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A molecular analysis of mouse development from 8 to 10 days *post coitum* detects changes only in embryonic globin expression

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Summary

The pattern of protein synthesis in 8-, 9- and 10-day *post coitum* (*p.c.*) mouse embryos was examined by 2-D gel electrophoresis of [^{35}S]methionine-labelled proteins. Of the 600–800 polypeptides detected only one, a $14\times10^3 M_r$ (14K) protein, was found to accumulate over this period. To isolate cDNA clones that potentially encode this protein, ^{32}P-labelled cDNA was synthesized from 9 and 10 days *p.c.* embryo poly(A)$^+$RNA, and used for the differential screening of an 8·5-day *p.c.* mouse embryo cDNA library cloned in λgt10. Six clones that hybridized strongly to the 10-day probe were purified and their inserts subcloned into plasmid vectors. Cross hybridization and restriction mapping of these inserts indicate that they fall into four distinct groups. Each of these hybridize with transcripts of approximately 600 nucleotides, which accumulate in the embryo from 9 to 10 days *p.c.* Expression was barely detectable in adult tissues and restricted to liver and spleen. Expression of one of these clones, 10.1, was examined by *in situ* hybridization of ^{35}S-labelled RNA probes to 8·5–12·5 day *p.c.* embryo sections. Strong hybridization was observed in yolk sac blood islands, fetal liver and embryonic erythrocytes, suggesting that 10.1A encodes an erythrocyte-specific protein. DNA sequence analysis indicates that the four classes of cDNA were derived from transcripts of the α1, ζ, βh1 and ε globin genes. Labelling of 10-day *p.c.* erythrocyte proteins with [^{35}S]methionine, followed by 2-D gel electrophoresis, clearly demonstrates that the most abundant polypeptide migrates to the same position as the 14K protein which accumulates from 8 to 10 days *p.c.* Thus the only abundant transcripts and corresponding proteins that change over a period of profound morphogenetic change correspond to globins of the newly established blood system.

Key words: mouse embryos, 2-D gels, cDNA screening, globin, protein synthesis.

Introduction

In the mouse embryo, the period of development from 8 to 10 days *p.c.* is marked by several major morphogenetic events, in particular the formation of somites and the onset of neural development. The structure of the 8-day embryo as seen in histological section is relatively simple. The embryo consists of epiblast cells, some of which migrate through the primitive streak in the posterior region of the embryo to form the embryonic mesoderm. At this time, the mesoderm shows no obvious somitic structure, although scanning electron microscopy suggests an underlying segmental organization (Meier & Tam, 1982). By 9 days *p.c.* the embryo is markedly more complex with a clear cephalocaudal organization. At the anterior end, upward migration of ectoderm and mesoderm cells gives rise to the head folds, from which the brain is formed later in development. In the trunk paraxial mesoderm is now clearly arranged into three to seven paired, epithelial blocks of cells, the somites, that are precursors for bone, muscle and dermal tissue of later stages. In addition, the heart tube is visible under the neural folds.

By 10 days *p.c.*, the embryo has rotated, externalizing the ectodermal tissue. 20 to 24 somites are now visible and these are undergoing differentiation into myotome, sclerotome and dermatome components. Enclosure of the neural folds is complete in the cephalic region and is proceeding posteriorly along the neural tube. Two to three branchial arches have formed under the cephalic region, and for the first time the forelimb bud is discernible as a swelling at the level of the 8th–12th somites. Internally, the

circulatory system is well established and rudiments for several organs including the liver, pancreas and thyroid are formed.

We are interested in the molecular changes that underlie the increasing complexity of the embryo from the late primitive streak stage to early organogenesis. To this end we have examined transcriptional and translational patterns over this period. Our results indicate only one clear difference in the mRNA and protein species synthesized. Since our analysis only discerns changes in the more abundant classes of mRNA and protein, it is most likely that morphogenesis in the 8- to 10-day *p.c.* mouse embryo is associated with changes in the rarer class of mRNA and protein.

Materials and methods

(A) *Mouse matings and embryo collections*

All mouse embryos used in this study were derived from matings between inbred CBA/Ca parents. Matings were set up on a reverse cycle, with the midpoint of the dark period at noon. Matings were assumed to occur at this time. Pregnant females were killed by cervical dislocation at noon 8, 9 and 10 days later. Embryos were dissected free of decidual and extraembryonic tissue in M2 medium (Quinn, Barros & Whittingham, 1982). 8-day egg cylinders were cut into embryonic and extraembryonic halves. Visceral endoderm that surrounds the embryonic cells at this time was not removed. Blood cells were collected from 10-day embryos as previously described (Kovach, Marks, Russell & Epler, 1967).

It should be noted that CBA embryos used in this study are developmentally retarded as compared to most other mouse strains (Theiler, 1972). Thus 8-, 9- and 10-day embryos are equivalent to 7·5-, 8·5- and 9·5-day embryos of other strains. Typically, 8-day embryos have a well-developed amnion and no obvious neural plate, 9-day embryos head folds and 3–7 somites, and 10-day embryos a closed anterior neuropore, 20–24 somites and forelimb buds.

(B) *[^{35}S]methionine labelling and two-dimensional gel electrophoresis of mouse embryo and blood cell proteins*

One to three embryos, or blood cells from twenty 10-day *p.c.* embryos, were incubated for 2 h at 37 °C in 200 μl of methionine-free RPMI, supplemented with 1 μCi ml^{-1} of [^{35}S]methionine (1360 Ci mm^{-1}, Amersham). Embryos (with developing hearts removed) or blood cell samples were then washed several times in PBSA and added directly to 20 to 100 μl of urea lysis buffer (O'Farrell, 1975), followed by vigorous vortexing. Incorporation of [^{35}S]methionine into trichloroacetic acid precipitable counts was determined. Either 3×10^5 cts min^{-1} (blood sample) or 1×10^6 cts min^{-1} (embryo samples) was loaded onto the isoelectric focusing gel (IEF). All 2-D gel procedures were essentially as described by O'Farrell (1975). We used a mixture of ampholytes for the IEF gel, which consisted of a 4:1 ratio of pH 5 to 7 (Serva) and pH 3 to 10 (Pharmacia), respectively. Proteins were resolved by SDS–polyacrylamide gel electrophoresis on 10 or 12·5 % polyacrylamide gels simultaneously calibrated by prestained molecular weight markers (BRL). Following electrophoresis gels were fixed, treated for fluorography (Laskey & Mills, 1975), dried and exposed to preflashed film (Fuji) at −70 °C.

(C) *RNA preparation from embryo and adult tissues*

RNA from embryo and adult tissue samples was prepared using the LiCl/urea (Auffray & Rougeon, 1980) or guanidinium hydrochloride (Maniatis, Fritsch & Sambrook, 1982) procedures. Poly(A)$^+$RNA from 9- and 10-day *p.c.* embryo total RNA was prepared by oligo dT cellulose chromatography (Aviv & Leder, 1972) or using Hybond mAP paper (Amersham). We estimated the total RNA content of 9- and 10-day embryos as 1·5 μg and 20–30 μg, respectively.

(D) *Screening of a mouse embryo cDNA library*

Approximately 50 000 λgt10 recombinant clones of an 8·5-day mouse embryo (C57BL/10ScSn/Nimr) cDNA library (the generous gift of Drs B. Hogan and K. Fahrner) were plated out and duplicate plaque lifts prepared by standard procedures (Benton & Davis, 1977). ^{32}P-labelled cDNA was prepared from 9- and 10-day mouse embryo poly(A)$^+$RNA as follows: 400 ng of RNA was mixed with 1 μl of 1 mg ml^{-1} oligo dT_{12-18} (Pharmacia), 26 μl sterile water and heated to 100 °C for 2 min. After allowing to cool, 10 μl OLB buffer (1 M-Hepes, 0·125 M-Tris–HCl, pH 6·6, 12·5 mM-$MgCl_2$, 10 mM-dATP, 10 mM-dGTP, 27 A_{260} units ml^{-1} random primers (Pharmacia), 0·16 % β-mercaptoethanol, 5 μl [^{32}P]dCTP, 5 μl [^{32}P]dTTP (3000 Ci mm^{-1}, New England Nuclear) and 10 units of AMV reverse transcriptase (Pharmacia) were added and the mixture incubated at room temperature for 1 h. Next, 4 μl of 5 mM-dATP, dGTP, dCTP and dTTP were added and incubation continued for a further 30 min at room temperature and 30 min at 37 °C. To remove RNA template, 1·2 units RNase H (Pharmacia) was added and incubated at 37 °C for 1 h prior to removal of unincorporated nucleotides on a Sephadex G50 column. These probes were hybridized to plaque lifts at 10^6 cts min^{-1} ml^{-1} for 36 h and then washed by standard procedures (Maniatis *et al.* 1982). Plaques corresponding to differentially expressed RNA were purified to homogeneity and their inserts excised and subcloned into pGEM3 as described (Maniatis *et al.* 1982).

(E) In situ *hybridization*

Procedures for *in situ* hybridization were as described by Cox, DeLeon, Angerer & Angerer (1984) with the following modifications. Embryos were fixed in 4 % paraformaldehyde in PBS at 4 °C overnight and then washed, dehydrated and embedded in paraffin wax (Fibrowax pastillated; Raymond Lamb). Sections of 6 μm thickness were cut, transferred to chrome alum-gelatin-coated slides and allowed to dry in the presence of silica gel desiccant for at least 15 h. Wax was removed and sections rehydrated (Cox *et al.* 1984), then sections were fixed with 4 % paraformaldehyde in PBS for 20 min and washed twice with PBS for 5 min

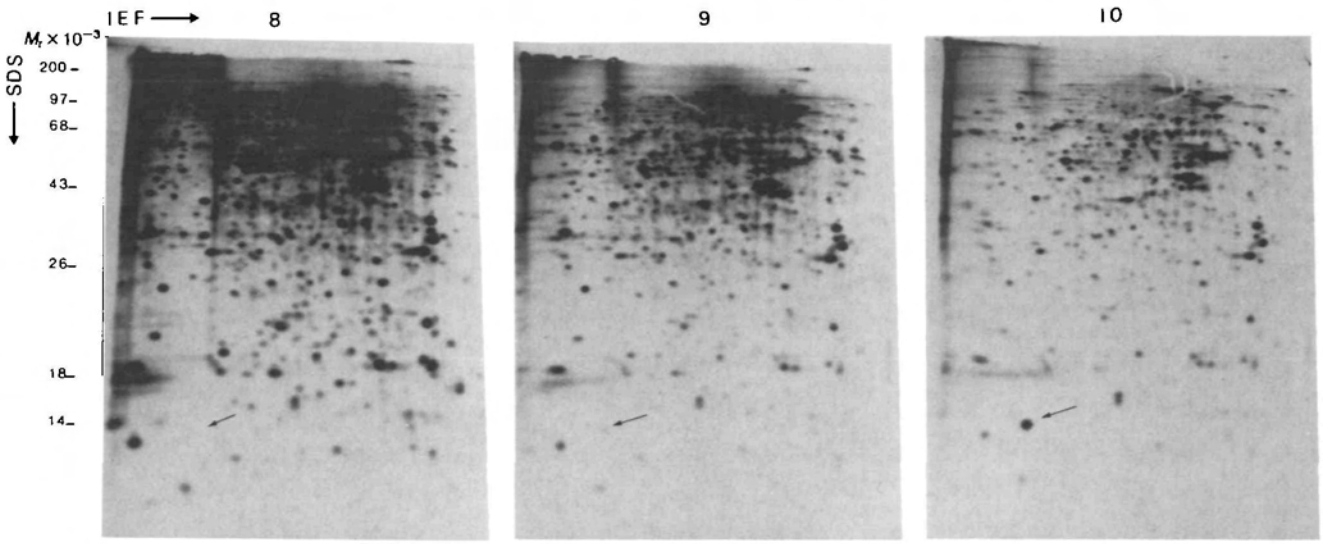

Fig. 1. Two-dimensional gels of [^{35}S]methionine-labelled embryo proteins. (A) 8-day *p.c.* embryos, (B) 9-day *p.c.* embryos, (C) 10-day *p.c.* embryos. The autoradiograph of 8-day labelled proteins (A) is deliberately overexposed to show the absence of the labelled 14 K polypeptide arrowed in B and C. Careful comparison of similar exposures shows no reproducible quantitative or qualitative differences in labelling of resolved polypeptides from different staged embryos. The direction of isoelectric focusing (IEF) and SDS–polyacrylamide gel electrophoresis (SDS) is indicated together with the position of prestained molecular weight markers run in parallel with embryo samples.

each. Sections were treated with 20 μg ml^{-1} proteinase K in 50 mM-Tris–HCl, pH 8·0, 5 mM-EDTA for 7·5 min, washed with PBS for 5 min and further fixed in 4% paraformaldehyde in PBS for 5 min. Following this, the slides were washed with PBS for 5 min and then treated with acetic anhydride and dehydrated as described (Cox *et al.* 1984). [^{35}S]UTP-labelled single-stranded sense and antisense RNA probes were prepared by standard procedures (Melton, Krieg, Rebagliati, Maniatis, Zinn & Green, 1984) from *Eco*RI inserts cloned in both orientations in pGEM 3, using T7 RNA polymerase (Boehringer) to transcribe the *Hind*III-linearized template DNA. Following removal of unincorporated nucleotides on a Sephadex G50 column, the probes were degraded to an average length of 100 bases (Cox *et al.* 1984) and ethanol precipitated. The probes were redissolved at a final concentration of 0·3 ng μl^{-1} kb^{-1} in 50% formamide, 0·3 M-sodium chloride, 20 mM-Tris–HCl, pH 8·0, 5 mM-EDTA, 10 mM-sodium phosphate, pH 8·0, 10% dextran sulphate, 1×Denhardt's, 0·5 mg ml^{-1} yeast RNA, 20 mM-DTT. Aliquots of this mix were used to hybridize sections at 50°C overnight and then washed and dehydrated (Cox *et al.* 1984). Autoradiography using Ilford K5 nuclear track emulsion was performed essentially as described by Angerer & Angerer (1981). Finally, sections were stained with 0·02% toluidine blue and mounted.

(F) *RNA blot analysis*

RNA preparations were fractionated on 1·5% agarose gels containing formaldehyde as described by Maniatis *et al.* (1982) and transferred to Genescreen (New England Nuclear). Crosslinking of RNA to the membrane was achieved by exposure to ultraviolet light (Church & Gilbert, 1984), ^{32}P-labelled probes were prepared from cDNA inserts as described (Feinberg & Vogelstein, 1984) and used at 10^6 cts min^{-1} ml^{-1} for overnight hybridization (Maniatis *et al.* 1982) followed by washing as recommended for Genescreen membranes by the manufacturer.

(G) *DNA sequencing of cDNA clones*

cDNA inserts isolated following differential screening of the 8·5-day λgt10 embryo library were subcloned into M13mp18 and 19 (Yanisch-Perron, Vieira & Messing, 1985) and sequenced in both orientations using the dideoxy chain termination method (Sanger, Coulson, Barrell, Smith & Roe, 1980) as modified by Biggin, Gibson & Hong (1983).

Results

(A) *Protein synthesis in 8-, 9- and 10-day* post coitum *mouse embryos*

[^{35}S]methionine incorporation into newly synthesized proteins of 8-, 9- and 10-day *p.c.* embryos was examined by 2-D gel electrophoresis. Fig. 1 illustrates a typical example of this analysis. The gels consistently and reproducibly resolved 600 to 800 polypeptides, almost all of which showed no quantitative or qualitative differences between 8 and 10 days. Only one protein of approximately 14×10^3 M_r (arrowed in Fig. 1) changed appreciably over this period. At 8 days *p.c.* this protein is not detected (Fig. 1A). It first appears at 9 days *p.c.* (Fig. 1B) and by 10 days *p.c.* the 14 K protein is one of the more abundant newly synthesized proteins (Fig. 1C).

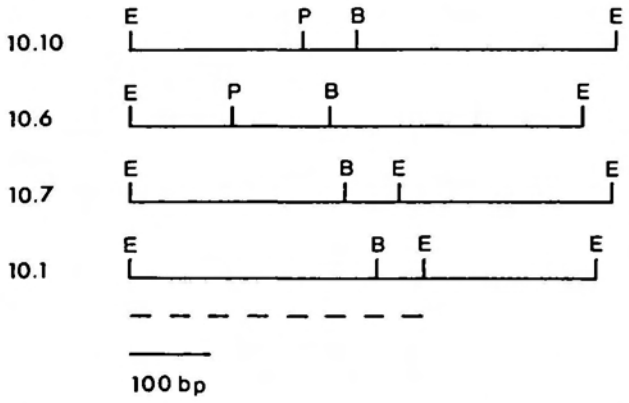

Fig. 2. Restriction maps of four different cDNA clones selected by differential screening of an 8·5-day *p.c.* λgt10 cDNA library with 9- and 10-day *p.c.* embryo ^{32}P-labelled cDNA. Restriction sites diagrammed are *Eco*RI (E), *Pst*I (P) and *Bam*HI (B). The broken line under 10.1 indicates the larger *Eco*RI fragment (10.1A) used as a hybridization probe in RNA blot (Fig. 3) and *in situ* hybridizations (Fig. 4).

(B) *Differential cDNA screening of an 8·5-day* p.c. *embryo cDNA library*

To attempt to isolate cDNA clones encoding the 14 K protein, we undertook differential screening of an 8·5-day *p.c.* mouse cDNA library with ^{32}P-labelled cDNA synthesized from 9 and 10 day *p.c.* embryo poly(A)$^+$mRNA to identify cDNA clones that hybridize strongly only to the 10-day probe. This approach assumes that the accumulation of the 14 K protein reflects an accumulation of mRNA encoding this protein from 9 to 10 days *p.c.* On screening 50 000 plaques, only six recombinants were identified that hybridized differentially with the 9- and 10-day probes. All of these showed strong hybridization specifically with ^{32}P-labelled 10-day cDNA.

Inserts from recombinant phage λgt10 were isolated and subcloned into the plasmid vector pGEM3, then restriction endonuclease mapped and tested for cross reactivity. These data indicate that the inserts fell into four distinct groups and restriction maps of the largest members of these are illustrated in Fig. 2. The only cross hybridization between these groups occurred at low stringency between the smaller (right in Fig. 2) *Eco*RI fragment of 10.1 and of 10.7.

(C) *RNA blot analysis of the expression of 10-day embryo-specific cDNA clones*

Expression of the RNAs corresponding to the isolated cDNA clones was examined in embryo and adult RNA. All four cDNA clones hybridize strongly to 10-day embryo transcripts of approximately 600 b, sufficiently large to encode 14 K proteins. An example of an RNA blot using the 10.1A fragment as a probe is shown in Fig. 3. The 600 b transcript (arrowed in Fig. 3) increases approximately tenfold from 9 to 10 days *p.c.* and in adult tissues is present in barely detectable amounts, only in liver and spleen. Hybridization of the same RNA blot to a control actin probe (Minty, Caravatti, Robert, Cohen, Daubas, Weydert, Gros & Buckingham, 1981) indicates that similar amounts of RNA are present in all lanes (Fig. 3, upper band in all tracks).

(D) In situ *hybridization of 10.1A to mouse embryo sections*

To further characterize the embryonic expression of one of these clones, ^{35}S-labelled single-stranded probes corresponding to each of the strands of 10.1A were hybridized to mouse embryo sections. We observed strong hybridization specific to one strand

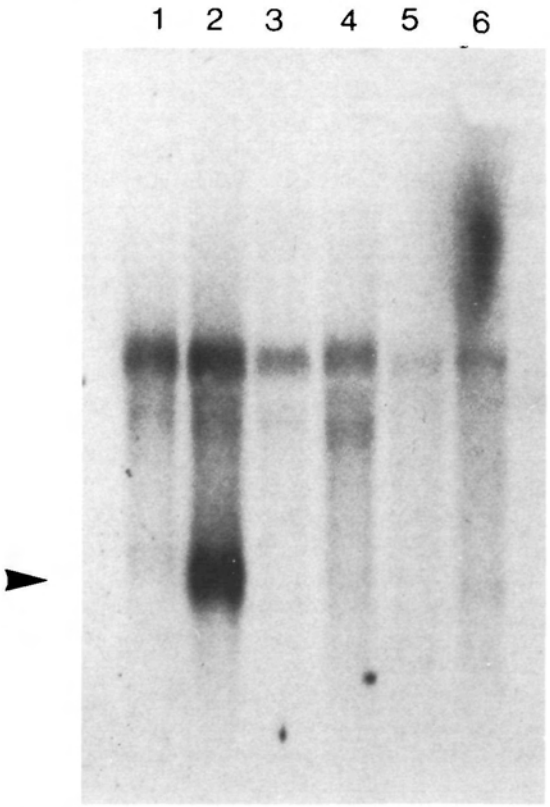

Fig. 3. RNA blot analysis of expression of 10.1 mRNA in embryonic and adult tissue. 10 μg of various embryo and adult fetal RNAs were electrophoresed through a denaturing agarose gel, transferred to Genescreen and hybridized first with 10.1A, then with a control actin coding probe (Minty *et al.* 1981) to verify that all lanes contain approximately equal amounts of undegraded RNA. Lane 1, 9-day *p.c.* embryo RNA; lane 2, 10-day *p.c.* embryo RNA; lane 3, adult liver RNA; lane 4, adult testis RNA; lane 5, adult kidney RNA; lane 6, adult spleen RNA. The lower band (arrowed, 600 nucleotides) represents specific hybridization with 10.1A and the upper band (2300 nucleotides) hybridization with the actin probe.

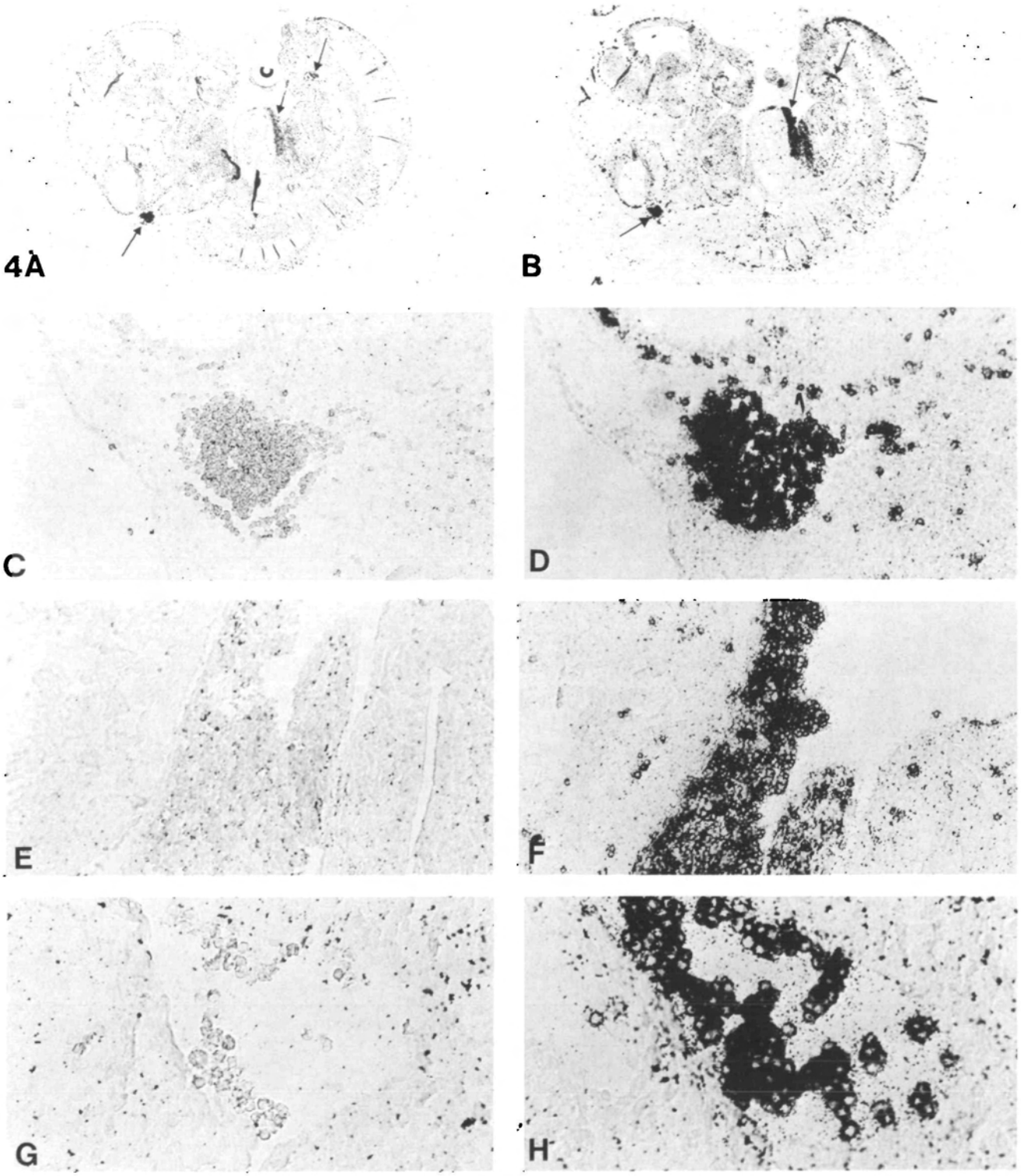

Fig. 4. *In situ* hybridization of ^{35}S-labelled strand-specific RNA probes to tissue sections of a 12·5-day *p.c.* embryo. (A,C,E,G) Hybridization with the presumed 'sense' strand shows no reaction. (B,D,F,H) Hybridization with the presumed 'anti-sense' strand shows strong hybridization. (A,B) Whole mounts of parasaggital sections of a 12·5-day *p.c.* embryo; (C–H) High-power views of arrowed areas in A and B; (C,D) blood cells in head region sinus; (E,F) developing fetal liver; (G,H) blood cells in the posterior region of the aorta. Strong strand-specific hybridization to blood cells (D,H) and haematopoietic tissue (F) is clearly visible.

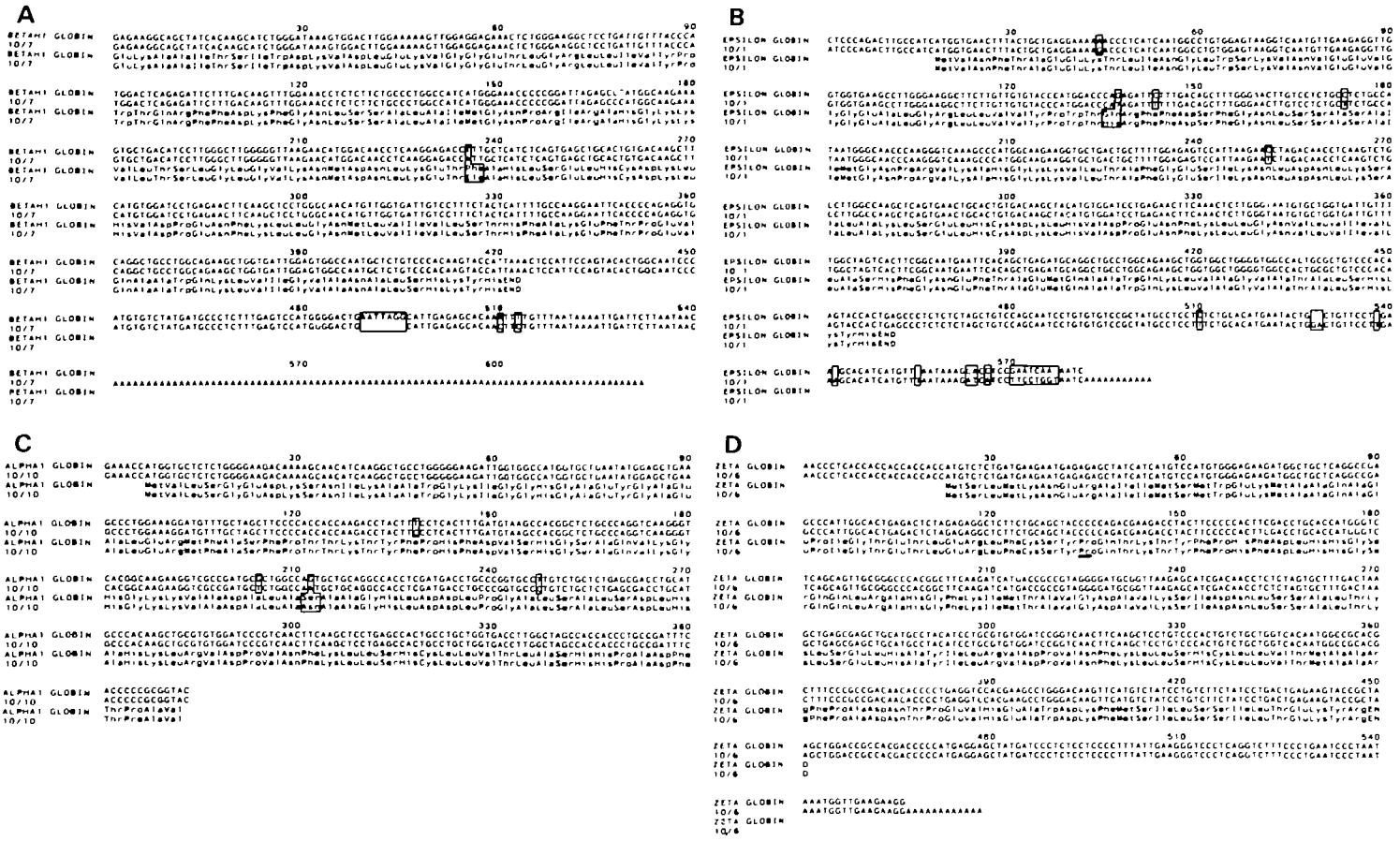

Fig. 5. Complete DNA and predicted amino acid sequences for cDNA inserts 10.7, 10.1, 10.10 and partial sequence of 10.6. Sequences are aligned to show nucleotide and amino acid homology with published sequence for βH1, ϵy_2, ζ and α1 globin genes, respectively.

only with blood cells and haematopoietic sites in sections of embryos from 8·5 to 12·5 days *p.c.* Examples of such a hybridization with 12·5 day embryos are illustrated in Fig. 4. Hybridization is only seen to fetal blood cells (Fig. 4D,H) and liver (Fig. 4F), which at this time is the fetal site of haemopoiesis. These results strongly suggest that the 10.1 cDNA clone recognizes an erythroid-specific transcript.

(E) *DNA sequencing of the 10-day embryo cDNA clones*

The isolated cDNA inserts were subcloned into the M13 phages mp18 and 19 and sequenced in both orientations. The complete sequence of three clones (10.1, 10.10, 10.7) and a partial sequence for the fourth clone (10.6) are shown in Fig. 5. Comparison of the sequences with those of known erythroid-specific genes identified all four cDNAs as products of globin genes (Fig. 5). Two are homologous to genes of the β-family. 10.1 to the embryonic epsilon gene (ϵy^2) and 10.7 to the embryonic βH1 gene. The other two are homologous to the α family, 10.10 to the embryonic zeta (ζ) gene and 10.6 to the embryonic- and adult-expressed α1 gene. It is interesting to note that comparison of the sequence between C57BL/10ScSnNimr mice used in this study and Balb/c from published data reveals differential rates of change in the different genes. Whereas ζ and 10.10 are identical, ϵy^2 and 10.1 show 23 base changes distributed throughout the coding and noncoding sequence.

(F) *Identification of the 14 K protein as the major newly synthesized protein of the embryonic erythrocyte*

From the above results, we would predict that if the 14 K protein that accumulates from 8 to 10 days of development is a globin encoded by one or more of the isolated cDNA clones, a similar 14 K protein should be the major protein synthesized by embryonic erythrocytes. To test this, embryonic erythrocytes were isolated from 10-day *p.c.* embryos, incubated with [^{35}S]methionine and labelled proteins resolved by 2-D gel electrophoresis (Fig. 6). The major labelled protein from embryonic erythrocytes migrates to an identical position as the 14 K protein that accumulates in 10-day embryos. Thus it is highly

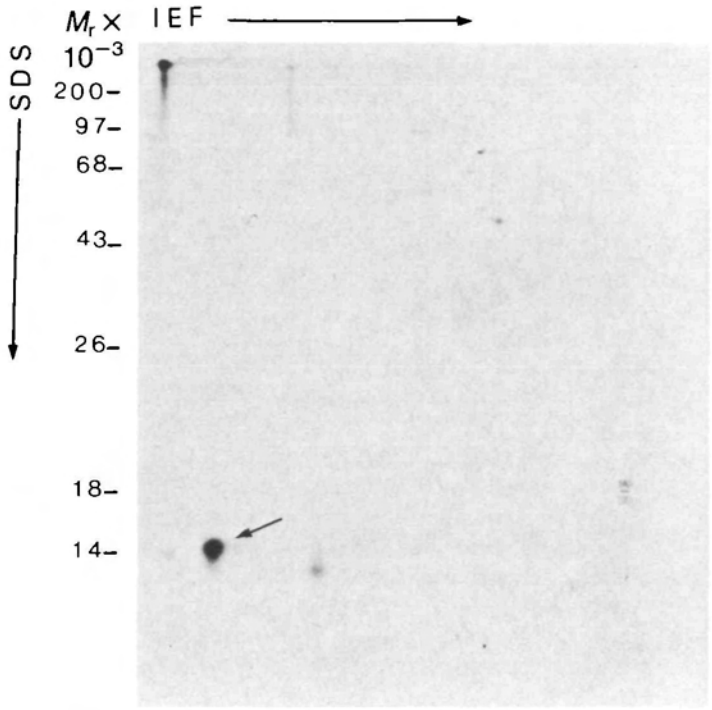

Fig. 6. Two-dimensional gel of [^{35}S]methionine-labelled erythrocyte proteins from 10-day *p.c.* embryos. The most-intensely labelled polypeptide (arrowed) migrates to an identical position as the 14 K polypeptide seen to accumulate from 8–10 days of embryonic development (Fig. 1).

likely that the 14 K embryonic protein is indeed a globin.

Discussion

The first erythrocytes in the mouse embryo arise as a result of erythropoiesis in the blood islands of the yolk sac mesoderm, around 9 days *p.c.* (Kovach *et al.* 1967; Fantoni, de la Chapelle, Rifkind & Marks, 1968). During the following 24 h, the heart undergoes a considerable increase in size and complexity, from a simple tube to a large beating structure. Coincident with the development of the heart, there is a rapid expansion of both the internal and external circulatory system of the embryo, thereby establishing an effective mechanism for the transfer of materials to and from the developing embryo. In the work reported here, we investigated changes occurring in mRNA and protein populations of mouse embryos over this period of 8 to 10 days *p.c.* The only detectable changes were the appearance of globin mRNA and protein, coincident with the onset of erythroid development.

Four groups of cDNA clones were isolated which correspond to two embryonic β-family genes, βh1 (Hill, Hardies, Phillips, Davis, Hutchinson & Edgell, 1984) and ϵy^2 (Hansen, Konkel & Leder, 1982), as well as two α-family genes, embryonic ζ (Leder, Weir & Leder, 1985) and embryonic/adult α1 (Nishioka & Leder, 1979). No cDNA clones were isolated encoding the embryo-specific βh0 globin, which presumably reflects the considerably lower abundance of βh0 transcripts over this period (Hill *et al.* 1984). The βh1, ϵy^2 and ζ globins are, in normal circumstances, only expressed in embryonic and early fetal erythrocytes. As the major site of erythropoiesis shifts from the yolk sac to the fetal liver from about 12 days *p.c.* (Kovach *et al.* 1967), there is a marked change in erythroid development. In contrast to yolk-sac-derived erythrocytes, hepatic erythrocytes are smaller, non-nucleated and express predominantly adult globins (Kovach *et al.* 1967). Thus from 12 days *p.c.* there is a gradual replacement in the peripheral blood, of yolk-sac-derived erythrocytes expressing embryonic globins, by liver-derived erythrocytes expressing adult globins.

Although the regulation of globin mRNA and proteins has been widely investigated in the established yolk sac and in later liver-derived embryonic and fetal erythrocytes, little is known of the onset of globin synthesis in the small numbers of haemocytoblasts which first appear at 8 days *p.c.* *In situ* hybridization as demonstrated here, provides a sensitive detection procedure for addressing this issue, and experiments are currently underway to examine the switching on of early globin genes.

The establishment of the embryonic blood system is one key developmental event occurring over the period of embryogenesis that we investigated. Other major morphogenetic changes include the onset of somitogenesis and neural tube formation. It seems likely that changes in the synthesis of specific mRNA and protein species also underly these processes. However, we were unable to detect any differences except those associated with erythroid development. Thus it would appear that molecules involved in these other processes are expressed at lower levels than can be detected using the procedures described. To isolate cDNA clones for low abundance mRNAs, we are currently developing more sensitive methods using 'subtractive' hybridization probes (Davis, Cohen, Nielsen, Steinmetz, Paul & Hood, 1984) for the differential screening of cDNA libraries. It is hoped that such probes will then allow us to identify changes in less-abundant stage-specific mRNA molecules over this interesting period of development.

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