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In situ hybridization reveals co-expression of embryonic and adult α globin genes in the earliest murine erythrocyte progenitors

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Summary

Murine erythropoiesis begins with the formation of primitive red blood cells in the blood islands of the embryonic yolk sac on day 7.5 of gestation. By analogy to human erythropoiesis, it has been thought that there is a gradual switch from the exclusive expression of the embryonic α-like globin (ζ) to the mature adult form (α) in these early mouse cells. We have used in situ hybridization to assess expression of these two globin genes during embryonic development. In contrast to what might have been expected, we find that there is simultaneous expression of both ζ and α genes from the very onset of erythropoiesis in the yolk sac. At no time could we detect expression of embryonic ζ globin mRNA without concomitant expression of adult α globin mRNA. Indeed, adult α transcripts exceed those of embryonic ζ in the earliest red cell precursors. Moreover, the pattern of hybridization reveals co-expression of both genes within the same cells. Even in the fetal liver, which supersedes the yolk sac as the major site of murine fetal erythropoiesis, there is a brief co-expression of ζ and α genes followed by the exclusive expression of the adult α genes. These data indicate an important difference in hematopoietic ontogeny between mouse and that of human, where ζ expression precedes that of α. In addition to resolving the embryonic expression of these globin genes, our results suggest that the embryonic α-like globin gene ζ may be physiologically redundant, even during the earliest stages of embryonic development.

Key words: hematopoietic lineages, globins, yolk sac, fetal liver.

Introduction

Erythropoiesis in the mouse begins with the production of a distinct population of red blood cells in the blood islands of the visceral yolk sac at day 7.5 postcoitum (p.c.). These *primitive* yolk sac red blood cells are large (about four times the volume of adult erythrocytes), nucleated and produce a distinctive set of embryonic hemoglobins (Craig and Russell, 1964; Russell and Bernstein, 1966; Barker, 1968; Chui et al., 1978). By day 11.5 p.c., there is a major transition in which these nucleated red blood cells diminish in number and the fetal liver becomes the major hematopoietic organ. Among the hepatic epithelial cells, erythroid precursors (erythroblasts) proliferate, differentiate and enter the circulation. The liver-derived, circulating erythrocytes (called *definitive* erythrocytes) are small, lack nuclei and express a distinctive set of adult hemoglobins, which continue to be synthesized in the adult organs of hematopoiesis, the spleen and bone marrow (Kovach et al., 1967; Fantoni et al., 1967; Gilman and Smithies, 1968; Barker, 1968; Rifkind et al., 1969; Wong et al., 1983).

The switch from embryonic to adult hemoglobins has long been recognized by the distinctive electrophoretic mobilities of the hemoglobins derived from primitive and definitive erythrocytes. In vitro culture experiments promoted the notion that there are two hematopoietic progenitor cells, one producing embryonic and the other, adult hemoglobin (Wong et al., 1986). If indeed there are two progenitor cell types, then both must originate in the yolk sac. This is so since cultured primitive erythrocytes derived from the embryo before the fetal liver is formed can synthesize adult hemoglobins (Cudennec et al., 1981; Wong et al., 1982).

Another method of following globin gene expression in early erythrocyte progenitor cells is to use in situ hybridization with gene-specific riboprobes. In this way, globin transcripts can be identified from the very onset of erythropoiesis and the 'switching' event can be pinpointed accurately, thereby documenting the stage at which embryonic globin gene expression is replaced by that of the adult. Here we have used this approach to study the expression of the -globin gene cluster.

The hemoglobin molecule is a heme-bearing tetramer made up of two heterodimeric subunits, each composed of an or -like chain and a or -like chain. In the mouse, the globin gene cluster resides on chromosome 7 and consists of three embryonic and two adult genes (in addition to two pseudogenes) (Leder et al., 1980; Edgell et al., 1981; Hansen et al., 1982; Hill et al., 1984). The -globin cluster, which resides on chromosome 11, consists of one

embryonic and two adult genes organized in the following 5 to 3 orientation: 1 2 (Leder et al., 1981). Three embryonic hemoglobins are produced in the yolk sac prior to fetal liver formation. Their subunit composition is as follows: EI(2: y2), EII(2 y2) and EIII(2 h1 2) (Fantoni et al., 1967; Edgell et al., 1981; Farace et al., 1984; Leder et al., 1985). Thus both and (eventually) are expressed in primitive erythrocytes.

In the present study, we have chosen to focus on the locus since specific and probes readily distinguish the expression of these related genes. (The transcripts of the two adult genes, 1 and 2, are indistinguishable and thus cannot be assessed separately.) By analogy to the human (Peschle et al., 1985), we expected to be expressed first, followed by the expression of . We were therefore surprised to find that this was not the case. From the onset of erythropoiesis, and gene transcripts are expressed simultaneously and, most importantly, in the same cells. Interestingly, adult gene expression always exceeds , suggesting that the entire locus is available for transcription in the primitive erythrocyte lineage.

Materials and methods

Sample preparation

Embryos and whole deciduas were isolated from CD1 mice at times indicated in the text. Gestational age was defined as the number of days postcoitum (p.c.), with the day of the vaginal plug recorded as day 0.5 (Rugh et al., 1990). Samples were fixed in freshly prepared 4% paraformaldehyde in PBS for 1-4 hour at 4°C and embedded as described in Zeller et al. (1983). Two micron sections were placed on gelatin-coated microscope slides and stored at −20°C until hybridization.

In situ hybridization

Slides with sections were brought to room temperature, dewaxed and hybridized to ^{35}S-labeled RNA probes overnight in a humidified box at 52°C. Hybridization buffer, washing and RNAase treatment followed the procedure described by Wilkinson et al. (1987). Slides were dipped in emulsion (Kodak NTB-2) and exposed for 3-10 days before developing.

Preparation of probes

Linearized plasmids provided templates for the synthesis of the sense and antisense ^{35}S-UTP-labeled probes using SP6 or T7 RNA polymerase. To make the riboprobe, a small *Pst*I/*Bam*HI fragment (Fig. 1) was subcloned from the genomic clone (Leder et al., 1981) into pGEM-3. The antisense riboprobe was synthesized using T7 polymerase to transcribe the plasmid linearized with *Hin*dIII; the sense riboprobe was similarly synthesized using SP6 polymerase on the same plasmid linearized with *Bam*HI. To make the riboprobe, an approximately 1 kb *Eco*RI/*Xba*I fragment was subcloned from a genomic clone (Leder et al., 1985) (Fig. 1) into the SP64 vector; antisense riboprobe was synthesized by using the SP6 polymerase to transcribe the *Ava*II linearized plasmid. The sense riboprobe was synthesized using the same fragment subcloned into pGEM-2, linearized with *Eco*RI and transcribed with SP6 polymerase.

Ribonuclease protection assay

Total RNA was isolated following the method of Chirgwin et al. (1979). Ribonuclease protection assays were performed as described by Krieg and Melton (1987). Radioactive probes (3×10^5 cts/minute) were hybridized overnight at 50°C to 2 μg of total RNA.

Results

Gene-specific riboprobes for α and ζ globins

In order to assess the expression of and globin genes independently, it was crucial to use probes that do not cross-hybridize. Although globin is quite different from (specifically, in 66 of 141 amino acids (Leder et al., 1985)), there are regions that are quite homologous. Therefore, we chose the dissimilar first exons of both genes together with their associated 5 untranslated sequences as gene-specific probes. The homology between the two -like genes is less than 50% in this region.

In Fig. 1 we demonstrate the specificity of these probes using the ribonuclease protection assay, an assay comparable in stringency to in situ hybridization. ^{32}P-labelled antisense probes and unlabeled sense RNAs corresponding to and transcripts were synthesized as indicated in Fig. 1A. As can be seen in Fig. 1B, hybridization of the antisense probe to the unlabeled sense RNA yielded the expected 200 bp protected fragment. Conversely, hybridization of the antisense probe to unlabeled sense RNA yielded no protected fragments. The same degree of specificity is exhibited by the probe (Fig. 1B).

This degree of specificity was maintained when total cellular RNA was used as the source of the unlabeled sense sequences. For example, when and probes were hybridized against total RNA extracted from day 9.5 and 10.5 p.c. embryos and from day 12.5 p.c. fetal liver, both probes protected the expected fragments (see Fig. 1C). Note that while ample expression of both genes is evident in the embryos, expression is greatly reduced in the fetal liver.

Expression of α and ζ globins in embryonic yolk sacs

Given the evident specificity of the probes, in situ hybridization was performed on early embryos with the intention of following and gene expression from the onset of erythropoiesis. As our earliest point, we chose day 6.5 p.c. embryos in which the mesoderm has just formed and begins to proliferate from the primitive streak. Using in situ hybridization, we also analyzed day 7.5 and 8.5 p.c. embryos and day 10.5 and 11.5 p.c. fetal livers. For each time point, we used sense riboprobes as negative controls. Although we present here only the results of a negative control hybridization with the sense probe for the day 7.5 p.c. embryo, similar hybridizations were done for all time points and were found to be similarly negative.

Fig. 2 shows sections of a day 6.5 p.c. egg cylinder embedded in the decidua. No hybridization can be seen at this time with the probe (Fig. 2B,D). Hybridization with the probe occurs only in maternal erythrocytes found in the decidua and not in the embryo. We conclude that, at this early time, erythropoiesis (as measured by the appearance of globin transcripts) has not yet begun. This finding is certainly in agreement with established data (Craig and Russell, 1964).

At day 7.5 p.c., in situ hybridization now reveals embry-

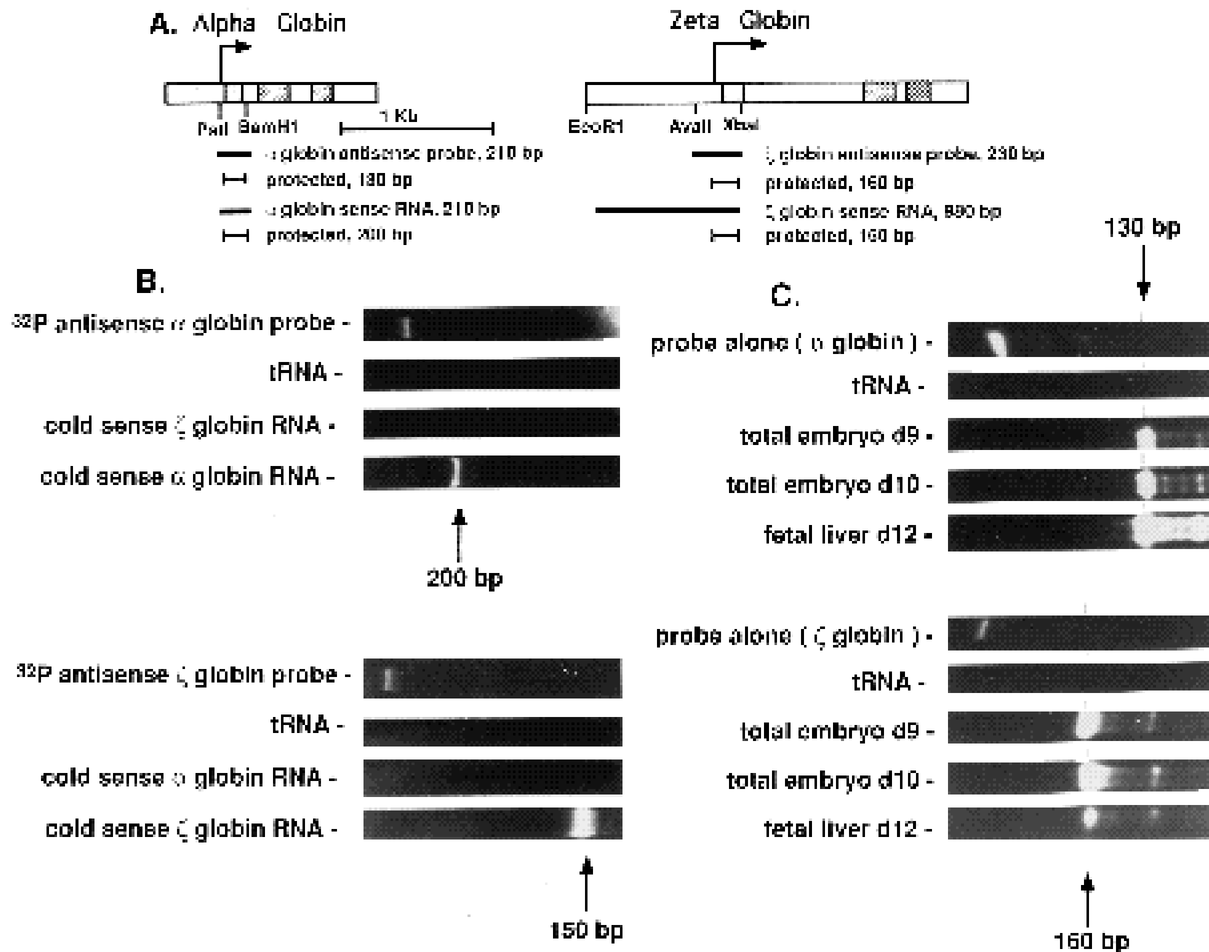

Fig. 1. Riboprobes and protected fragments for the mouse and globin genes. (A) Diagrammatic representations of the and globin genes and their three exons (filled blocks), displaying the restriction sites used in probe construction. Heavy horizontal lines show the extents of the antisense and sense probes used, while the thin lines indicate the expected ribonuclease-protected fragments. Note the size differences between the predicted sense and antisense protected fragments. (B) Lack of cross-hybridization between the and riboprobes. ^{32}P-labeled antisense riboprobes were hybridized against unlabeled sense riboprobes for both and globin. Protected fragments are only observed in the appropriate lane for both the globin antisense riboprobe (top panel) and the globin antisense probe (bottom panel). Approximately 1 ng of unlabeled sense riboprobes were used in this experiment. (C) Expression of and globins in day 9.5 and 10.5 p.c. embryos (including visceral yolk sacs) and day 12.5 p.c. fetal livers. Ribonuclease protection assays were performed using the antisense probe (top panel) and the antisense probe (bottom panel). In this experiment, 2 μg of total RNA was used for each sample; exposure time was 18 hours. The tRNA sample was included as a negative control.

onic cells expressing both and globin mRNAs (Fig. 3). The expressing embryonic cells are confined to the blood islands of the yolk sac, which at this stage of development is a small structure limited to the central portion of the egg cylinder. As can be seen in Fig. 3 (A,B,E,F), the embryonic patterns of and hybridization are very similar, with the exception of the hybridization of the probe to the maternal erythrocytes in the decidua. As expected, no hybridization could be seen with the sense probes (Fig. 3C,D,G,H).

Our results thus confirm that the onset of erythropoiesis (as measured by the initiation of globin mRNA synthesis) occurs around day 7.5 p.c. Unexpectedly, however, we find that and genes are co-expressed at this time. Furthermore, it is noteworthy that hybridization with the probe is more intense than hybridization with the probe. Since both and probes are approximately the same size (cf. Fig. 1), labeled to the same specific activity and have a similar uridylic acid content (42 uridylic acid residues in the probe and 37 in the), the probes per se are not likely to account for this difference. This difference must therefore reflect the levels of the respective transcripts.

At day 8.5 p.c., the visceral yolk sac is well developed, enveloping the entire fetus with interlaced blood islands (Fig. 4). Hybridization using and probes to these sections reveals high expression of both globin mRNAs in these structures. As before, both genes exhibit very similar patterns of expression, with the globin gene more intensely expressed. As would be expected at this stage of development, some hybridization can also be seen inside the embryo, representing circulating erythrocytes.

In an effort to detect the presence of mRNAs in single

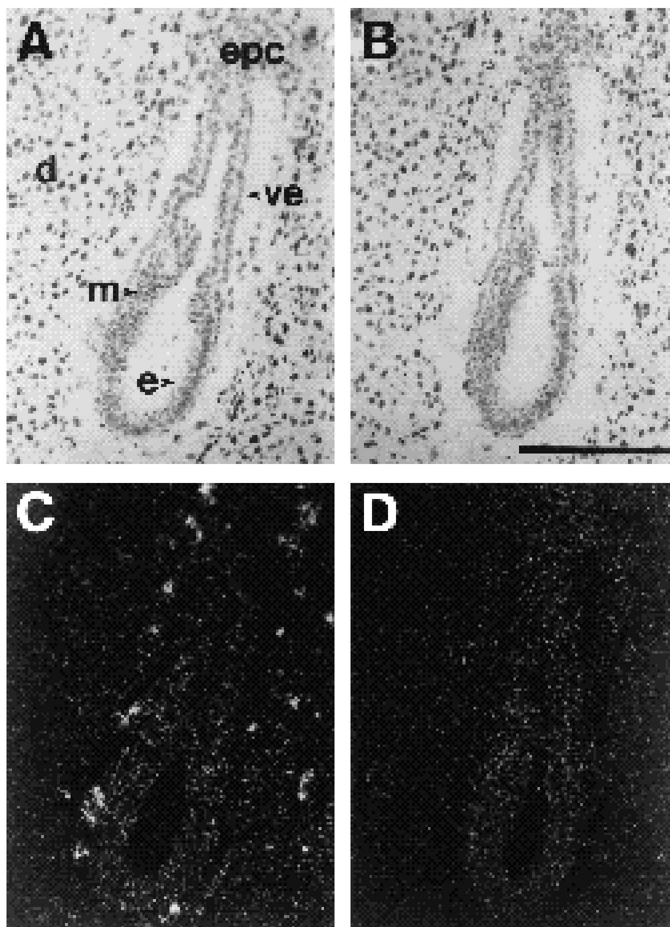

Fig. 2. Expression of and globins at day 6.5 p.c., examined by in situ hybridization. Two micron sections were cut sagittally through an early-primitive-streak-stage embryo contained within its decidual capsule. Bright-field and dark-field views are shown of adjacent sections hybridized with antisense (A and C) and antisense (B and D) riboprobes. The hybridizing grains observed in (C) are all located outside the embryo and correspond to globin transcripts present in maternal erythrocytes. Abbreviations: **d**, decidua; **epc**, ectoplacental cone; **e**, embryonic ectoderm; **m**, newly formed mesoderm emerging from the primitive streak; **ve**, visceral endoderm. The scale bar in (B) indicates 100 μm.

cells, we examined the large nucleated yolk sac erythrocytes under higher magnification. Since primitive erythrocytes are approximately 8 microns in diameter, it is not surprising to find the same cell in several sequential 2 micron sections. For example, the same cells (marked with arrows in bright field and dark field) can be seen in the two sections shown in Fig. 5. Both hybridize with (Fig. 5C), as well as (Fig. 5D) probes. Additional examples (not shown) also indicate that individual erythrocytes can indeed express both and mRNAs, suggesting that two distinct hemoglobins might be produced by a single cell.

Consistent with the interpretation noted above, sections through blood islands from day 10.5 p.c. embryos show strong hybridization to both probes (Fig. 6). Although individual cells cannot be scored, it is clear that a great proportion of the cells in the blood islands are erythropoietic cells expressing both and globin mRNA. Once again stronger hybridization is seen with the probe. It can also be observed that expression is confined to the nucleated erythrocytes of the blood islands and is not found in the underlying visceral endoderm cells or in the surrounding mesoderm.

Expression of α and ζ globins in fetal liver

Soon after the fetal liver is formed at day 10.0 p.c., it becomes an important source of definitive erythrocytes. These are smaller than the primitive cells of the yolk sac and are not nucleated (Kovach et al., 1967; Fantoni et al., 1967). Although globin is expressed in the fetal liver (Fig. 1C), it is not clear from ribonuclease protection assays whether this expression is due to liver-derived definitive cells, or whether it is due to yolk sac-derived primitive erythrocytes circulating through the fetal liver.

To resolve this issue, we noted that yolk sac-derived erythrocytes can be distinguished by their more intensely eosinophilic staining cytoplasm in haemotoxylin- and eosin-stained sections. Such sections of day 10.5 p.c. fetal liver hybridized to and probes are shown in Fig. 7. As expected, many cells show high levels of globin mRNA expression while many fewer cells express mRNA. Although it is not always easy to distinguish between a liver-derived erythroblast and a yolk sac-derived erythrocyte, the cells marked with short arrows in Fig. 7 clearly represent yolk sac-derived cells, while the cells marked with long arrows clearly represent liver-derived cells. Here again one sees that both the primitive and the definitive erythrocytes express the and mRNAs.

By day 11.5 p.c., the fetal liver is the major hematopoietic organ of the embryo and hybridization of the probe to liver sections reveals a very high level of gene expression. This is visualized in the dark-field illumination shown in Fig. 8C. Hybridization with the probe is largely confined to the blood vessels and likely represents circulating primitive erythrocytes (Fig. 8D). Thus, expression of globin mRNA in liver-derived erythrocytes is minimal at this stage at best.

Discussion

Although the globin genes were among the first mammalian genes to have been cloned and characterized (Leder et al., 1980; Maniatis et al., 1981), identifying the *cis*-acting sequences that governed their expression proceeded rather slowly until the use of transgenic mice permitted the identification of distant segments of DNA important for their control (Grosveld et al., 1987; Behringer et al., 1990; Enver et al., 1990). Even though this has been a successful approach, the study of the expression of human globin genes in mice may have limitations since there are substantial differences between the mouse and human loci. For example, duplicated fetal -like genes are absent in the mouse (Leder et al., 1980; Edgell et al., 1981).

Despite the similar organization of the globin loci in mouse and human, our results indicate that they differ in the programmed temporal expression of the -like genes.

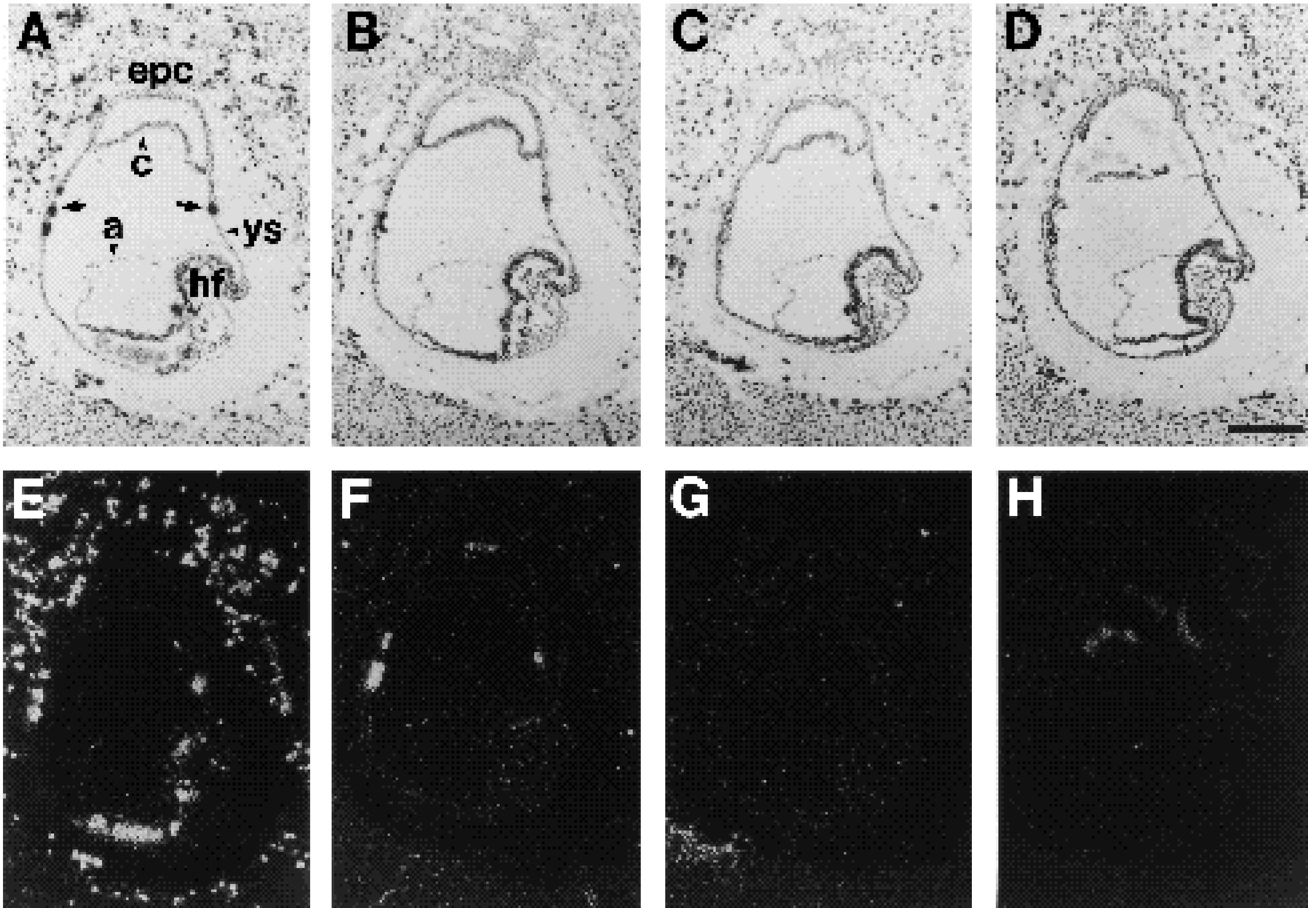

Fig. 3. Expression of and globins at late day 7.5 p.c. Adjacent parasagittal sections of a head-fold-stage embryo within the decidual capsule are displayed. Shown are bright-field and dark-field views are shown of hybridization to antisense (A and E) and antisense (B and F) riboprobes and, as negative controls, hybridization to sense (C and G) and sense (D and H) riboprobes. The arrows in A point to the newly formed blood islands of the visceral yolk sac, which hybridize positively to both the and antisense probes. As in Fig. 2, the substantial hybridization seen in E in the decidua corresponds to globin transcripts present in maternal erythrocytes. However, the patchy hybridization within the embryo results from contamination by maternal blood during dissection. Abbreviations: **a**, amnion; **c**, chorion; **epc**, ectoplacental cone; **hf**, head fold; **ys**, visceral yolk sac. The scale bar in D represents 100 μm.

While the and globin mRNAs are simultaneously expressed from the onset of embryonic erythropoiesis in the mouse, this appears not to be the case in the human. Here the best evidence indicates that only the embryonic protein is found in the five week embryo, after which synthesis of the adult protein commences (Peschle et al., 1985). Transgenic mice harboring major segments of the human locus under the regulation of the human globin locus control region (LCR) express these genes according to the human rather than the mouse pattern of temporal expression (Albitar et al., 1991). For example, by day 11.5 of mouse gestation, the human transgene is highly expressed while only a trace of human mRNA is detectable. This suggests that the human temporal pattern of expression is encoded in *cis* on the transgene (Albitar et al., 1991). It also implies that, although the mouse can recognize the regulatory signals on the human DNA, these signals must either be absent at the mouse locus or differently arranged so as to bring about the mouse pattern of expression.

As we have noted above, our in situ hybridization studies detect apparent levels of transcripts that are much higher than those of . If the entire -globin locus were equally available for transcription, one might have expected twice as many transcripts as , since there are two genes and only one . However, the very high level of expression in yolk sac blood islands at day 8.5 and 10.5 p.c. appears to exceed this two-fold expectation. Furthermore, our results with early embryos are consistent with the ribonuclease protection assays of Whitelaw et al. (1990), who observed high / expression ratios in later stage embryos. In this regard, Lee et al. (1991) have recently described a translational regulation of embryonic hamster globin gene expression in which cytoplasm derived from yolk sac erythrocytes inhibits the translation of adult, but not embryonic globin mRNA. Such a regulatory mechanism might dampen adult hemoglobin biosynthesis in primitive erythrocytes, notwithstanding the high level of adult mRNA present within these cells. Indeed, with this observation in mind, embryonic globin mRNA expression in the five week human embryo should be re-evaluated, given the possibil-

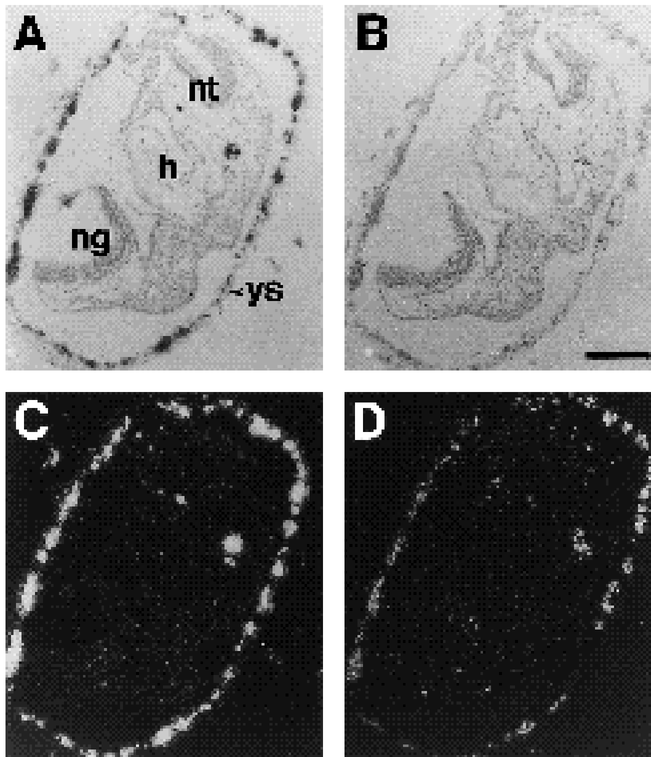

Fig. 4. Blood island expression of   and   globins at day 8.5 p.c. Adjacent transverse sections of an embryo and visceral yolk sac are displayed. Bright-field and dark-field views are shown of hybridization to   antisense (A and C) and   antisense (B and D) riboprobes. Hybridization within the embryo is due to circulating nucleated erythrocytes. Note that the area of the neural tube shown at the top of the sections is somewhat distorted due to a dissection artifact. Abbreviations: **h**, heart; **ng**, neural groove; **nt**, neural tube; **ys**, yolk sac. The scale bar in B represents 100 μm.

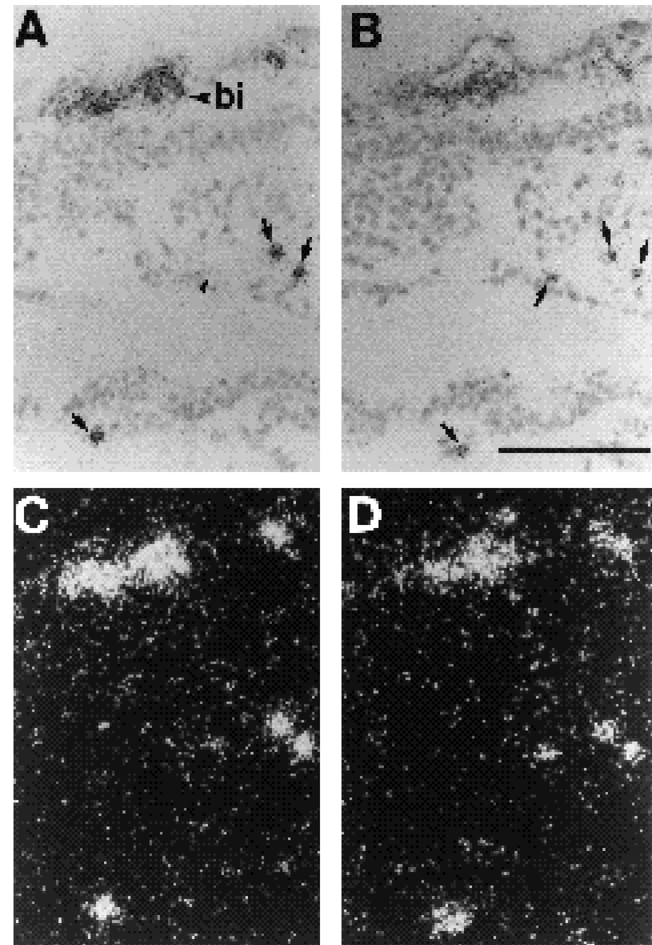

Fig. 5. Co-expression of   and   globins in individual nucleated erythrocytes at day 8.5 p.c. Shown are higher-power views of an embryo and visceral yolk sac, comparable in developmental stage to that displayed in Fig. 4. Bright-field and dark-field views are displayed of hybridization to   antisense (A and C) and antisense (B and D) riboprobes. The arrows point to expressing cells that are present in both adjacent two micron sections. Abbreviation: **bi**, blood island. The scale bar in B represents 50 μm.

ity that   mRNA is expressed, but not translated, at this early stage.

The notion that there might be two distinct hematopoietic lineages emerged when it was shown that primitive erythrocytes from the yolk sac produce embryonic hemoglobins that are electrophoretically distinguishable from those of the definitive adult cells of the fetal liver (Craig et al., 1964; Kovach et al., 1967; Fantoni et al., 1967; Barker, 1968). Subsequent in vivo and in vitro studies have shown that erythropoietic cells of the fetal liver are derived from the primitive cells of the yolk sac (Johnson and Moore, 1975; Chui et al., 1981; Wong et al., 1982). Therefore, if the two-lineage model is correct, the primitive cells must harbor both lineages (Wong et al., 1986).

Although the two-lineage model is plausible, one can easily interpret the experimental data on the basis of a single progenitor. According to this model, the primitive yolk sac erythrocytes colonize the liver rudiment and are induced there to differentiate along the definitive pathway giving rise to cells expressing adult hemoglobins. Indeed, diffusible factors produced by the liver rudiment and capable of inducing erythroid differentiation have been described (Cudennec et al., 1981). It has also been suggested that as gestation proceeds, even the primitive nucleated erythrocytes begin to produce adult hemoglobins (Brotherton et al., 1979; Chui et al., 1978), supporting the notion that there is a temporal switch within a single progenitor.

Additional evidence for a single progenitor comes from a transplantation experiment that indicates that primitive yolk sac hematopoietic cells can behave as progenitors to repopulate definitive fetal and adult hematopoietic organs and eventually to produce adult hemoglobin. Toles et al. (1989) used primitive stem cells from the yolk sac of normal, 9 day p.c. embryos (carrying the Hbb^{s} allele) to populate the hematopoietic organs of 11-15 day p.c. mutant *W* fetuses (carrying the Hbb^{d} allele). When analyzed after birth, some of the recipient mice were found to carry definitive red cells producing the donor hemoglobin. Thus, yolk sac donor cells harbor progenitors capable of responding to stimuli in the more mature recipient and of giving rise to

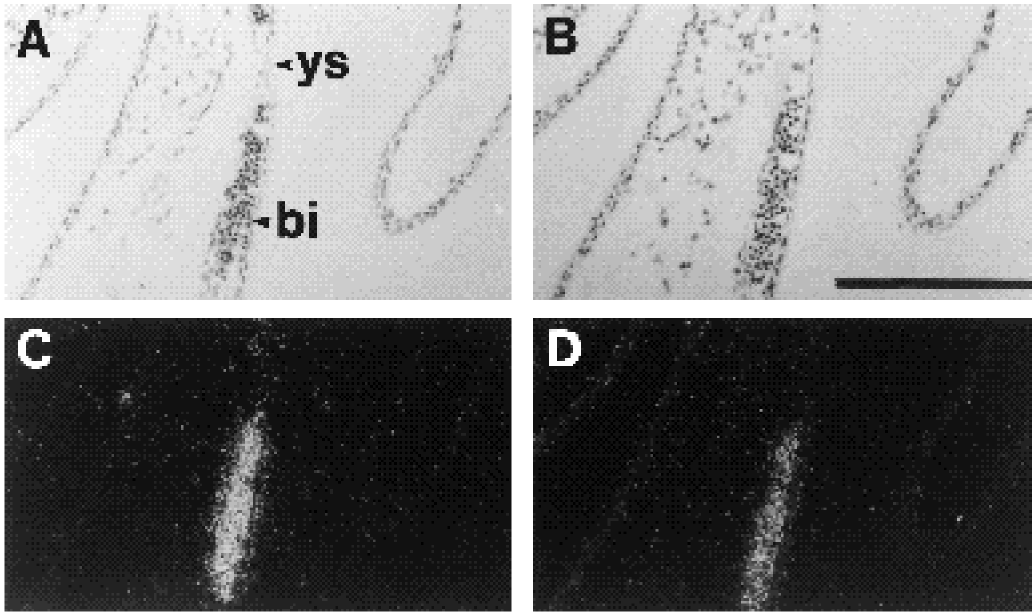

Fig. 6. Blood island expression of and globins at day 10.5 p.c. Shown are bright-field and dark-field views of hybridization to antisense (A and C) and antisense (B and D) riboprobes. Abbreviations: **bi**, blood island; **ys**, visceral yolk sac. The scale bar in B represents 100 μm.

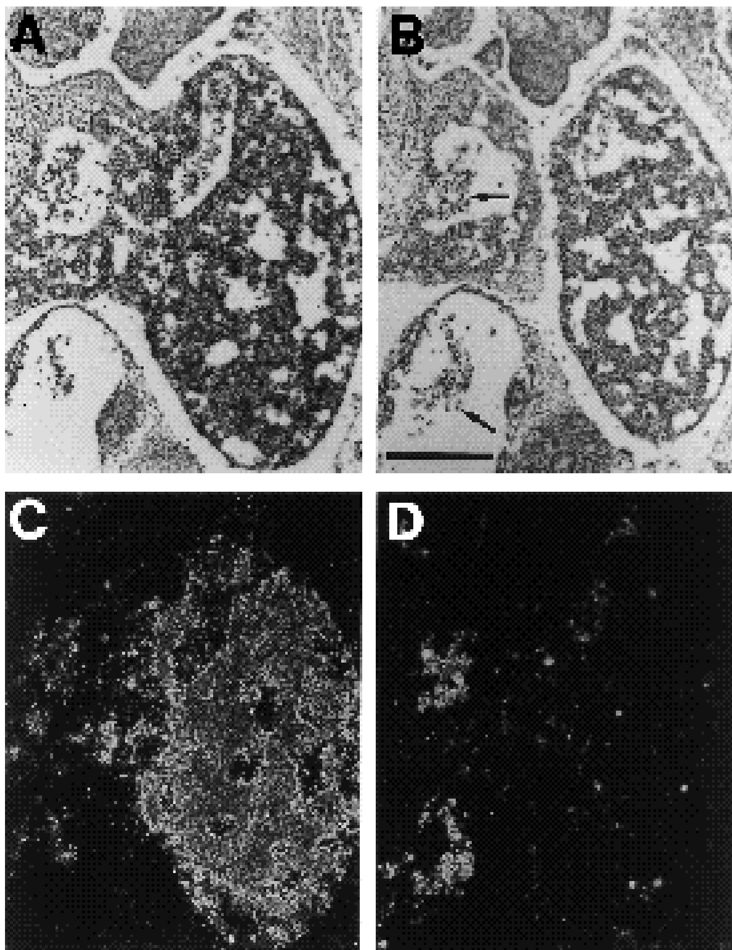

Fig. 8. Expression of and globins in day 11.5 p.c. fetal livers. Bright-field and dark-field views of hybridization to antisense (A and C) and antisense (B and D) riboprobes are shown. The arrows in B point to clusters of circulating nucleated erythrocytes. The scale bar in B represents 100 μm.

definitive adult erythrocytes expressing the adult hemoglobins.

We have approached the lineage question using in situ hybridization, a technique that allows gene expression to be evaluated in individual cells drawn from the developing organism. Moreover, using 2 micron sections, large primitive erythrocytes can be evaluated with different probes over several sections. Since primitive cells produce the

embryonic hemoglobins E1, E2 and E3, and since these contain mixtures of and chains (see above), we considered the possibility that gene expression might mark one lineage and another. If expression of the globin genes were to proceed initially with expression, then with both and expression, and then exclusively with expression, a single, linear erythroid developmental pathway would be simplest to imagine. On the other hand, if and were to be expressed concomitantly, but in different cells, a branched developmental pathway with parallel progenitors would seem more likely. As noted above, we found that both and globin genes were co-expressed in the same cells from the onset of erythropoiesis, an observation that fits well with a single linear erythroid progenitor pathway. These observations also suggest that individual cells might simultaneously produce not just and globin gene transcripts, but, together with globin gene expression, might contain more than one type of hemoglobin molecule.

Overlapping production of embryonic and adult globins is a phenomenon that is not unique to the mouse. A similar situation was described in the hamster by Boussios et al. (1982), who showed that yolk sac erythroid cells express predominantly embryonic hemoglobin, but express a small amount of the adult hemoglobin as well. Conversely, hamster hepatic erythrocytes express both adult and small amounts of embryonic hemoglobin. Recall that in the day 10.5 p.c. mouse fetal liver we detected both and globin transcripts in what appear to be definitive erythroblasts. By day 11.5 p.c., however, the definitive cells of the fetal liver no longer express the globin gene, while abundant transcripts were detected. At the same time, large circulating red blood cells (primitive cells) continue to express both and mRNAs. These patterns are compatible with a single, multipotential progenitor lineage emerging from the yolk sac and differentiating along a linear, definitive pathway in the hepatic environment.

In summary, our results show that erythroid precursors maintain gene expression while switching off expression of the gene. Thus, while the embryonic globin gene is expressed for the most part in primitive erythrocytes (with brief expression in hepatic erythroblasts) and is thus truly an *embryonic* gene, the so-called adult gene is also *embryonic* since it is expressed from the onset of erythropoiesis in the yolk sac. Such early expression of the gene raises the possibility that the embryonic gene is a redundant feature of erythropoiesis and may not be required for embryonic development in the mouse. Appropriate insertional mutations, accomplished by homologous recombination in transgenic mice, could clearly resolve this point.

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Fig. 7. Expression of and globins in day 10.5 p.c. fetal liver. Bright-field views of hybridization to antisense (A) and antisense (B) riboprobes on non-adjacent sections are displayed. These sections are heavily stained with hematoxylin and eosin to emphasize the eosin staining of the nucleated erythrocytes. At this stage, hybridization to both antisense probes is evident for nucleated erythrocytes (short arrows) as well as for presumptive liver erythroid cells (long arrows). The scale bar in B represents 50 μm.

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