# Sequence Differences between Mouse Embryonic $y$- and Adult $\beta$-Globin Messenger Ribonucleic Acids†

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ABSTRACT: Poly(A)-containing RNA was isolated from yolk sac cells of $BDF_1$ hybrid mice on the 12th day of gestation. In vitro translation of the poly(A)-containing RNA by a messenger RNA (mRNA) dependent reticulocyte lysate system revealed that this RNA contained a high concentration of mRNAs from two embryonic globin chain subunits, $y$-1 and $y$-2. Cross hydridization of complementary DNA (cDNA) to the embryonic globin mRNAs with adult reticulocyte globin mRNAs and with cloned cDNAs for adult $\alpha$- and $\beta$-globin chain mRNAs showed that the sequence of embryonic globin $y$-chain mRNA differed from that of adult $\beta$-chain mRNA.

Many animal species are known to synthesize various globin chain subunits sequentially during embryonal development. During human fetal development, for example, although $\delta$-chain synthesis continues at a low level throughout fetal and neonatal life, there is a switchover from $\gamma$-chain to $\beta$-chain synthesis. In humans, the $\beta$-like globin genes have been shown to be closely linked (Tuan et al., 1979). An analogous change of globin chain expression from the embryonic to the adult type occurs in mice during development. However, in mice embryonic globins are synthesized in yolk sac cells of the fetus, and the adult type $\beta$ chain is synthesized in the fetal liver (Fantoni et al., 1967, 1968), whereas in humans both the fetal type $\gamma$ chain and the adult type $\beta$ chain are synthesized in the liver. The adult type $\alpha$- and $\beta$-chain mRNAs of mice have been characterized, and their expressions have been investigated in detail (Gilman, 1972), but little is known about the fetal globin chains of mice. Characterization of embryonic $y$-globin genes seems of particular interest, because little is known about their molecular properties or their expression and repression (Gilman, 1976). In studies on the mechanism of switch from $y$-globin expression to $\beta$-globin expression during mouse embryonic development, it is essential to use nucleic acid probes that are specific for the respective globin genes.

This paper describes the separation and characterization of embyronic $y$-chain globin mRNAs and their relation to $\beta$-chain mRNA.

## Materials and Methods

*Preparation of Mouse Yolk Sac Cells.* Pregnant C57BL/6 female mice that had been mated with male DBA/2 mice were obtained from the Fuji Animal Farm (Yamanashi Prefecture, Japan). The start of pregnancy was taken as the day of appearance of a vaginal plaque (day 0). On day 12 of pregnancy, the embryos' ($BDF_1$ embryos) yolk sac erythroid cells were isolated from yolk sac blood islands as described by Kovach et al. (1967). The yolk sac cells were stored at −80 °C until used for extraction of RNA.

Other inbred or cross-bred mouse strains are from our own colonies at the Cancer Institute (Tokyo, Japan).

*Analysis of Hemoglobin Chains.* The subunits of fetal and adult globins were separated by electrophoresis on 12% polyacrylamide gel containing 8 M urea–5% acetic acid and 2% Triton X-100 by the method of Franklin & Zweidler (1977). Preelectrophoresis was carried out with 0.5 M cysteamine in 5% acetic acid at a constant voltage (100 V dc) until the current became steady (usually after one night). Lysates of yolk sac cells or adult mouse reticulocytes were prepared as described in the figure legends. Briefly, the cells were lysed in $H_2O$–$CCl_4$ (4:1 v/v) by gentle pipetting and then centrifuged at 10000$g$ for 30 min at 4 °C. The supernatant was then subjected to electrophoresis. For this, about 10 $\mu$g of hemoglobin sample was put into each slot of the slab gel, and electrophoresis was carried out at 100 V dc, under a constant voltage. Globin chains from either total red cell lysates or hemoglobin purified by isoelectric focusing were analyzed after denaturation of the lysate with 4 M urea, 5% acetic acid, and 5% $\beta$-mercaptoethanol.

Hemoglobin chains separated as described above were also analyzed by two-dimensional gel electrophoresis. In the first dimension the samples were analyzed by isoelectric focusing disc gel electrophoresis (Monte et al., 1976). For this, a mixture of 8% polyacrylamide gel containing 4% ampholine, pH 6–8, 0.005% riboflavin, and 0.06% *N,N,N′,N′*-tetramethylethylenediamine was polymerized by UV irradiation overnight. The anodal buffer was 0.02 M $H_3PO_4$ and the cathodal buffer was 0.1 M NaOH. Electrophoresis was carried out at a constant voltage of 200 V for 30 min, then at 400 V for 60 min, and finally at 600 V for 30 min. The hemoglobin was detected by converting it to cyanomethemoglobin by treatment with KCN (0.067 $\mu$g/$\mu$g of protein) and scanning the gel at 520 nm. After this, the gel was dialyzed against 4 M urea–5% acetic acid–5% $\beta$-mercaptoethanol for 2 h and then placed on the Triton–acidic urea gel described above. Electrophoresis in the second dimension was carried out as described by Rovera et al. (1977).

*Preparation of Yolk Sac Globin mRNA.* The yolk sac cells were thawed, homogenized in the extraction buffer described below, and centrifuged at 10000$g$ at 4 °C for 1 min. Then the total cytoplasmic RNA in the supernatant was extracted with $CHCl_3$–phenol (1:1 v/v) and $CHCl_3$–isoamyl alcohol (50:1 v/v). The extraction buffer consisted of 0.5% Nonidet P-40 (NP-40) in 50 mM Tris, pH 8.0, 10 mM $MgSO_4$, 100 mM NaCl, 20 mM EDTA, 0.25% sodium dodecyl sulfate ($NaDodSO_4$), 0.5% diethyl pyrocarbonate, 20 $\mu$g of poly(vinyl sulfate), and 35 $\mu$g/mL spermidine.

The RNA was precipitated with ethanol, and poly(A)-containing RNA was prepared as described previously (Kameji et al., 1977). The yield of poly(A)-containing RNA was about 2% of the total cytoplasmic RNA, which was similar to that

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0006-2960/80/0419-3967$01.00/0 

of globin mRNAs from mouse reticulocytes. The poly(A)-containing RNA was placed on a linear gradient of 15–30% sucrose containing 100 mM NaCl, 10 mM EDTA, and 10 mM Tris, pH 7.4, and centrifuged in the SW-27 rotor of a Beckman L5-65 ultracentrifuge at 24000 rpm for 22 h at 4 °C. In this way 30–40 μg of 9S RNA was obtained from $10^8$ yolk sac cells collected from about 600 embryos. Adult globin mRNAs were obtained similarly from the spleen cells or reticulocytes of anemic mice.

*Analysis of Translation Products.* Cell-free translation was performed with messenger RNA dependent rabbit reticulocyte lysate pretreated with calcium-dependent micrococcal nucleases (Pelham & Jackson, 1976) to digest endogenous mRNAs. This lysate was obtained commercially from New England Nuclear Corp. (Boston, MA) as a Translation Kit (Catalog No. NEK-001). Translation was carried out following the assay protocol in the kit. Briefly, the reaction mixture of 25 μL contained 2.0 μL of mRNA (about 0.5 μg), 10 μL of reticulocyte lysate, and 13.0 μL of translation mixture, consisting of 8.5 μL of L-[$^{35}$S]methionine, 2 μL of cocktail (see translation cocktail in the instruction in the kit), 2 μL of 1 M $CH_3COOH$, and 0.5 μL of 32.5 mM $(CH_3COO)_2Mg$.

Translation was assayed by measuring the incorporation of [$^{35}$S]methionine as described by Pelham & Jackson (1976). When about 0.5 μg of fetal globin mRNA or 0.5 μg of adult globin mRNA was added to the translation mixture, about a ninefold incorporation of labeled methionine was observed.

The newly synthesized globin chains were analyzed after denaturation of the lysate with 4 M urea, 5% acetic acid, and 5% β-mercaptoethanol. The denatured sample was centrifuged at 10000 rpm for 5 min, and a sample of the supernatant (about $10^6$ cpm) was subjected to electrophoresis using the Triton–acidic urea gel system as described above. The gel was then dried in vacuo and exposed on X-ray film (Kodak XRP-5) at −80 °C for 12 h.

*cDNA–RNA Hybridization.* cDNA for the purified poly(A)-containing RNA was prepared with avian myeloblastosis virus (AMV) reverse transcriptase as described by Kameji et al. (1977), with minor modifications. The reverse transcriptase was obtained from Resources and Logistics, National Cancer Institute, through the U.S.–Japan Cooperative Cancer Research Program. Briefly, 9S yolk sac poly(A)-containing RNA (1 μg) was incubated for 90 min at 42 °C in a reaction mixture (50 μL) consisting of 50 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2$, 100 mM NaCl, 10 mM dithiothreitol, 1 mM dATP, dTTP, and dGTP, 100 μCi of [$^{32}$P]dCTP (350 Ci/mmol, New England Nuclear Corp.) (Monahan et al., 1977), 0.1 $A_{260}$ unit of oligo$(dT)_{12-18}$, 1 μg of yolk sac poly(A)-containing RNA, 20 units of AMV reverse transcriptase, and 10 μg of actinomycin D. The reaction was stopped by adding 0.1% NaDodSO$_4$, and the mixture was subjected to 0.1 mM NaCl and 5 mM EDTA. Labeled cDNA was extracted with phenol–chloroform (1:1 v/v). The preparation was boiled for 5 min in 0.1 N NaOH and neutralized with HCl, and cDNA was precipitated with ethanol. The specific radioactivity of [$^{32}$P]cDNA was about $5 \times 10^7$ cpm/μg, calculated assuming that [$^{32}$P]cDNA was incorporated into one-fourth of the total DNA sequences.

Hybridization was carried out in a final volume of either 10 or 20 μL in capillary tubes (Clay Adams Co., Parsippany, NJ), containing [$^{32}$P]cDNA (about 1000 cpm/assay). The salt concentration and hybridization temperature were as described in the figure legends. The ratio of RNA to cDNA and the concentration of RNA in the mixture were usually kept constant throughout the experiment. After we added all the reagents, the reaction mixture was heated at 95 °C for 10 min, rapidly cooled to 0 °C, and then brought to the hybridization temperature. After incubation, the amount of hybridization DNA was measured by digestion of single strands with $S_1$ nuclease. For this the mixture was incubated with 4 mL of a solution of 0.3 M NaCl, 0.03 M sodium acetate, pH 4.5, 3 mM $ZnCl_2$, 20 μg of single-stranded calf-thymus DNA, and 1000 units of $S_1$ nuclease (Ryffel & McCarthy, 1975) at 37 °C for 2 h. The cold 5% $Cl_3AcOH$-insoluble material was collected on a GF/C filter (Whatman Ltd.), and its reactivity was counted in a liquid scintillation counter. Usually 5 to 10% of cDNA was resistant to $S_1$ nuclease, and this amount was subtracted as the background level from observed values in hybridization experiments.

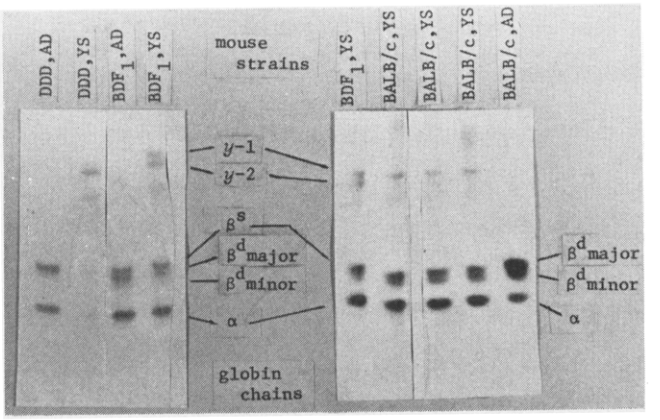


FIGURE 1: Electrophoretic analysis of embryonic globin subunits. DDD, $BDF_1$, and BALB/c are mouse strains. Hemoglobin lysates of adult peripheral blood (AD) and yolk sac cells (YS) were applied to Triton–acidic urea gel (about 10 μg of protein/slot) and subjected to electrophoresis as described under Materials and Methods. DDD and BALB/c mice were used to replace C57BL/6 and DBA/2 mice, respectively, because the latter two strains had poor reproduction rates. DDD has embryonic *y*-2 and adult $\beta^S$ globin subunits; BALB/c has embryonic *y*-1 and adult $\beta^D$ globin subunits.

## Results

To obtain a sufficient amount of embryonic globin mRNAs from yolk sac cells for study, we used $F_1$ hybrids of male DBA/2 mice and female C57BL/6 mice, usually called $BDF_1$ mice, since the cross gives a high reproduction rate and pregnancy is well maintained in the mothers. Different strains of mice are known to have specific *y*-globin chain patterns and adult β-globin chain patterns. In fact, strains of mice can be classified into two types on the basis of this specificity: one type of mice, the D type including DBA/2 and BALB/c mice, has an embryonic *y*-1 type chain and adult $\beta^D$ type chain ($\beta^D$ major and $\beta^D$ minor), and the other type, the S type including C57BL/6 mice, has an embryonic *y*-2 chain and adult $\beta^S$ type chain (Gilman, 1976). Because of the codominance of the *y*-1 and *y*-2 alleles, $F_1$ hybrids of DBA/2 (*y*-1 type) and C57BL/6 (*y*-2 type) should synthesize both *y*-1 and *y*-2 chains.

*Two Dimensional Gel Separation of Embryonic Globin Subunits.* The population of yolk sac cells from 12-day embryos was morphologically homogeneous, consisting of fetal erythrocytes with pycnotic nuclei and a few larger erythroblastic cells. A lysate of $BDF_1$ yolk sac cells of a 12-day embryo was analyzed by electrophoresis in polyacrylamide gel containing urea and Triton X-100. Under these conditions, $\beta^D$ major and $\beta^D$ minor globin chains are clearly separated. Slowly migrating bands were also clearly detected (Figure 1). Evidence that these slowly migrating bands, denoted as *y*-1 and *y*-2 in the graphs, were embryonic hemoglobin subunits was obtained by two-dimensional gel electrophoresis. For this

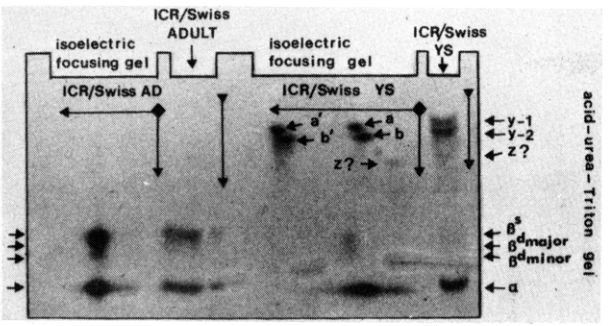


FIGURE 2: Two-dimensional gel analysis of fetal globin subunits. Adult and embryonic hemoglobins, 70 $\mu$g each, were subjected to isoelectric focusing at pH 6–8 for 2 h at 4 °C. Then the gel was dialyzed against a mixture of 4 M urea and 5% acetic acid and subjected to electrophoresis in the second dimension in gel containing urea and Triton X-100. Long arrows indicate the directions of electrophoresis. a and b are $y$-1 and $y$-2, respectively, which comigrate with the $\alpha$ subunit in isoelectric focusing. a′ and b′ are possibly $y$-1 and $y$-2, respectively, which bind to $x$ or another subunit. A faint but consistent band moving faster in Triton–acidic urea gel than $y$-1 and $y$-2 is thought to be the $z$ subunit ($z$? in the figure).

the yolk sac lysate was separated in the first dimension by isoelectric focusing, and hemoglobin was converted to cyanomethemoglobin and detected by its absorbance at 520 nm. The material was then subjected to electrophoresis in the second dimension in a Triton–acidic urea gel system. As shown in Figure 2, the two slowly migrating bands (a and b) were comigrated with $\alpha$-globin subunits, indicating that they were components of hemoglobin. They were identified as $y$-1 and $y$-2 subunits, respectively, by comparison with yolk sac lysate of D- and S-type mouse strains, which give only the $y$-1 and $y$-2 bands, respectively (Gilman, 1976). Lysates from the $BDF_1$ embryos used in this experiment showed the bands of both $y$ chains. Figure 2 suggests that three bands, those of $\alpha_2\beta_2$, $\alpha_2 y_2$, and possibly $\alpha_2 z_2$, can be separated by isoelectric focusing.

Electrophoresis in a 15% polyacrylamide gel containing $NaDodSO_4$ showed that the two bands have the same molecular weight as those of the $\alpha$-chain subunits (data not shown).

*Synthesis of Embryonic y-Globin Subunits in Yolk Sac Cells.* When yolk sac cells from embryos on day 12 of gestation were labeled in vitro with [$^{35}$S]methionine and analyzed by polyacrylamide gel electrophoresis (Figure 3), preferential synthesis of $y$-globin chains was observed. Thus, the yolk sac cells at this embryonic stage have abundant $y$-chain messenger RNA.

*Separation and Translation of Yolk Sac Cell Poly(A)-Containing RNA.* Total cytoplasmic RNA was extracted from the yolk sac cells of the embryos as described above, the poly(A)-containing RNA was separated by poly(U)–Sepharose column chromatography. This poly(A)-containing RNA amounted to about 2% of the total cytoplasmic RNA. On sucrose density gradient centrifugation, this poly(A)-containing RNA gave a major peak at around 9 S with small amounts of contaminating ribosomal RNA. This suggests that yolk sac cell mRNA has a rather simple composition, possibly containing much globin mRNA, like that of reticulocytes. The total poly(A)-containing RNA was translated in an mRNA-dependent rabbit reticulocyte lysate, and the products were analyzed by Triton–acidic urea gel electrophoresis as described above. The translation products of yolk sac cell mRNAs gave bands of $y$-1, $y$-2, and $\alpha$ and $\beta$ chains (Figure 4). After the rabbit reticulocyte lysate was incubated alone without added mRNA, no bands were detectable in the gels, indicating that

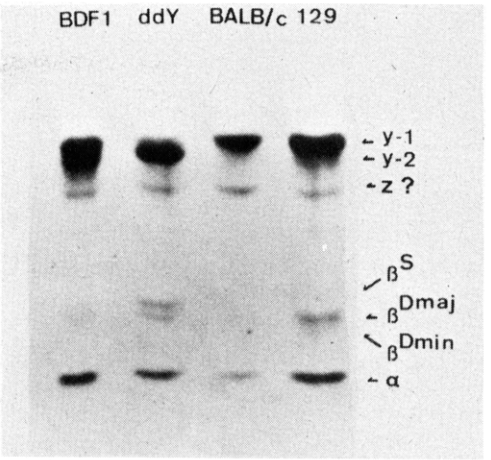


FIGURE 3: Electrophoretic analysis of hemoglobin subunits synthesized by embryonal yolk sac cells. Yolk sac cells ($10^8$ cells) from each mouse strain were incubated in methionine-free medium containing 200 Ci of [$^{35}$S]methionine for 40 min. After incubation, the specific radioactivity of the total yolk sac protein reached about 1000 cpm/$\mu$g. Samples ($10^6$ cpm) were subjected to electrophoresis. The gel shows that embryonic $y$ subunits can be synthesized in yolk sac cells maintained in vitro for a short period. The ddY strain is a random-bred strain, possessing both $\beta^S$ and $\beta^D$ subunits, but $y$-1 expression is less than $y$-2 in this gel, for unknown reasons. Strain 129 is an inbred strain possessing embryonic $y$-2 and adult $\beta^D$ subunits, $\beta^D$ minor being very faint.

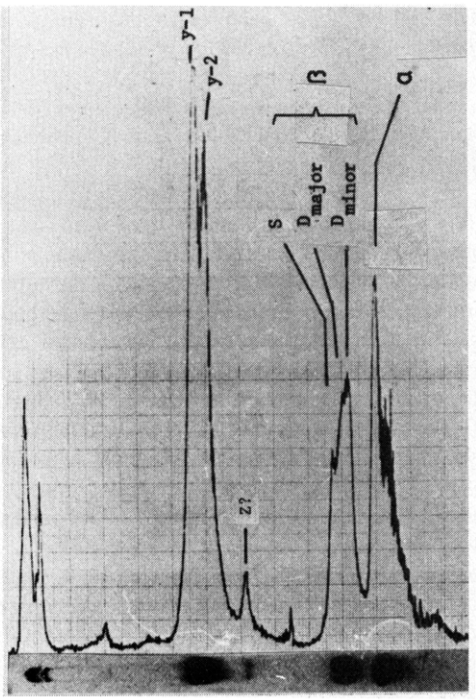


FIGURE 4: Cell-free translation of yolk sac 9S poly(A)-containing RNA. 0.5 $\mu$g of 9S poly(A)-containing RNA from yolk sac cells was added to an mRNA-dependent rabbit reticulocyte lysate. After translation, the products labeled with [$^{35}$S]methionine were subjected to electrophoresis and autoradiography as described under Materials and Methods.

the bands were dependent on the addition of the yolk sac mRNA. When globin mRNAs from mouse reticulocytes were added to the same in vitro translation system, only the $\alpha$ and $\beta$ chains were synthesized. Thus, poly(A)-containing RNA from mouse yolk sac cells contains mRNAs for $y$ chains. Gilman first noted the existence of two types of strain-specific $y$-chain polypeptides, and we first demonstrated the existence of two types of $y$-globin mRNAs. The pattern of translational products was essentially similar to that of the labeled products

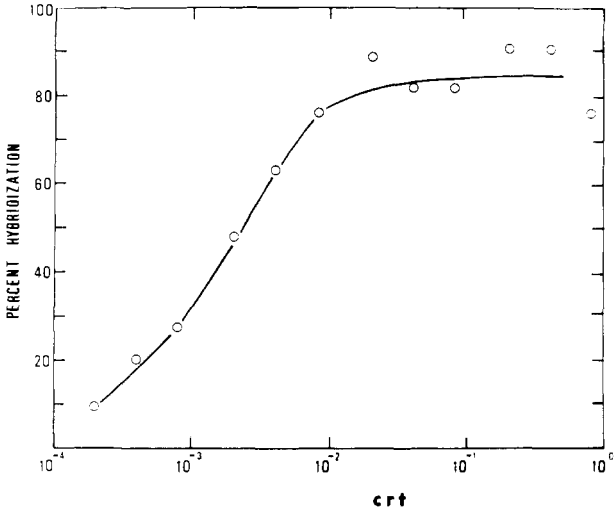


FIGURE 5: Hybridization of yolk sac cDNA with yolk sac mRNAs. Hybridization of yolk sac cDNA and its template yolk sac 9S poly(A)-containing RNA in 0.41 M phosphate buffer, pH 6.8, at 68 °C. Extent of hybridization is thought to be dependent on concentration of globin mRNAs in 9S poly(A)-containing RNA.

synthesized in the intact yolk sac cells, as shown in Figure 3. Yolk sac mRNAs thus contain a large proportion of mRNAs for $y$ chain.

Electrophoresis of the translation products of these yolk sac cell mRNAs by a wheat germ extract system, which has no globin background, also showed the presence of $\alpha$- and $\beta$-globin chains as well as $y$-globin chains.

*Synthesis of Complementary DNA to Yolk Sac Cell mRNAs.* Complementary DNA (cDNA) probes are useful in analysis of the composition of mRNA and its expression. In this work, we prepared cDNA using avian myloblastosis virus (AMV) reverse transcriptase for 9S yolk sac cell poly(A)-containing RNA. The efficiency of back transcription of embryonic yolk sac cell messages was similar to that of adult globin messages. The yolk sac cell cDNA was about 9 S, like adult globin cDNA. On hybridization of this cDNA with template RNA, 80% of the single kinetic component was saturated at a relatively low $C_rt$ (Figure 5). This could mean that the population of globin mRNA sequences is very high in the yolk sac cell poly(A)-containing RNA, supporting our results on in vitro translation. To know the content of the specific mRNA in total cytoplasmic RNA, the same cDNA probe was hybridized with total cytoplasmic RNA. The $C_rt_{1/2}$ value between the cDNA probe and total cytoplasmic RNA was 50-fold higher than that between the probe and 9S poly(A)-containing RNA, showing that the content of the specific mRNA was around 2% of the total RNA (data not shown).

*Comparison of y-Chain mRNA with β-Chain mRNA Sequences.* The amino acid sequences of non-$\alpha$-globin subunits, $\beta$, $\gamma$, and $\delta$ subunits in humans for example, are very similar, and thus these subunits have been thought to have a common ancestory (Hunt & Dayhoff, 1976). However, little similarity was found between the sequence of $\gamma$-chain mRNA from thalacemic patients and the sequence of $\beta$-chain mRNA (Wilson et al. 1978; Lanyon et al., 1975), although the sequences of $\beta$- and $\delta$-chain mRNAs were very similar (Lawn et al., 1978). In mice the amino acid sequences of $\beta$ and $y$ subunits are very similar (Gilman, 1976), and it seemed interesting to compare their mRNA sequences. This comparison was made in two ways. One way was to hybridize cDNA for fractionated poly(A)-containing RNA homologously with its template RNA and heterologously with adult reticulocyte

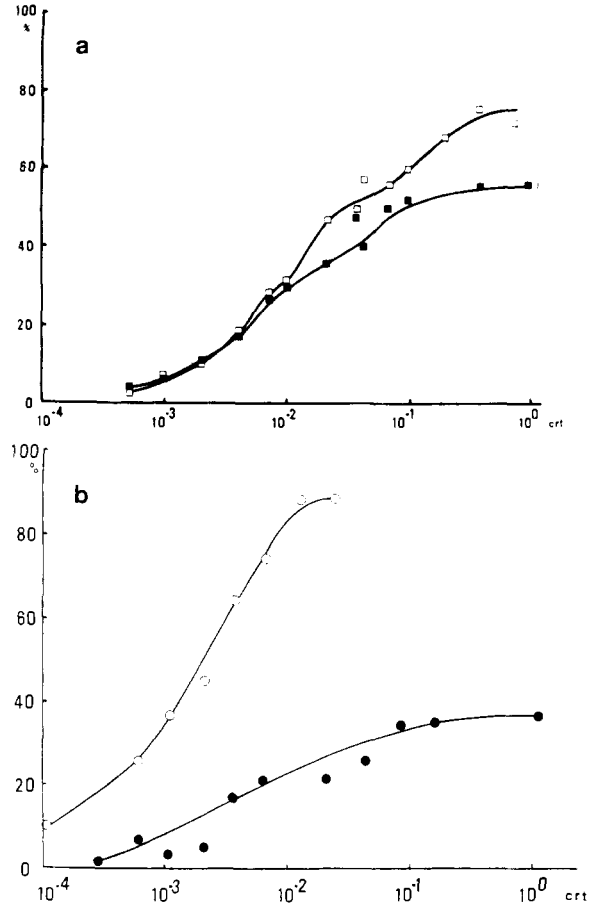


FIGURE 6: Cross hybridizations between adult and embryonic globin cDNAs and adult and embryonic globin mRNAs. (a) Hybridizations of adult globin cDNA and yolk sac cDNA with yolk sac mRNA. Both cDNAs could hybridize equally well to yolk sac mRNA: (□) reticulocyte cDNA ($cDNA_{\alpha+\beta}$) × yolk sac mRNAs ($\alpha + \beta + y$-mRNA); (■) yolk sac cell cDNA ($cDNA_{\alpha+\beta+y}$) × yolk sac mRNAs ($\alpha + \beta + y$-mRNA). (b) Hybridizations of adult globin cDNA and yolk sac cDNA with adult globin mRNA. The binding of adult globin mRNA to yolk sac cDNA is much less than that to adult globin cDNA, indicating little in the sequences of $y$-cDNA and $\beta$-cDNA: (○) reticulocyte cDNA ($cDNA_{\alpha+\beta}$) × reticulocyte mRNAs ($\alpha + \beta$-mRNA); (●) yolk sac cell cDNA ($cDNA_{\alpha+\beta+y}$) × reticulocyte mRNAs ($\alpha + \beta$-mRNA). Hybridizations were done in 0.3 M phosphate buffer, pH 6.8, at 68 °C.

globin mRNA, and compare the results with those of cDNA for adult reticulocyte globin mRNA homologously with its template RNA and heterologously with the 9S yolk sac cell poly(A)-containing RNA. The saturation value for the cross hybridization of yolk sac cell cDNA with adult reticulocyte globin mRNA was 36%, which was lower than the homologous hybridization saturation value of 60% (Figure 6b). Therefore, 40% of yolk sac globin mRNA might have embryonic type messages.

The other method of comparison was to cross-hybridize yolk sac cDNA with cloned mouse $\alpha$- and $\beta$-globin cDNAs in plasmids. Nitrocellulose filters with either $\alpha$- or $\beta$-plasmid DNA were hybridized with $^{32}$P-labeled yolk sac cDNA in lower cots and washed with solutions of different salt concentrations at different temperatures. The extent of hybridization is summarized in Table I. Adult reticulocyte globin cDNA containing equal amounts of $\alpha$ and $\beta$ sequences hybridized equally well with $\alpha$- and $\beta$-plasmid DNA, whereas yolk sac cell cDNA showed 10 times higher hybridization with $\alpha$-plasmid DNA with $\beta$-plasmid DNA, indicating the difference of sequences between adult $\beta$-subunit mRNAs and embryonic $y$-subunit mRNAs.

Table I: Filter Hybridization of Adult and Embryonic Globin cDNAs with Cloned $\alpha$- and $\beta$-DNAs[a]

| cDNA | plasmid | |
|---|---|---|
| | pCR1-$\alpha$ | pCR1-$\beta$ |
| $\alpha + \beta$[b] | 1959[d] | 3300 |
| $\alpha + \beta + y$[c] | 2100 | 331 |

[a] Samples of adult and embryonic globin cDNAs with the same specific radioactivity ($5 \times 10^7$ cpm/$\mu$g) were prepared by using [$^{32}$P]dCTP. The two cloned DNAs were sonicated and then denatured with alkali. The DNAs were then fixed on nitrocellulose filters. cDNA samples, about 10 000 cpm each, were hybridized to the filter DNAs in 6 × SSC (standard saline-citrate) at 68 °C for 24 h, and then filters were washed with 0.1 × SSC at 68 °C. Adult reticulocyte globin cDNA ($\alpha + \beta$) hybridized equally well with both $\alpha$- and $\beta$-DNA, whereas yolk sac cDNA ($\alpha + \beta + y$) showed 10 times higher hybridization with $\alpha$-DNA than with $\beta$-DNA, indicating the difference in sequences between $\beta$- and $y$-mRNAs. [b] Anemic spleen globin mRNA. [c] Yolk sac cell globin mRNA. [d] Counts per minute.

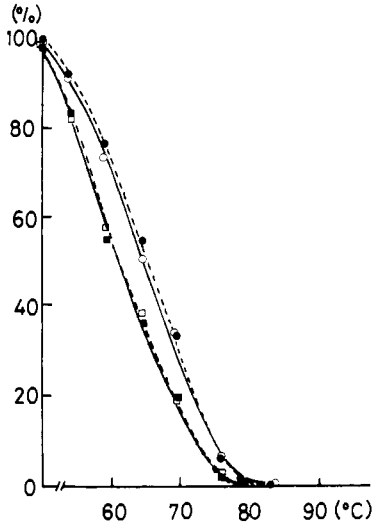


FIGURE 7: Melting profiles of hybrids of adult and embryonic globin cDNAs with cloned $\alpha$-DNA and cloned $\beta$-DNA. Thermal denaturation of cDNA-cloned DNA hybrids on the filter used in the experiment for Table I was carried out in 0.1 × SSC. There was no difference in melting temperatures between the two $\alpha$-DNA hybrids, nor any difference between the two $\beta$-DNA hybrids. $T_m$s of the former hybrids were 3 °C lower than those of the latter hybrids: (○) reticulocyte cDNA × cloned $\alpha$-DNA; (●) yolk sac cell cDNA × cloned $\alpha$-DNA; (□) reticulocyte cDNA × cloned $\beta$-DNA; (■) yolk sac cell cDNA × cloned $\beta$-DNA.

The thermal denaturation curves of the hybridized cDNA–DNA on the filters in the above experiment are shown in Figure 7. The half melting temperatures ($T_m$s) of the yolk sac and adult reticulocyte cDNAs hybridized with $\alpha$-plasmid DNA were almost the same (67 °C), indicating the presence of the same $\alpha$-globin mRNA sequences in the yolk sac cells and reticulocytes. The $T_m$ values of the two hybrids with $\beta$-plasmid DNA were also identical. These results indicate that the plasmid-cloned adult $\alpha$- and $\beta$-DNAs hybridize equally well to $\alpha$- and $\beta$-globin cDNAs from reticulocytes, and to those of yolk sac cells, showing similar degrees of mismatching in both hybrids. This suggests that the results obtained in Table I were actually based on the fact that yolk sac cell cDNA contains embryonic $y$-subunit cDNA, while the adult reticulocyte cDNA does not. It is not known why in this experiment the $T_m$s for $\beta$-plasmid DNA hybrids were about 3 °C lower than those for $\alpha$-plasmid DNA hybrids. Possibly this was due to a difference in the populations of strain-specific $\beta$-chain mRNAs, such as $\beta^D$ major, $\beta^D$ minor, and $\beta^S$ mRNAs.

Mouse $y$-chain genes thus do not seem to be homologous with $\beta$-chain genes, because globin cDNA formed by yolk sac cells could not bind well to cloned $\beta$-DNA, and because different saturation values were obtained on hybridization of yolk sac cell cDNA–yolk sac cell mRNAs and yolk sac cell cDNA–adult reticulocyte mRNAs.

*Concentrations of Respective Globin mRNAs in Yolk Sac Cells.* When yolk sac cell cDNA was hybridized homologously with template mRNA as described above, the saturation value was as high as 60% (Figure 6a). Thus, the globin mRNA concentration in 9S poly(A)-containing RNA seems to be about 60%. When reticulocyte globin cDNA was hybridized with yolk sac cell mRNAs, the saturation value was 36%, and this value indicates the level of globin mRNAs homologous to $\alpha$- and $\beta$-mRNAs in yolk sac cells (Figure 6b). The difference between the above values, i.e., 24%, may correspond to the concentration of non-($\alpha + \beta$)-globin mRNAs, which here are possibly $y$-mRNAs. The kinetics of hybridization of yolk sac cell cDNA with reticulocyte mRNAs was compared with that of hybridization of reticulocyte cDNA with reticulocyte mRNA (Figure 6b). The $C_rt_{1/2}$ values of the two hybridizations were similar, giving a rationale for the above comparison. Biphasic kinetics were observed on hybridization of reticulocyte cDNA with yolk sac mRNA and yolk sac cell cDNA with reticulocyte mRNAs (Figure 6a). The early portion of the curve is possibly that of $\alpha$-mRNA hybrids, whereas the later portion, with about 1 log higher $C_rt_{1/2}$ values, is that of $\beta$-mRNA hybrids. This suggests that yolk sac cells contain about 10 times more $\alpha$-mRNA than $\beta$-mRNA.

From these results we conclude that the percentages in each globin mRNA in the total poly(A)-containing RNA of yolk sac cells are roughly as follows: 33% of $\alpha$-mRNA, 3% of $\beta$-mRNA, and 24% of $y$-mRNA.

## Discussion

The switching over of expression of globin chains during development appears to be more complex in mice than in humans, judging from the strain differences in the expression of $y$-globin chains in mice and their possible close relation to the expression of $\beta$-globin chains. Fantoni et al. (1967) reported that in mice the first species of hemoglobin, E1 ($x_2y_2$), appears before day 8 of gestation, later being replaced by E2 ($\alpha_2y_2$) and E3 ($\alpha_2z_2$), and finally by the adult type ($\alpha_2\beta_2$) from day 12 of gestation. E1, E2, and E3 hemoglobins are synthesized in yolk sac erythroid cells, whereas adult type hemoglobin is synthesized in the fetal liver.

It is conceivable that the mouse $y$ chain is analogous to the human $\gamma$ chain, both being expressed in early fetuses before expression is switched to the $\beta$ chain (Gilman, 1976; G-I. Soma et al., unpublished observations). In many mouse strains examined, there are two types of $y$ chain, $y$-1 and $y$-2, and expression of these chains is closely related to that of the adult type $\beta$ chain.

Probes for individual gene sequences are necessary for analysis of the mechanism of switchover of expression of $\beta$-related globin genes. We are interested in this switching over of expression of these globin subunits in mice, because it may well reflect certain essential mechanisms of eukaryotic gene expression. In addition, we decided to study the expression of the $y$-globin chain because a sufficient quantity of experimental material for study could be obtained from mouse embryos.

To isolate mouse embryonic $y$-globin sequences, we first attempted to purify the corresponding mRNAs. In the present work, we could obtain 9S poly(A)-containing RNA with a high percentage of $y$-globin mRNA from yolk sac cells of 12-day $BDF_1$ embryos. Fantoni et al. (1968) and Terada et al. (1971)

reported that the amount of embryonic mRNA is greatest in the yolk sac cells of 12-day embryos. We confirmed this observation in preliminary studies on the recovery of RNA and on labeling of proteins in isolated intact yolk sac cells at different stages of embryonic development (G-I. Soma et al., unpublished observation).

Smear preparations of yolk sac cells from embryos at 12 days of gestation showed a variation in the extent of erythroid differentiation, although all the embryos were exactly the same age. This variation seems to be reflected in a variation in the proportion of globin mRNAs in the total poly(A)-containing RNA: the proportion varied from 55 to 85% of the total poly(A)-containing RNA. This variation may further explain the extent of homologous cDNA–RNA hybridization in Figures 5 and 6a.

In vitro translation of yolk sac cell poly(A)-containing RNA resulted in the synthesis of $y$-globin chains as well as $\alpha$- and $\beta$-globin chains (Figure 4). The distribution of these globin chains could be estimated by [$^{35}$S]methionine labeling, since the $y$ chain has at least one methionine residue per polypeptide (Gilman, 1976), while the $\alpha$ and $\beta$ chains have two methionine residues. From the results in Figure 3, the ratio of $\alpha$:$\beta$:$y$-1:$y$-2 was estimated in $BDF_1$ mice as 3:1:2:2. In many cases, the ratio of $\alpha$ to $\beta$ chains is 1:1 in erythroid cells. If the syntheses of $\alpha$ and $y$ chains in yolk sac cells are linked, the ratio of ($y$-1 + $y$-2) to $\alpha$ should be about 1:1, but since the exact number of methionine residues in the $y$ chain is unknown, the extent of this linkage in mice embryos is uncertain.

Translation of the $\beta$ subunit was in fact observed in experiments with yolk sac cells (Figures 3 and 4), but it is uncertain whether yolk sac cells actually contain some $\beta$-globin mRNA at a certain stage of fetal development, or whether the $\beta$-globin mRNA in the preparations was a contaminant derived from circulating adult-type erythroid cells of the fetal liver. In preliminary experiments, comparison of the globin chain patterns of fetal liver erythroid cells with those of yolk sac cells during fetal development suggested that the proportions of expression of $\beta^D$ major, $\beta^D$ minor, and $\beta^S$ chains in the fetal liver were quite different from those in the yolk sac cells in several inbred and random-bred mouse strains (G-I. Soma et al., unpublished observations).

The $\beta$-globin subunit was also obtained on in vitro translation of fetal globin messages using mRNA-dependent rabbit reticulocyte lysate. In this case, mRNAs for the $\alpha$ and $\beta$ chains are present in the rabbit reticulocyte lysate, and they might be stimulated by the input mouse mRNAs. However, under our conditions of electrophoresis, the rabbit $\alpha$ chain migrated more slowly than the mouse $\alpha$ chain, and the rabbit $\beta$ chain overlapped the $\beta^S$ chain, but was separated from the $\beta^D$ major and $\beta^D$ minor chains. Therefore, the $\alpha$- and $\beta$-chain bands observed on the gel could be identified as mouse $\alpha$ and $\beta$ chains, not rabbit $\alpha$ and $\beta$ chains. The high ratio of $\alpha$ to $\beta$ chains observed was also further evidence that these bands were from mouse mRNAs, not rabbit mRNAs, since in rabbit reticulocytes the ratio of $\alpha$ to $\beta$ messages is about 1:1.

cDNA was prepared for 9S poly(A)-containing RNA of yolk sac cells from 12-day embryos, and also for 9S poly(A)-containing RNA from adult reticulocytes. As described above, the yolk sac cDNA or ($\alpha$ + $\beta$ + $y$) cDNA could hybridize well with both yolk sac cell and reticulocyte globin mRNAs, but the saturation value for the former was 24% more than that for the latter, whereas if the sequence of $y$-globin mRNA is very similar to that of $\beta$-globin mRNA, the two saturations should be the same (Figure 6b). In these experiments, hybridization was carried out in 0.3 M phosphate buffer, pH 6.8, at 68 °C, conditions which permit a moderate degree of mismatching. Thus, in fact, there may be even less similarity in the nucleic acid sequences of the $\beta$ and $y$ chains than that indicated by the experimental results. This conclusion that there is little similarity in the nucleic acid sequences is supported by hybridization and thermal denaturation experiments on cDNA-cloned DNA hybrids, which indicated that $y$-cDNA could not bind to $\beta$-DNA (Table I and Figure 7).

At the protein level, however, more than 60% of the amino acid sequences of the $y$-globin chain appeared to be the same as that of the $\beta$ chain (Gilman, 1976). This finding suggested that the genes of the two globin subunits were from a common ancestor, although the complete amino acid sequencing of the embryonic globin chains is yet to be done. This is definitely in contrast to the lack of similarity in their mRNA sequences. In human globin chains, there is about 75% similarity in the amino acid sequences of the $\gamma$ and $\beta$ chains, but there was little similarity between the nucleic acid sequences of the $\gamma$ and $\beta$ chains (Wilson et al., 1978). Whether there is a difference between mouse $\beta$-mRNAs and $y$-mRNAs in the third letters of the respective codons will be clarified by sequencing the plasmid-cloned $y$-chain DNA and comparing the results with the nucleic acid sequences of the cloned $\beta$-chain DNA. This project is currently in progress.

The relation between the strain-specific $y$-1 and $y$-2 globin subunits is not well understood. Humans have two $\gamma$-chain genes per haploid genome, which differ from each other by a single amino acid at position 136 from the N terminus (Schroeder et al., 1968).

Our preparation of fetal yolk sac mRNA contained two $y$-chain mRNAs. It will be possible to use these mRNA preparations of the two $y$ genes for molecular cloning, if these genes are in fact distinct, and for studies on the mechanism of switchover of globin gene expression during embryonal development.

After a lysate of yolk sac cells was separated on Triton–acidic urea gel, a band was detected between those of $y$- and $\beta$-globin (Figures 1 and 3). Two-dimensional polyacrylamide gel electrophoresis suggested that this band was that of a non-$\alpha$-embryonic globin subunit (a very faint but consistent band in Figure 2). It seems probable that this band is that of the embryonic $z$-globin subunit, which is expressed almost synchronously with $y$-globin. This band was also detected on in vitro translation of yolk sac mRNA, although in much smaller amounts than $y$-globin (Figure 4). Therefore, the content of $y$-globin mRNA calculated from the cross hybridization of cDNA to adult globin mRNA (as shown in Figure 6a,b) may have included that of $z$-globin mRNA in a smaller amount. We did not examine the similarity in the sequences of $y$- and $z$-globin mRNA.[1]

## Acknowledgments

The authors are grateful to Michiko Uematsu and Naoko Obara for their skillful technical assistance in isolation of yolk sac cells and in gel analysis of hemoglobin and also to Takayasu Matsugi, D.V.M., for supplying mouse embryos.

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[1] We have recently cloned the embryonic $y$-globin chain gene(s) (Ikawa et al., 1979). Preliminary experiments on cross hybridization of the cloned $y$-DNA confirm that the $\beta$-globin gene and $y$-globin gene have little similarity.

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# Studies on Reactivity of Human Leukocyte Elastase, Cathepsin G, and Porcine Pancreatic Elastase toward Peptides Including Sequences Related to the Reactive Site of $\alpha_1$-Protease Inhibitor ($\alpha_1$-Antitrypsin)†

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ABSTRACT: The reaction of human leukocyte (HL) elastase, porcine pancreatic (PP) elastase, and human cathepsin G has been studied with peptide substrates. Most of the peptides had a prolyl residue at $P_2$, since several serine proteases have been shown to productively bind such substrates only with the proline at the subsite ($S_2$) adjacent to the primary substrate binding site ($S_1$). HL elastase prefers a valyl residue over Ala or Met at $S_1$ while PP elastase prefers an Ala. With both elastases, extension of the peptide chain results in significant increases in $k_{cat}/K_M$. With the appropriate substrates, HL elastase is as reactive as PP elastase. Cathepsin G shows a preference for Phe over Met at $S_1$ and a preference for Phe over Ala or Leu at $S_1'$. Extension of the peptide chain yields little increase in rate, and thus the $k_{cat}/K_M$ values observed with cathepsin G are not as large as those of the other enzymes. The $\alpha_1$-protease inhibitor ($\alpha_1$-PI) reactive site has recently been shown to have the sequence -Ala-Ile-Pro-Met*Ser-Ile-Pro-Pro-, where the asterisk indicates the bond cleaved ($P_1$–$P_1'$) when $\alpha_1$-PI·protease complexes are split. The octapeptide Ac-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro-$NH_2$ and the analogue with a $P_1'$ Thr instead of Ser were synthesized. All three enzymes bound to and cleaved the peptides at the $P_1$ Met bond. The $K_M$ values were in the millimolar range, showing that this particular sequence alone does not account for the tight binding of serine proteases to $\alpha_1$-PI. The $k_{cat}$ values, a measure of the ease with which certain types of bond formation between proteases and $\alpha_1$-PI would occur, were higher for the $P_1'$ Ser octapeptide than for the Thr analogue, indicating that relatively minor amino acid substitutions in the $\alpha_1$-PI reactive site will profoundly influence its reactivity toward various proteases. Inactivation of $\alpha_1$-PI in the lung by oxidation and the resulting protease imbalance is the currently accepted model for the development of emphysema. In the majority of cases studied, oxidation of the $P_1$ Met residue of simple peptides to the sulfoxide resulted in decreased binding to the enzymes studied, and a decreased $k_{cat}/K_M$. Reduction of substrate effectiveness was greatest with HL elastase for the $P_1'$ Ser peptides compared to the $P_1'$ Thr peptides. Reactive site substitutions could effect the degree to which oxidation is damaging to the inhibitor and may be one possible explanation for the greater susceptibility to emphysema of some individuals with normal $\alpha_1$-PI.

The granule fraction of human leukocytes contains major amounts of two serine proteases, HL[1] elastase and cathepsin G. HL elastase has been implicated in the proteolysis of lung elastin which is observed in pulmonary emphysema (Mittman, 1972; Turino et al., 1974; Hance & Crystal, 1975). Cathepsin G is also capable of attacking elastin and other connective tissue components. Granulocyte serine proteases are normally involved in the turnover of dead lung tissue and in the destruction of invading bacteria (Blondin et al., 1978) and only

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[1] Abbreviations used: Aala, $-NHN(CH_3)CO-$; MeO-Suc, methoxysuccinyl; Suc, succinyl; $\alpha_1$-PI, $\alpha_1$-protease inhibitor ($\alpha_1$-antitrypsin); PP, porcine pancreatic; HL, human leukocyte; NA, 4-nitroanilide.

0006-2960/80/0419-3973$01.00/0 