig. 1. May-Grunwald-Giemsa-stained primitive (PE) and definitive (DE) erythroid cells of chicken embryos. (A) PE and DE cells in whole peripheral blood of a 7-day embryo. (B) and (C) PE and DE cells respectively, isolated from the blood of the same 7-day embryo. (D) PE cells exclusively present in whole peripheral blood of a 5-day embryo.

0·5 % in phosphate buffer (PB) and NaCl to reconstitute PBS conditions in the sample. The haemolysate was centrifuged at 30 000 g for 20 min at 4 °C. The pellet was extracted once more with PBS, leaving it colourless and transparent indicating that all haemoglobins are extracted. The haemoglobin concentration was measured at 540 nm (Zeiss PMQ 11 spectrophotometer) in a 10 mm pathway cuvette, using A1% = 8·63.
Labelling of the cells

The cells (3 x 10^7 cells/ml) were incubated in 1 ml Eagles minimal essential medium, without leucine and glutamine (Flow Labs Ltd, Irvine, Scotland). Glutamine (2 mm), streptomycin and penicillin (0.2 ml/100 ml) and heat-inactivated chicken serum 1/10 (v/v) were added just prior to use. The cells were preincubated for 30 min at 38°C in a shaking waterbath, before addition of 25 μCi/ml [3H]leucine (spec. act. 1 μCi/μl) or 50 μCi/ml [14C]leucine (spec. act. 340 mCi/mmol, freeze-dried before use) both from the Radiochemical Centre, Amersham. The labelling was stopped after 1-2 h of incubation at 38°C, by adding a tenfold volume of ice-cold PBS, followed by immediate centrifugation (1000g, 10 min, 4°C) and two washings with PBS. Incorporation of labelled material into acid-precipitable protein was at least linear up to two hours of incubation.

Immunofluorescence (IF)

Thoroughly air-dried cytocentrifuge preparations of blood cell suspensions in 5% BSA in PBS, pH 7.8 were fixed at room temperature for 5 min in acetone-methanol (9/1, v/v), dipped in PBS and reacted with FITC-labelled specific antibody preparations for 45 min at 37°C in the dark. The antibody was applied in several concentrations to find the optimal condition, which varied. Blanks were made by using corresponding concentrations of non anti-haemoglobin antibodies. Slides were washed extensively in PBS on a magnetic stirrer in the dark, air-dried, mounted in glycerol-PBS (9/1, v/v) and photographed at once with a fluorescence microscope (Zeiss).

Illumination HBO lamp, excitation filter BP 455-1490; farb filter FT 510, sperfilter BP 520-560, film Kodak ektachrome 160, exposure time 1 min.

Antibodies

Rabbits were injected with highly purified globins (Schalekamp et al. 1976), 1 mg in 0.5 ml Freund Adjuvant Complete (DIFCO) and boostered as previously described (Schalekamp, 1972). Antisera were absorbed by affinity chromatography. Anti-αA antiserum was freed from antibodies cross reacting with αD and βA globin, by batch-wise absorption with Hb D, which was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala). Coupling was according to the manufacturer's manual and in a ratio of 30 mg haemoglobin to 1 gm dry sepharose. The αA globin specific antibodies were subsequently adsorbed on a Hb A-sepharose column and eluted with glycine buffer (0.2 m-glycine-HCl, 0.5 m-NaCl, pH 3-0). Similarly anti-αD antibodies were prepared by batch-reacting the serum of rabbits injected with purified αD globin, with a Hb A-sepharose and subsequent isolation of the specific antibodies on a Hb D-sepharose columns; anti-α,α' globin antiserum was prepared in rabbits injected with purified α' globin and absorbed with
sepharose to which total adult haemolysate was coupled. It was further isolated by adsorption to a Hb P-sepharose column and subsequent elution. This antibody did not distinguish αα and αα'. The antibody preparations were brought to neutral pH, dialysed and diluted with PBS prior to use. Their specificity and titre was controlled by IF and RIA.

For the use in IF studies, to 10–25 mg of a specific antibody preparation in 1 ml 0.1 M-sodium carbonate buffer, pH 9.5 (CB), 0.5 % FITC (Baltimore Biol. Lab.), freshly dissolved in 1 M-CB, was added slowly, to a ratio of 20 μg fluorochrome per mg protein. The reaction was conducted in the dark at room temperature on a magnetic stirrer for 2 h. The antibody was purified over G25 sepharose (Pharmacia, Uppsala) in PBS (pH 7.2) and used as fresh as possible (storage if necessary in aliquots at −20°C in the dark). The F/P ratio as estimated following Nessler analysis (see Nairn, 1964, p. 40) was 2.5 in our preparations.

Radioimmunoassay (RIA)

Highly purified Hb A, D or P, 2.5–5 μg in 5 μl 0.01 M-PB, pH 7.6 was diluted with 5 μl 0.5 M-PB, pH 7.6 and coupled to 5 μl sodium iodide-125 (spec. act. 15 mCi 125I/μg iodide; concentration 100 mCi/ml, The Radiochemical Centre, Amersham), by adding 5 μl chloramine T (Fluka AG), 1 % in 0.01 M-PB, pH 7.6. After 1 min at 0°C, the reaction was stopped with 10 μl Na2S2O5 (1 % in 0.1 M-PB, pH 7.6). After addition of 200 μl NaI (1 % in 0.1 M-PB) and 50 μl 1 % BSA, the haemoglobins were purified over a 15 × 1.0 cm column of Biogel P2 (BIO RAD) in PBS, which was pretreated with 2 ml 2 % BSA in PBS. Labelling with hypochlorite, lactoperoxidase or the Bolton-Hunter reagent did not yield better results. The specific activity of our preparations was between 50 and 95 mCi/mg measured on the base of protein concentration or between 40–80 mCi/mg measured on the base of immunogenic haemoglobin activity in RIA. The initial binding of fresh preparations was around 60 %, with a blank around 3 %. However, this initial binding declined sharply by freezing at −20°C. Therefore the labelled antigen preparations and also the standards had to be kept in the cold room and were not used when older than a week. This made the method very time consuming and we are now looking into other methods of labelling and preservation.

Equilibrium radioimmunoassays of the double antibody type were performed. The samples (CS), 100 μl of known total haemoglobin concentration, were diluted in 1 % BSA in PBS and compared to standard dilutions of 100 μl non-labelled Hb A, D and P in a range of 0.1–100 ng/100 μl, in the presence of 10⁴ c.p.m. (CT) of the corresponding labelled haemoglobin in 100 μl 0.1 % BSA in PBS, and 100 μl of the specific antibody, optimally diluted in 0.25 % normal chicken serum (NCS). Blanks (CO) were prepared by using 0.25 % NCS instead of the specific antibody. Initial binding (Cl) was found when buffer replaced the sample or standard dilution. After 48 h at 4°C, 100 μl of the
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second antibody, donkey anti-rabbit IgG (Wellcome) was added, at a dilution (mostly 1/15, v/v), which produced complete precipitation of the rabbit IgG within 24 h at 4 °C. The sample was centrifuged (1750 g, 20 min, 4 °C), washed with PBS and recentrifuged. Precipitate was counted in an automatic gamma counter (Nuclear Chicago). The percentage binding was calculated as (CS–CO) ÷ CT. The haemoglobin concentrations were calculated from the appropriate standard curves as percentage of the total haemoglobin concentration of the sample. For example, the most concentrated sample of DE cell haemolysate in the upper panel of Fig. 4 contains 25 ng Hb/100 μl (measured at 540 nm). This scores 27% anti-α^A bound counts, a percentage which corresponds to 11.3 ng/100 μl standard Hb A. So we calculate the proportion of α^A containing haemoglobin to be 11.3/25 × 100 = 45%. Each sample was measured in 3–4 dilutions in triplicate. The lower limit of detection with this method was around 2 ng/100 μl sample for Hb A and D, and 5 ng/100 μl sample for Hb P, when the sensitivity is defined as Cl ± 2 SD, at the level of initial binding. The coefficients of variation were sometimes as high as 20%, when flat standard curves had to be included. This was at times unavoidable as the labelled and standard haemoglobin preparations proved to be very unstable, as mentioned above. The recovery of 10 ng Hb A, D or P, which was added to the homologous standard dilution series, was greater than 90%. The cross reactivity of 100 ng Hb A, D or P, which was added to the heterologous standard dilution series was less than 5%, except for Hb P, which cross reacted in an assay of D/anti-α^D sometimes up to 7%, but this may be due to the contamination of some of the Hb P preparations with a small amount of Hb M, which contains α^D.

Chromatofocussing (CF)

The method was performed according to the instructions of the manufacturer (Pharmacia, Uppsala). Haemolysate, 1–35 mg Hb, in 1–1.5 ml lysate, radioactivity between 10^5 and 10^8 dpm (ratio dpm 3H/14C at least 10) was dialysed overnight in two changes of 25 mM-ethanolamine, pH 8.6 and separated over a 0.9 × 57 cm column of PBE 94, equilibrated with 25 mM-ethanolamine, pH 9.5, using polybuffer 96 at starting pH 7.0. The radioactivity of 100–500 μl of each fraction was counted in 2–8 ml Pico-Fluor™15 (Packard) in an autoprogrammed Mark III liquid scintillation counter (Searle).

Concentration

Fractions were concentrated in Minicon B 15 concentrators (Amicon Corporation, Danvers, USA). This method leads to considerable (up to 70%) loss of haemoglobin. Therefore to the very dilute radioactive samples, cold haemoglobin fractions were sometimes added, prior to concentration.
Isoelectric focussing (IEF)

Samples, 5–10 μl, containing 1–3 μg/μl haemoglobin, were electrophoresed in agarose/sorbitol in a pH 6.5–9 gradient of pharmalyte, during 1500–1800 Volt/hr. Apparatus and method were Pharmacia (Uppsala). The gels were dried, stained with Coomassie R-250 and photographed. Radioactive fractions containing approximately $10^3$ dpm/5 μl were recorded by exposure to a Kodak XO mat AR film.

Urea starch gel electrophoresis (USG)

The gels (8 m-urea, 50 mM-mercaptoethanol, 20 mM-Ba-lactaat buffer, pH 3.2) were prepared as described by Gilman & Smithies (1968). Haemolysate, 50–150 μg Hb/50 μl ($10^4$–$10^6$ dpm), was electrophoresed vertically (anode up) for 50–70 h at 5V/cm in a cold room. The globins migrated between 12–18 cm. The gels were sliced into two halves and subsequently fixed. Both halves, inside up, were stained with amidoblack. One halve was kept to photograph, the other was sliced in blocks, 1 cm x 1 mm, with a gel slicer (The Mickle Lab., Surrey). The gel slices were solubilized in 1 ml Soluene 350 (Packard) for 7 h at 50°C and the radioactivity was determined in 10 ml Dimilume 30 (Packard). Amidoblack did not disturb the readings.

Fig. 2. Globin analysis by USG, comparing the haemolysates of 5-day PE cells, isolated 8-day PE cells, isolated 8-day DE cells, adult DE cells and a natural (8-day total) and artificial (8-day PE + DE) mixture of 8-day PE and DE cells. The artificial mixture contained [3H]leucine-labelled 8-day PE cells (PE*) and [14C]leucine-labelled 8-day DE cells (DE**). The right panel represents the radiogram.
RESULTS

Globin types present in purified PE and DE cells

Pure early PE cells or late DE cells can be readily obtained from developmental stages in which only one cell type occurs, i.e. embryos younger than 5 days for PE cells and hatched animals for DE cells; at intermediate

Fig. 3. Immunofluorescent-stained erythroid cells. (A) A 2-day embryo. The embryo was spread as a whole, which causes a thick and therefore somewhat misty appearance. (B, C and D) Peripheral blood of a 4-, 7- and 12-day embryo respectively. The antibody in all cases was fluorescein-labelled specific anti-α^a,α'. Only PE cells stain.
stages, during the switch from PE to DE cells, where a mixture of both cell types occurs, the two populations can be separated by the use of a velocity sedimentation gradient at unit gravity (Fig. 1). USG analysis (Fig. 2) of haemolysates shows the presence of the globins α^A, α^D, α^π, α^π', β^e and β^ρ in PE cells from 5- as well as 8-day embryos. A β^A globin is not resolved between the β^e and β^ρ globins. The haemolysate of DE cells of 8-day embryos shows a globin pattern virtually identical to that of DE cells derived from hatched chickens. The presence of α^A, α^D and β^A globin is pronounced whereas β^e, if present, is overshadowed by β^A. No α^π or α^π' globin is evident in either DE cell preparation.

Both cell types were analysed in IF studies with antibodies specific for the globins α^A, α^D and α^π,α'. The specificity of the antibodies was established as indicated in the method section. Anti-α^π antibody showed abundant fluorescence in PE cells from 2- to 12-day embryos, the earliest and oldest stages investigated (Fig. 3). Early DE cells from 7-day embryos show some faint fluorescence with this antibody: whether this is an artefact is unclear, but DE cells derived from later embryonic stages showed less fluorescence with the same antibody. Anti-α^A and anti-α^D showed fluorescence throughout all embryonic stages in PE as well as in DE cells, but the intensity of fluorescence was highest in DE cells when reacted with anti-α^A serum (results not shown).

Quantification of α^A, α^D and α^π or α^π' - containing haemoglobins in total haemolysates of the individual cell types was performed by RIA. The lower limit of this method ranges from 2-5 ng/100 μl sample. As the average amount of haemoglobin per cell is around 40 pg (Bruns & Ingram, 1973), the method is able to detect a particular globin in a few hundred cells. We have not yet fully exploited this possibility, due to the technical difficulties described in the methods. However the method is promising and will be of special value for cell culture techniques. From the studies performed already, we show some results in Fig. 4 (see method section for details). DE as well as PE cells from 8-day embryos contain α^A globin, but to a different amount (upper panel), even so both cell types contain α^D globin (middle panel). It may be noted here that the amount of α^D globin in 8-day DE cells is 48%. In our earlier studies (Schalekamp et al. 1972) we calculated the amount of α^D containing Hb D in DE cells from newly hatched to adult animals, on the basis of chromatographic analyses and found a sliding percentage of 33–19%. The higher amount of α^D

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Fig. 4. RIA of α-type globins in 8-day PE and DE cells. Three specific anti-α type antibody preparations were used, anti-α^A, anti-α^D and anti-α^π,α' (upper, middle and lower panel, respectively). Standard curves were made with fresh-purified haemoglobins, containing the appropriate globin (Hb A, Hb D and Hb P, respectively). Calculations were performed as described in Materials and Methods. The method determines globins; no discrimination between haemoglobins containing the same globin is possible. Thus the sum of Hb E + A + P'', Hb D + M and Hb P + P' respectively is estimated in this type of assay.
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- Standard A ~ 100 %
- DE cells ~ 45 %
- PE cells ~ 25 %
- sensitivity ~ \( Cl \pm 2 \) s.d. (n = 22)

- Standard D ~ 100 %
- DE cells ~ 48 %
- PE cells ~ 11 %
- sensitivity ~ \( Cl \pm 2 \) s.d. (n = 14)

- Standard P (n = 2) ~ 100 %
- Standard P' (n = 2) ~ 100 %
- DE cells ~ < 2 %
- PE cells ~ 80 %
- sensitivity ~ \( Cl \pm 2 \) s.d. (n = 13)

Total haemoglobin concentration in ng/100 µl
in these earlier DE cells is in part explained by the presence of Hb M ($\alpha^D\beta^e$) in these cells (Figs 5, 6). On the basis of RIA, adult DE cells contain around 20 % Hb D (results not shown). The amount of $\alpha^{n-n'}$ globin (lower panel) in the young DE cells is 2 % or less. Interestingly in PE cells, the calculated sum of haemoglobins exceeds 100 %, which is, we think, an indication that free $\alpha$-type globins are present in the lysates.

Late PE cells synthesize increased amounts of adult $\alpha$ globins

The globin synthesis in PE and DE cells from embryos of various stages was measured by labelling the isolated cells in the presence of $[^3H]$ or $[^14C]$leucine. The haemoglobins were analysed by CF and the globins by USG (in our hands electrophoresis in starch-gels was superior to that in polyacrylamide gels; the method recommended by Tobin et al. 1979). The radioactivity carried by each globin was estimated by slicing and counting the gels. Fig. 5 compares 5- and 8-day erythroid cells: the results (upper panel) show that 8-day DE cells synthesize Hb A ($\alpha^A\beta^A$), Hb D ($\alpha^D\beta^A$) and Hb E ($\alpha^A\beta^e$) and perhaps also Hb M ($\alpha^D\beta^e$). The presence of Hb M in these cells, which has not been noted previously, was confirmed by IEF autoradiography (Fig. 6). In contrast, PE cells from 8 day embryos (Fig. 5, upper panel) synthesize Hb E ($\alpha^A\beta^e$), Hb P' ($\alpha^{n-}\beta^p$), Hb M ($\alpha^D\beta^e$) and Hb P'' ($\alpha^A\beta^p$). No Hb D ($\alpha^D\beta^A$) is made; the synthesis of Hb A ($\alpha^A\beta^A$) and P ($\alpha^A\beta^p$) is doubtful. The new Hb P'' was deduced from our present study as indicated in Fig. 7. PE cells from 5 day embryos (Fig. 5, lower panel) synthesize Hb E ($\alpha^A\beta^e$), Hb P ($\alpha^{n-}\beta^p$), Hb P' ($\alpha^{n-}\beta^p$) and Hb M ($\alpha^D\beta^e$). Perhaps some Hb P'' ($\alpha^A\beta^p$) is also present in the shoulder to the left of Hb P. However this peak was too small to do USG analysis.

We here emphasize that the haemoglobins, carrying the adult-type $\alpha$ globins, $\alpha^A$ and $\alpha^D$, (i.e. Hb E, Hb P'' and Hb M) are synthesized in late (8-day) PE cells at a relatively high rate in comparison to PE cells of a 5-day embryo, in which relatively more radioactivity is assembled in the embryo-specific Hb P and Hb P' (Fig. 5; compare PE trace in upper and lower panel). The shift is most obvious for Hb P'', but it is also true for Hb M and Hb E, as could be further demonstrated by the direct comparison of PE cells from 5- and late 8-day embryos, using the same techniques. The CF radiogram (Fig. 8) shows relatively more radioactivity in the adult-type $\alpha$ globin carrying Hb E, P'' and

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Fig. 5. CF of the radiolabelled haemoglobins of PE and DE cells at two stages of development. Upper panel: Cochromatofocussing of unlabelled 8-day total haemolysate (solid line), $[^3H]$leucine-labelled 8-day PE cell haemolysate (...., dpm $\times 10^{-4}$) and $[^14C]$leucine labelled 8-day DE cell haemolysate (----, dpm $\times 10^{-3}$). Pooled fractions (■) were analysed in USG (insert). Lower panel: Cochromatofocussing of 5-day unlabelled and $[^3H]$leucine-labelled PE cell haemolysate. In the USG (insert), purified Hb A and Hb D from adult animals are present for comparison.
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Fig. 6. IEF of 10μg of the haemolysates of 8-day DE cells, 8-day PE cells and 8-day total haemolysate and of approximately 5μg of the fractions isolated by chromatofocussing as shown in Fig. 5 (upper panel). Left panel: Coomassie Blue staining. Right panel: autoradiogram of the CF fractions. Only 14C-labelled fractions, that is 8-day DE cell fractions, show up at the exposure time used (one week at −70°C). Thus 8-day DE cells show synthesis of Hb E, Hb A, Hb M and Hb D and not of Hb P, Hb P' or Hb P''.

M, derived from the PE cells of the older embryos. However the total overall haemoglobin synthetic activity of PE cells is 20-fold lower at 8 day compared to 5 day, even when both cell preparations are cultivated in the same session. Thus one could conclude that these ‘older’ PE cells make less haemoglobin, but what is made is predominantly adult-type α globin rather than embryonic. The change in globins present in each of the haemoglobin fractions (as analysed by USG) is visualized in Fig. 9. This confirms the shift in the αA, αD/αα,α'' globin synthesis ratio in PE cells from days 5-8 and demonstrates that the relatively higher radioactivity in the Hb E, Hb P' and Hb M peaks is indeed due to the presence of relatively more of the adult-type α globins.

Globin imbalance in PE cells

The switch in globin synthesis from αα and αα'' to αA and αD in the PE cells is accompanied by an imbalance in the ratio of α- to β-type globins synthesized (Fig. 9). In 5-day PE cells the imbalance is already present; the ratios being αα/βP < 1 in Hb P, αα''/βP = ± 1 in Hb P', αA/βE > 1 in Hb E and αD/βE > 1 in Hb M. But in 8-day PE cells it is more pronounced. Such an imbalance is not seen in the USG radiogram of the Hb A (Fig. 7) or Hb D (not shown) produced by 8-day DE cells. Since the leucine content of the α globins is 15–16 per mole as compared with 17–18 for the β globins (Schalekamp et al. 1976) and the incorporation of radioactive leucine is linear for at least 2h, the globin imbalance is unlikely to be due to labelling artefacts. However in USG, but also in polyacrylamide gels, the colour intensity of globin bands, especially the β-type, seem to fade sometimes, indicating that these globins are not very stable under the electrophoretic conditions used. Nevertheless, although
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electrophoretic artefacts cannot be altogether excluded, the globin imbalance observed is pronounced and reproducible and may therefore be genuine.

The presence of a pool of free, unassembled α-type globins in PE cells is also supported by the fact that the whole haemolysate of 5-day PE cells (Fig. 9, upper panel) shows a much higher ratio of α to β than expected from consideration of the ratios of the two globins in the purified haemoglobins (Fig. 9, Hb P' trace).

It has proved difficult to determine whether the adult globin, β, is also synthesized by older PE cells, since the candidate haemoglobins that might contain it are minor haemoglobins. Haemoglobins A and D, which do contain β, are not found in late PE cells. Thus as yet we have not found positive evidence, for adult β globin synthesis in PE cells.

DISCUSSION

The diversity of chicken globins and their occurrence in the various haemoglobins

β-type globins

In this study we show that the adult β globin in Hb A can in fact be distinguished from the β globin in Hb P and Hb P' under conditions of USG electrophoresis where β, β and β globin are resolved clearly (Fig. 2). However, even under these improved resolving conditions the β globins of adult Hb A and Hb D and of foetal Hb D are indistinguishable, as are the β globins in Hb E and Hb M.

![Diagram](image.png)

Fig. 7. Radiogram of the globins in the Hb P' + A + P fraction shown in Fig. 5 (upper panel, insert). DE cells from an 8-day embryo (—, dpm x 10^-2) make Hb A as shown by 14C-radioactivity in the α and β globin region. PE cells from the same embryo (--, dpm x 10^-3) show 3H-radioactivity in the α and β globin region, which led us to the deduction of a Hb P' (α β). Hb P (α β) is present, as shown by the protein-stained image (Fig. 5), but is no longer synthesized, as there is no radioactivity in the α globin region.
In our earlier, mainly immunological studies (Schalekamp et al. 1972) we postulated a different $\beta$ globin in Hb A and Hb D, in agreement with Muller (1961), Moss & Thompson (1969) and Moss & Hamilton (1974), but contested by Bruns & Ingram (1973), Brown & Ingram (1974), Matsuda et al. (1973) and Vandercasserie et al. (1975). We also distinguished on immunological evidence a foetal form of Hb D, but this was never confirmed. Our present biochemical studies now resolve these differences in the literature.

Our finding of an identical $\beta^e$ globin in Hb M and Hb E is in agreement with Chapman et al. (1982a, 1982b), who have recently sequenced both globins. As a whole, the $\beta$-type globins appear very much alike, much more so than the $\alpha$-type globins: Chapman et al. (1981, 1982a) calculate a divergence of only 13% in aminoacid residues between adult and embryonic $\beta$-type globins; whereas the embryonic $\alpha$-type globin $\alpha^e$ differs in 43% of the residues from each of the adult $\alpha$-type globins. This may have contributed to the contradictory reports on the $\beta$-type globins.

Studies on the chromosomal arrangement of the chicken $\beta$-type globin genes (Dodgson et al. 1979; Dolan et al. 1981; Ginder et al. 1979; Richards et al. 1979; Stalder et al. 1980) suggest the presence of four structural $\beta$-type genes, clustered together and designated $\beta$ 1–4 in downstream order. Genes $\beta$ 1, 3 and 4 code for the globins $\beta^o$, $\beta^A$ and $\beta^e$ respectively. The $\beta$ 2 gene may represent the $\beta^H$ or $\beta^e$ gene, coding for the $\beta$-type globin in Hb H or Hb M (Brown & Ingram, 1974) respectively; or it may be a pseudogene.

The $\alpha$-type globins

Our present studies fail to reveal any difference in the $\alpha^D$ globin present in Hb D and Hb M, confirming our earlier work (Schalekamp et al. 1972, 1976). Recently Chapman et al. (1982b) found six scattered aminoacid differences between their sequence of $\alpha^D$, isolated from Hb M, and the sequence Takei (1975) published for $\alpha^D$ from Hb D, which Chapman et al. attribute to sequencing errors.

Our present studies also reveal a common $\alpha^A$ globin in Hb A and Hb E, as we found before. Interestingly, the same globin is also a constituent of the newly reported embryonic Hb P'. Chapman et al. (1982a) report 22 more or less scattered aminoacid differences between $\alpha^E$ and $\alpha^A$ sequence published by Matsuda (1971) for a Japanese white Leghorn breed. However, this substitution would not lead to a different electrophoretic or chromatographic behaviour.

The existence of a second $\alpha^A$-related globin has also been inferred from studies with a globin cDNA recombinant believed to correspond to an anaemic shock-induced $\alpha^S$ gene (Salser et al. 1979; Liu & Salser, 1981; Richards et al. 1980). However Dodgson et al. (1981) studying normal reticulocytes, presumes them all to represent the one adult $\alpha^A$ globin gene. Taken together, the analysis
Fig. 8. CF of the radiolabelled haemoglobins in PE cells at two stages of development. Total 8-day unlabelled haemolysate was cochromatofocussed with $[^{3}H]$leucine-labelled 8-day PE cell haemolysate (\ldots, dpm $\times 10^{-4}$) and $[^{14}C]$leucine-labelled 5-day PE cell haemolysate (\ldots, dpm $\times 10^{-3}$). Pooled fractions, as indicated at the bottom of the graph (■) were analysed on USG (insert) T represents original total mix.

of the $\alpha$ globin gene cluster (Deacon et al. 1980; Dodgson et al. 1981; Engel & Dodgson, 1978, 1980; Reynaud et al. 1980; Richards & Wells, 1980; Salser et al. 1979) points to the presence of only three structural $\alpha$ globin genes: $\alpha^\pi$,$\pi'$, $\alpha^D$, $\alpha^A$/$\alpha^S$. Indeed, the coexistence of an $\alpha^A$ and $\alpha^S$ sequence has never been reported: A combination of natural haemoglobin polymorphisms and technical errors is most likely the reason for the discrepancies.

Chapman et al. (1982a) reported an $\alpha^E$ globin to be present in DE cells in the peripheral blood of phenylhydrazine-induced anaemic young chickens, but not in the bone marrow. An interesting implication of her studies could be that cells from the bone marrow of young anaemic chickens and from the peripheral
Radioactivity — 8 d PE

Total haemolysate

Hb E

Hb P'

Hb P(+)P'

Hb P'

Hb M(+)P'

slice nr
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Blood of some adult anaemic chickens contain only Hb D. However, this has not been reported by others (Stino & Washburn, 1970; Godet et al. 1970).

The embryonic α-type globins, αα and αβ, first distinguished by ourselves (Schalekamp et al. 1972) and later by Brown & Ingram (1974), are present in the major embryonic haemoglobins, Hb P and Hb P', and differ markedly in their chromatographic and electrophoretic properties. This may be due to a single alanine/glutamine interchange (Chapman et al. 1980). However, since the α cluster of the chicken genome contains only one αα gene (see above), we are inclined to attribute the difference in biochemical properties to post-translational modifications, rather than to a difference in primary structure. Translational factors can proportionally modulate the display of these globins as shown by translation of one and the same poly (A+) mRNA preparation in different cell-free systems (manuscript in preparation).

Globin synthesis by PE and DE cells

DE cells contain only Hb E, Hb A, Hb D, and Hb M but no Hb P, Hb P', or Hb P'' as judged by CF, IEF and USG analyses: this is supported by globin chain analysis and by IF and RIA which shows the presence of αα and αβ in these cells, but less than 2% αα or αβ. This conclusion is in agreement with Cirotto et al. (1975), Shimizu (1976) and Mahoney et al. (1977). However Chapman & Tobin (1979) and Tobin et al. (1979) using IF, have claimed to find Hb P or Hb P' in early DE cells. Their results may be explained by unsuspected βε-antibodies – raised by contamination of their Hb P fractions with Hb M – which cross react with the Hb M (αD, βε) or Hb E (αα, βε) in young DE cells. Therefore on balance we think that the presence of αα or αβ in DE cells remains very doubtful. On the other hand the presence of βε in DE cells is unmistakable from our work and is in agreement with the work of Wood & Felsenfeld (1982), who found the βε gene to be active in blood cells of 14-day embryos.

PE cells contain both adult and embryonic α-type globins as judged by IF, RIA and USG. This corroborates our previous evidence (Schalekamp, 1972, 1976). However the novel interesting finding is that the haemoglobins containing adult α-type globins (Hb E, Hb P'' and Hb M) are synthesized preferentially in PE cells of 'older' embryos.

In fact, Cirotto et al. (1975) have suggested that Hb P and Hb E/M are present in different PE populations. However we have not been able to

Fig. 9. Radioograms of the globins synthesized by 5- and 8-day PE cells, analysed by USG as shown in Fig. 8 (insert). The DE cell pattern (shaded area) is included from another analysis for comparison. Upper panel: Mixture of the haemolysates of [14C]leucine-labelled 8-day PE and [3H]leucine-labelled 5-day PE cell haemolysate. Lower panels: the haemoglobin fractions isolated by CF. The radioactivity is in dpm × 10^-2 per 1 mm slice.
distinguish two PE cell populations either by our cell separation or IF studies, using specific anti-α\textsuperscript{a.α'} antibody.

The haemoglobin switch, is it clonal?

As discussed above, DE cells clearly synthesize adult globins and have almost certainly switched off the synthesis of embryonic globins. PE cells also synthesize, adult α\textsuperscript{A} and α\textsuperscript{D} globin, but the synthesis is preferentially stimulated in late PE cells from embryos in which the switch from primitive to definitive lineage is occurring. Unfortunately, the presence of the adult β\textsuperscript{A} globin in PE cells of these embryos cannot yet be established for technical reasons. However, the finding by Stalder et al. (1980) that the adult β\textsuperscript{A} globin gene in 5-day PE cells is as susceptible to DNA-ase I as the embryonic β-type genes, suggests that this adult gene is (pre-)activated in these cells (Weintraub & Groudine, 1976). The presence of active adult globin genes in PE cells is also suggested by the finding that adult-type haemoglobins appear when PE cells are cultured in vitro (Pine & Tobin, 1976). Similarly Chui et al. (1979) found that mouse yolk-sac-derived primitive nucleated erythroid cells started to synthesize adult haemoglobins in vitro. Papayannopoulou et al. (1979) and Stamatoyannopoulos et al. (1981) showed the co-existence of both foetal and adult haemoglobin in clonal cultures from an adult human erythropoietic cell line and most recently Peschle et al. (1983) claim gradual reprogramming from embryonic to foetal and finally adult haemoglobin synthesis in a single population of erythroid progenitors. Such findings argue against a clonal model for the explanation of haemoglobin switching in favour of environmental stimulation of globin gene transcription, at different stages of the erythroid cell proliferation or differentiation.

We presented a model elsewhere (Schalekamp et al. 1982, 1983). In essence we hypothesize a two-stage process: stage one is the commitment of an erythroid cell line, which brings the globin gene clusters as a whole in a permissive condition, such that during each cell cycle, each of the globin genes in the cluster is activated automatically in ontogenic order. The second stage then involves the active transcription of the gene upon an environmental stimulus. In this model, cells that are older, i.e. separated by many cell divisions from the stem cell, which have a relatively long cell cycle, transcribe only the later, that is the adult, globin genes. PE cells, which are known to arise as an early cohort and to have a short cell cycle (Ingram et al. 1979) transcribe preferentially, but not exclusively, the embryonic globin genes. This implies that PE and DE cells are not essentially different with respect to their haemoglobin potentialities; nor do they necessarily derive from separate erythroid precursor cells. Related ideas have been advanced by Tobin et al. (1981), Stalder et al. (1980), Comi et al. (1980) and Fucci et al. (1983). Our hypothesis is in keeping with the notion that in vivo DE cells may arise from the yolk sac (Lucas & Jamroz, 1961; Beaupin et al. 1979). Both cell types also may
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arise in vitro from the same primitive or even extraembryonic tissue fragment or cell culture (Hagopagian & Ingram, 1971; Chan & Ingram, 1973; Pine & Tobin, 1976; Chui et al. 1979; Zagris, 1980). Cudennec et al. (1981) and Ripoche & Cudennec (1983) demonstrated the capacity of mouse yolk-sac haemopoietic cells to produce definitive erythrocytes upon triggering with humoral diffusible factors. The production of adult haemoglobin by foetal erythropoietic tissue transplanted in adult sheep (Zanjani et al. 1979) is an example of the same phenomenon at a later stage of development. The converse has also been noted, where upon stress in sheep (Barker et al. 1979), baboons (DeSimone et al. 1979, 1982) and perhaps also in chickens (Chapman et al. 1982a) foetal globins reappear in adult animals, in which the foetal lineage cells are thought to be extinguished. The stochastic expression of foetal haemoglobin in adult human erythroid cell clones has been very convincingly argued by Stamatoyannopoulos et al. (1981).

It is now known that in many animals and in humans, two globin gene regions exist in which the \( \alpha \) and \( \beta \) globin genes are clustered together in downstream ontogenic order. This knowledge makes it easier to imagine the existence of a mechanism which favours activation of the genes in ontogenic order. However in chickens the order of the genes in the \( \beta \) cluster is different; the two embryonic \( \beta \) genes flank the adult (Dolan et al. 1981). This could plea against a ‘call the roll’ mechanism of activation. On the other hand chicken embryos display two embryonic \( \beta \) globins at the same time and one may speculate that looping of DNA globin domains during the activation process is performed to accomplish this.

Whatever the mechanism acting during haemoglobin switching may turn out to be, there is a hint from our and other’s work, that environmental factors can modulate the expression of globin genes.

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(Accepted 4 June 1984)