Hemoglobin Synthesis in Siderocytes of Flexed-tailed Mutant (f/f) Fetal Mice

By David H. K. Chui, George D. Sweeney, Margaret Patterson, and Elizabeth S. Russell

Mice carrying two mutant genes at the f locus on chromosome 13 have severe anemia during fetal development, characterized by hypochromic and microcytic red blood cells laden with many iron granules. Ultrastructural studies reveal that the excessive nonheme iron accumulated in mutant (f/f) fetal erythrocytes is present within mitochondria, similar to those seen in human sideroblastic anemia. The level of free erythrocyte protoporphyrin in f/f fetal red cells is similar to that in normal (+/+) fetal red cells. Furthermore, in f/f fetal reticulocytes β-chain synthesis is decreased compared with α-chain synthesis. Exogenous hemin fails to alter significantly globin synthesis in f/f cells, while it preferentially stimulates α-chain synthesis in +/+ cells. Protein synthesis in mutant reticulocytes is also more resistant than that in normal reticulocytes to the inhibitory effect of three heme synthesis inhibitors, i.e., isoniazid, 2,2'-bipyridine, and ethanol. These results are consistent with the hypothesis that in fetal mutant f/f red cells there is diminished globin chain production, leading to hypochromic and microcytic anemia. In addition, there is a relative excess of intracellular free heme pool due to decreased utilization. Iron accumulation in these erythroid cells may be secondary to the hemoglobin synthetic derangement. The present study also demonstrates that sideroblastic anemia recognized by identical morphologic criteria is a heterogeneous group of disorders with different basic biochemical abnormalities.
free erythrocyte protoporphyrin in \( f/f \) fetal red cells on day 18 of gestation is similar to that in normal red cells. Globin-chain synthesis in these mutant fetal mouse erythroid cells is unbalanced, displaying decreased \( \beta \)-chain synthesis. Furthermore, protein synthesis is more resistant in mutant than in normal reticulocytes to the inhibitory effect of three heme synthesis inhibitors.

**MATERIALS AND METHODS**

**Animals**

Adult mutant FL/\( I^{Re} \)-\( f/f \) and congenic normal FL/\( I^{Re} \)-\( +/- \) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. Mice of the same genotype were mated together and female mice were examined daily for vaginal plugs. The morning when the plug was found was designated day 0 of gestation.

**Cells and Tissues**

On the mornings of days 16 - 20 of gestation, pregnant mice were sacrificed by cervical dislocation. Each conceptus was removed and placed in either Krebs-Ringer phosphate (KRP) buffer, pH 7.4, or Waymouth medium [MB 725/I; Grand Island Biological Co. (Gibco), Grand Island, N.Y.] in some of the earlier experiments. Embryos were then carefully dissected free from the placentas, washed, and gently blotted dry with filter paper. Embryo weight was measured on a Mettler balance. Fetal blood from each embryo was collected with disposable micropipets (Drummond Scientific Co., Broomall, Pa.). Hemoglobin concentration was assayed by the Drabkin method. Red blood cell count and mean erythrocyte corpuscular volume were determined with a Coulter particle counter Model B attached to a channel analyzer (Coulter Electronics, Hialeah, Fla.) as previously described in detail.

**Light and Electron Microscopy**

Fetal erythrocyte smears were prepared by a cytocentrifuge (Shandon Scientific Co., London, England). Reticulocyte preparation was done by the new methylene blue staining method. Non heme iron granules in erythrocytes were visualized using the Prussian blue staining technique.

Fetal erythrocytes were fixed in cold 1\( \% \) phosphate-buffered glutaraldehyde (pH 7.2), postfixed with osmium tetroxide, dehydrated in graded alcohol, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate followed by citrate as previously described. These sections were examined in a Philips 301 electron microscope.

**Free Erythrocyte Protoporphyrin Assay**

Free erythrocyte protoporphyrin was determined with a micromethod. Fetal erythrocytes obtained from each day 18 \( +/- \) or \( f/f \) fetus were suspended in saline to a hemoglobin concentration of approximately 10 g/dl; 10 \( \mu l \) of this suspension was added with vigorous agitation (Vortex mixer) to 2 ml of a mixture of ethyl acetate and glacial acetic acid (2:1). Then 2 ml of 0.5 N HCl was added, and the mixture was shaken thoroughly and centrifuged at 500 g for 5 min. After removal of the organic phase, 1 ml of the acid phase was taken into a quartz 10 x 10 mm cuvette for fluorometry. Fluorescence measurements were made in an Aminco-Bowman spectrophotofluorometer equipped with a R446 photomultiplier. The excitation wavelength was adjusted for maximum emission using a protoporphyrin standard prepared freshly for each day’s work and quantitated by absorptiometry prior to final dilution. The emission spectrum was recorded over the range of interest; measurements depended upon the ratio of unknown to standard fluorescence at approximately 608 nm.

**Globin Synthesis**

Normal or mutant embryos of the same gestational age were bled into culture medium and the fetal red blood cells were pooled and subsequently divided into equal aliquots. These cell suspensions were then centrifuged, and each of the cell pellets, about 30 60 \( \mu l \) in volume, was resuspended in 1 ml of culture medium. This medium was made up of the following: 1 ml KRP
buffer; 1 ml of heat-inactivated fetal calf serum (Gibco) which had been previously dialyzed for 24 hr against KRP buffer; 40 μl of supplemented Eagle's basal medium amino acid 50× concentrated solution (Gibco); and 4 mg of glucose. In some earlier experiments, the fetal blood was collected in Waymouth medium and the cell pellets were resuspended in 150–250 μl of Waymouth medium.

The cell suspensions were then incubated for 15 min at 37°C in a fully humidified atmosphere. Then 25–50 μCi of 4,5-3H-L-leucine (specific activity 60.0 Ci/m mole), purchased from New England Nuclear (NEN) Corp., Boston, Mass., were added and the samples were further incubated for up to 2 hr and gently agitated once every 15 min. In some experiments, 5 μCi of 14C-L-leucine (specific activity 315 Ci/mole) from NEN were used as the radioactively labeled precursor. After labeling in vitro, the cells were washed three times with cold saline. One milliliter of nonisotopic freshly prepared adult mouse hemolysate containing approximately 30 mg of hemoglobin was added to each sample. Globin was prepared by adding the mixture of labeled fetal erythrocytes and adult hemolysate directly to 60–80 ml of cold acid-acetone. α and β globin chains were separated and recovered by column chromatography on carboxymethylcellulose (CMC) in 8 M urea, using a linear phosphate gradient from 0.01 to 0.025 M as previously described.11,12

Radioactivity was determined by liquid scintillation counting using 1 ml of eluate and 10 ml of Aquasol (NEN) in a Packard Tricarb scintillation counter. More than 90% of the radioactivity put on the column was recovered, of which at least 80% was eluted under the globin peaks. Under the present experimental conditions, 3H-leucine incorporation into globin chains was linear for at least 30 min.

Protein Synthesis

In this procedure 5 mg of protein obtained after the acid-acetone step was dissolved in 5 ml of distilled water. Subsequently, 0.8 ml of 20%, trichloroacetic acid was added to 1.0 ml of the aqueous protein solution, which was then heated to 90°C for 30 min. After cooling, the precipitates were collected on Millipore filters and washed further with 5% trichloroacetic acid. The precipitates were redissolved with 0.2 ml of concentrated formic acid before adding 10 ml of Aquasol for scintillation counting.

In other experiments, the aforementioned aqueous protein solution was dialyzed for 48 hr in six changes of 1–2 liters each of 0.5% formic acid at 4°C; 1 ml of the dialyzed solution was then added to 10 ml of Aquasol for radioactivity determination. Preliminary experiments showed that there was no significant difference between these two methods in determination of 3H-leucine incorporation into protein in fetal reticulocytes.

Preparation of Chemicals

Crystalline bovine hemin was obtained from the Sigma Chemical Co., St. Louis, Mo. Hemin (31 mg) was dissolved in a solution of 1 ml of 1 N NaOH, 1 ml of 1 M Tris, and 2 ml of culture medium. Both hemin solution and blank solution (identical composition as the hemin solution save for the absence of hemin) were carefully titrated to pH 7.4. Appropriate and equal volumes of these solutions were added to the cell-suspension incubation mixtures.

Isoniazid (INH) was obtained as Rimifon (25 mg/ml) from Hoffmann-LaRoche, Vaudreuil, Que. 2,2'-Bipyridine was purchased from Eastman Kodak Co., Rochester, N.Y., and dissolved in water to a concentration of 30 mM at room temperature. Absolute ethanol was purchased from Consolidated Alcohols, Toronto, Ont. Appropriate amounts of these chemicals were added to the incubation mixture. No change in pH was observed as a result of these additions.

RESULTS

Anemia and Red Cell Morphology

Flexed-tailed mutant (f/f) mouse fetuses in our stock developed normally and, on day 18 of gestation, their body weight (1.05 ± 0.02 g; n = 21) was almost identical to that of congeneric normal (+/+) fetuses (1.10 ± 0.01 g; n = 21). Nevertheless, at the same gestational age, these mutant fetuses were se-
Table 1. Circulating Blood Values in Day 18 Embryos

<table>
<thead>
<tr>
<th>Parameter</th>
<th>+/+</th>
<th>f/f</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10^12/liter)</td>
<td>3.58 ± 0.06</td>
<td>2.43 ± 0.04</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>13.1 ± 0.2</td>
<td>6.6 ± 0.1</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>40.2</td>
<td>23.0</td>
</tr>
<tr>
<td>Corpuscular values</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>112.3 ± 1.4</td>
<td>94.8 ± 1.1</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>36.6</td>
<td>27.2</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>32.6</td>
<td>28.7</td>
</tr>
</tbody>
</table>

Red blood cell count (RBC) done by Coulter Counter and data collected from 42 +/+ and f/f fetuses. Hemoglobin (Hb) measured by the Drabkin method in 42 fetuses. Mean corpuscular volume (MCV) determined by the Coulter Counter attached to a channel analyzer; mean values obtained from 50 embryos. Data expressed as mean ±SEM. For all three parameters, values of +/+ are very significantly different from that of f/f (p < 0.001). Hematocrit (PCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated from RBC, Hb, and MCV.

Very anemic with hemoglobin concentrations in the blood approximately half of that in the normal fetuses. The circulating f/f erythrocytes were hypochromic and microcytic (Table 1). The majority of red blood cells in normal (77%) and mutant (98%) fetuses were reticulocytes, as demonstrated by staining with new methylene blue.

Most of the f/f fetal erythrocytes had iron-containing granules (Fig. 1). Electron microscopic investigation revealed that the iron-rich material was present within mitochondria (Fig. 2). The degree of iron deposit varied. In some mitochondria, none or very little iron accumulation could be identified ultrastructurally (Fig. 2a). In others, the mitochondrial structure appeared to have broken down as a result of the massive infiltration by iron (Fig. 2b). Iron granules were rarely detected in mitochondria of +/+ erythrocytes as previously described. There was no significant difference in the levels of free erythrocyte protoporphyrin determined by fluorometry, between normal (15.4 ± 1.2 μg/g Hb; n = 20) and mutant (16.2 ± 1.8 μg/g Hb; n = 9) fetal erythrocytes.

Globin-Chain Synthesis

In +/+ fetal erythrocytes, equal amounts of α and β globin chains were synthesized. In contrast, f/f erythrocytes synthesized less β chain than α chain on day 18 of gestation (Fig. 3). In order to ascertain that the unbalanced globin-chain synthesis in f/f was not due to an experimental artifact during the globin-chain preparation and isolation, a double-labeling experiment was done using 3H-leucine label for +/+ cells and 14C-leucine label for f/f cells. After labeling, these cells were pooled and processed together. The result confirmed that in f/f, relatively less β chain was synthesized than α chain (Fig. 4). Moreover, the α and β globin chains synthesized in mutant cells were chromatographically identical to their counterparts synthesized in normal cells. In another experiment, the effect of duration of radioactive precursor labeling upon α/β synthetic ratio in f/f was examined. As shown in Fig. 5, more α chains were formed relative to β chains at all time points under study.
Fig. 1. Light micrographs of cytocentrifuge smears of circulating day 18 +/+ (a) and f/f (b) fetal erythrocytes stained for iron with Prussian blue. Note that almost all of the mutant erythrocytes contain iron granules which are stained blue. ×1000.

Table 2 summarizes the data which indicate balanced globin-chain synthesis in normal circulating fetal erythrocytes from day 16 to day 20 of gestation. At the same time, unbalanced globin-chain synthesis was observed in mutant cells, although there appears to have been a trend toward balanced synthesis during the latter part of gestation.

Effect of Exogenous Hemin on Globin Synthesis

Total globin-chain synthesis was enhanced in both +/+ and f/f fetal erythrocytes by the addition of hemin at a wide range of concentrations. In
Fig. 2. Electron micrographs of circulating day 18 f/f fetal erythrocytes. (a) Many minute electron-dense particles, presumably iron containing, are dispersed within the mitochondrion. (b) Massive infiltration of mitochondria by iron-containing substance. Note the presence of cristae within the mitochondria. ×43,700.

Fig. 3. Chromatography of globin chains synthesized by day 18 +/+ (A) and f/f (B) fetal erythrocytes. Cells were labeled with 3H-leucine for 90 min. Non-isotopic adult hemolysate was added before globin chains were prepared and isolated. (See Materials and Methods.) There were more +/+ than f/f fetal erythrocytes collected for this set of experiments, which accounted for the higher incorporation of 3H-leucine into globin chains in +/+ cells.
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Fig. 4. Chromatography of globin chains synthesized by day 18 +/+ and f/f fetal erythrocytes. Normal cells (a) were labeled with $^3$H-leucine for 90 min, and mutant cells (o) with $^{14}$C-leucine for 90 min. After labeling, cells were pooled and washed, and globin chains were then prepared and isolated. (See Materials and Methods.)

Fig. 5. Effect of duration of labeling upon α/β synthetic ratio in day 18 f/f fetal reticulocytes. Seven equal aliquots of cells were labeled with $^3$H-leucine for a period 90 sec to 120 min. Globin chains were isolated by CMC-urea column chromatography. (See Materials and Methods.)

Fig. 6. Effect of added hemin at various concentrations upon α/β synthetic ratio in day 18 +/+ (a) and f/f (o) fetal reticulocytes. After preincubation with hemin for 15 min, $^3$H-leucine was added and labeling continued for another 30 min. Globin chains were isolated by CMC-urea column chromatography. (See Materials and Methods.) Three different sets of experiments were performed with cells of each genotype. r: correlation coefficient.
Table 2. Globin-Chain Synthesis in Circulating Fetal Erythrocytes

<table>
<thead>
<tr>
<th>Day of Gestation</th>
<th>+/+ α/β</th>
<th>No. of Experiments</th>
<th>f/f α/β</th>
<th>No. of Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>1.10 ± 0.02</td>
<td>3</td>
<td>1.40 ± 0.05</td>
<td>3</td>
</tr>
<tr>
<td>17</td>
<td>1.01</td>
<td>1</td>
<td>1.25 ± 0.06</td>
<td>2</td>
</tr>
<tr>
<td>18</td>
<td>0.95 ± 0.05</td>
<td>4</td>
<td>1.48 ± 0.09</td>
<td>7</td>
</tr>
<tr>
<td>19</td>
<td>0.97</td>
<td>1</td>
<td>1.24 ± 0.03</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>0.93 ± 0.10</td>
<td>2</td>
<td>1.10 ± 0.08</td>
<td>4</td>
</tr>
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</table>

Cells were labeled with either 3H-leucine or 14C-leucine for 90 min. Globin chains were isolated by CMU-urea column chromatography. See Materials and Methods. Data expressed as mean ±SEM.

seven experiments, +/+ cells incubated with hemin at various concentrations from 4.5 × 10⁻⁶ M to 1.0 × 10⁻¹ M synthesized globin chains at a rate of 126% of cells not exposed to added hemin. In another seven experiments with f/f cells under similar experimental conditions, the stimulation averaged 125% (data not shown). However, in +/+ cells, hemin preferentially stimulated α globin-chain synthesis more than β. Thus, the α/β synthetic ratio in day 18 +/+ cells increased progressively with increasing hemin concentrations in the incubation mixture (Fig. 6). In another set of experiments using day 15 +/+ cells, the α/β synthetic ratio was 1.12 without hemin, but increased to 1.21 with 3.4 × 10⁻³ M hemin, and to 1.44 with 3.4 × 10⁻⁴ M hemin. On the other hand, the α/β synthetic ratio in day 18 f/f cells was 1.3, which was not significantly altered by the addition of exogenous hemin (Fig. 6).

Table 3. Effect of INH and Hemin on Protein Synthesis in Day 18 Fetal Blood

<table>
<thead>
<tr>
<th>Exp</th>
<th>Incubation Mixture</th>
<th>+/+ cpm</th>
<th>Percent of Control</th>
<th>f/f cpm</th>
<th>Percent of Control</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>434,000</td>
<td>100</td>
<td>210,000</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>INH 1.7 × 10⁻³ M</td>
<td>211,000</td>
<td>49</td>
<td>258,000</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>Hemin 3.0 × 10⁻⁴ M</td>
<td>550,000</td>
<td>127</td>
<td>314,000</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Hemin and INH</td>
<td>692,000</td>
<td>159</td>
<td>460,000</td>
<td>219</td>
</tr>
<tr>
<td>B</td>
<td>Control</td>
<td>358,000</td>
<td>100</td>
<td>126,000</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>INH 8.7 × 10⁻³ M</td>
<td>59,000</td>
<td>16</td>
<td>44,000</td>
<td>35</td>
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<tr>
<td></td>
<td>Hemin 5.2 × 10⁻⁴ M</td>
<td>466,000</td>
<td>130</td>
<td>139,000</td>
<td>110</td>
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<tr>
<td></td>
<td>Hemin and INH</td>
<td>508,000</td>
<td>142</td>
<td>176,000</td>
<td>140</td>
</tr>
<tr>
<td>C</td>
<td>Control</td>
<td>379,000</td>
<td>100</td>
<td>293,000</td>
<td>100</td>
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<tr>
<td></td>
<td>INH 8.7 × 10⁻³ M</td>
<td>79,000</td>
<td>21</td>
<td>155,000</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>Hemin 5.5 × 10⁻⁴ M</td>
<td>495,000</td>
<td>131</td>
<td>310,000</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>INH and Hemin</td>
<td>567,000</td>
<td>150</td>
<td>573,000</td>
<td>196</td>
</tr>
</tbody>
</table>

In experiments A and B, equal aliquots of circulating day 18 +/+ fetal erythrocytes were first incubated with either blank solution or hemin for 15 min before INH was added to the appropriate samples. Incubation was continued for another 30 min before ³H-leucine was added for pulse labeling, which lasted for another 20 min. The protein solutions subsequently prepared were then dialyzed for 48 hr with 0.5% formic acid before radioactivity incorporation was determined (See Materials and Methods). In experiment C, cells were first incubated with INH for 15 min, before hemin was added to the appropriate samples. Incubation was continued for another 30 min before ³H-leucine was added for pulse labeling which lasted for another 20 min. Similar experiments were done using f/f fetal erythrocytes. The number of +/+ cells used was more than that of f/f cells.
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Fig. 7. Effect of INH upon α/β synthetic ratio in day 18 +/+ (solid line) and f/f (dashed line) fetal reticulo-cytes. After preincubation with INH for 30 min, 3H-leucine was added and labeling continued for another 20 min. Globin chains were separated by column chromatography. (See Materials and Methods.) Two different sets of experiments were performed with cells of each genotype.

Effect of INH on Globin Synthesis

In order to examine the effect of heme deficiency on globin synthesis in +/+ and f/f cells, INH was added to the incubation mixture of the fetal red cells, and the subsequent effect upon protein synthesis was determined by 3H-leucine incorporation. As illustrated in Table 3, INH at the appropriate concentrations markedly decreased protein synthesis in both +/+ and f/f erythrocytes. However, the inhibitory effect of INH could be totally reversed by the addition of exogenous hemin to the cell incubation mixture. Indeed, the addition of both INH and hemin resulted in the greatest number of counts incorporated.

The α/β synthetic ratio of both normal and mutant fetal erythrocytes decreased in the presence of a sufficient amount of INH, indicating that in red cells obtained from embryos of both genotypes, α-chain synthesis was more susceptible than β-chain synthesis to inhibition due to the lack of intracellular free heme (Fig. 7). Nevertheless, total globin synthesis in f/f cells was less vulnerable to the inhibitory effect of INH than that in +/+ cells. Thus, INH at a concentration of 1.7 × 10^-3 M had little effect (106%) upon total globin synthesis in mutant erythrocytes. Similar amounts of INH suppressed the total globin synthesis of normal erythrocytes to a level of 26% of the control value (Fig. 8).

Effect of 2,2'-Bipyridine and Ethanol on Protein Synthesis

The effect of 2,2'-bipyridine and ethanol on protein synthesis in fetal reticulo-cytes was examined. Similar to the data obtained with INH, protein
Fig. 9. Effect of 2,2'-bipyridine upon protein synthesis in day 18 +/+ (•, solid line) and f/f (•, dashed line) fetal reticulocytes. After preincubation with 2,2'-bipyridine for 60 min, 3H-leucine was added and labeling continued for another 20 min. The globin solutions subsequently prepared were precipitated with 20% trichloroacetic acid before radioactivity incorporation was determined. (See Materials and Methods.) Data were calculated as follows:

\[
\text{Protein synthesis with 2,2'-bipyridine} \times 100.
\]

Protein synthesis without 2,2'-bipyridine

Two different sets of experiments were done with +/+ cells, and three different sets of experiments with f/f cells.

synthesis in f/f fetal reticulocytes was much more resistant to the inhibitory effects of these two heme synthetic inhibitors than +/+ cells. As shown in Fig. 9, 2,2'-bipyridine at a concentration of 0.4 mM suppressed protein synthesis in mutant fetal erythrocytes to only 90\% of control value. On the other hand, a similar amount of this iron-chelating agent inhibited protein synthesis in normal cells to 45\%.

Fig. 10 demonstrates that 0.8 M ethanol suppressed protein synthesis to only 72\% of control value in f/f cells, but 36\% of control value in +/+ cells.

Fig. 10. Effect of ethanol upon protein synthesis in day 18 +/+ (•) and f/f (•) fetal reticulocytes. After preincubation with ethanol for 30 min, 3H-leucine was added and labeling continued for another 20 min. The globin solutions subsequently prepared were precipitated with trichloroacetic acid before radioactivity incorporation was determined. (See Materials and Methods.) Data were calculated as follows:

\[
\text{Protein synthesis with ethanol} \times 100.
\]

Protein synthesis without ethanol

Two different sets of experiments were done with cells of each genotype.
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DISCUSSION

The present study confirms that flexed-tailed mutant \( f/f \) mice have severe anemia during fetal development, characterized by hypochromic and microcytic red blood cells laden with many iron granules. The content of free erythrocyte protoporphyrin in mutant red cells on day 18 of gestation is normal as determined by the fluorometric technique. On the other hand, free erythrocyte protoporphyrin level in iron deficient red blood cells is markedly elevated. Similarly, protoporphyrin accumulates in hepatocytes when iron incorporation into protoporphyrin is inhibited by a ferrochelatase inhibitor. Taken together, these results suggest that there is no demonstrable defect in the availability of elemental iron or its incorporation into protoporphyrin to form heme in \( f/f \) fetal erythroid cells.

Hemin has been shown to be capable of stimulating globin synthesis in heme-deficient reticulocytes to as much as 270\( \% \) above the control value. On the other hand, hemin stimulates globin synthesis in mutant fetal blood cells to only 25\( \% \) above control value, which is identical to the stimulatory effect of hemin upon normal fetal red cells. These observations suggest that mutant fetal erythrocytes are not heme deficient.

INH is a potent heme synthesis inhibitor and is believed to act at the step involving pyridoxal phosphate. 2,2'-Bipyridine interferes with heme formation by virtue of being an iron-chelating agent. Ethanol also blocks heme synthesis in erythroid cells, the mechanism of which has not yet been clearly identified. Nevertheless, the inhibitory effects of all three agents upon globin synthesis can be reversed by the addition of exogenous heme. Protein synthesis in \( f/f \) fetal reticulocytes has been shown to be more resistant to the inhibitory effect of all three heme synthesis inhibitors than is that in \(+/+\) fetal reticulocytes. These data suggest that there is a relative excess of free heme present in mutant cells. During mammalian fetal development, delta aminolevulinic acid (ALA) synthetase, the first and rate-limiting enzyme in heme biosynthesis, appears not to be susceptible to repression by the end-product heme to the same degree as in adult animals.

Hemin has been shown to enhance incorporation of amino acids into both globin chains in normal fetal mouse reticulocytes. Moreover, the degree of augmentation of α-chain formation is more marked than that of β-chain, and is dependent on the concentration of hemin added. These data provide further evidence that heme plays an important role in coordinating globin-chain biosynthesis in intact erythroid cells. In some human pathologic states associated with heme deficiency, α-globin-chain synthesis is depressed compared to β. Hemin has also been shown to stimulate selectively the translation of α-globin messenger ribonucleic acid (mRNA) injected into Xenopus oocytes. In \( f/f \) mutant fetal reticulocytes, α-chain synthesis is already more active than β, and it has been shown that this disparity is not further accentuated by the addition of exogenous heme. These results lend further support to the suggestion that there is an excess of free heme present in \( f/f \) fetal reticulocytes.

The \( f \) locus is on chromosome 13, while the α-globin gene locus is on chromosome 11 and the β-globin gene locus is on chromosome 7. Therefore, the primary structures of globin chains synthesized in \( f/f \) mutant erythrocytes are identical to the normal. The \( \alpha/\beta \) synthetic ratio in normal fetal erythrocytes
has been demonstrated to be close to unity as assessed by radioactive leucine incorporation. There are 17 leucine residues in both α and β mouse globin chains,\textsuperscript{24,26} and the data indicate balanced globin-chain synthesis during normal fetal development. On the other hand, in f/f fetal erythrocytes, the β chain is synthesized at a rate significantly less than the α chain. One possible explanation is that there is a decreased amount of β-globin mRNA present in these mutant cells. Alternatively, the imbalance in globin-chain synthesis may be secondary to the presence of excessive intracellular free heme.

Hypochromic and microcytic anemia is associated with inadequate hemoglobin synthesis due to deficiency either in heme formation or in globin chain production. The data of the present investigation indicate that there is a relative excess of free heme in mutant cells. Therefore, it is reasonable to suggest that one effect of the mutant f gene product is to interfere with the normal expression of globin genes during fetal erythropoiesis, leading to decreased production of globin chains, hypochromic microcytic anemia, and a relative excess of intracellular free heme pool due to decreased utilization. Iron accumulation observed in these fetal erythroid cells may be another effect of the hemoglobin synthetic derangement. In β thalassemia, iron accumulation in erythroid cells is also a prominent feature of the disease.\textsuperscript{27,28} Further unravelling of the effects of this single locus f mutation in the mouse may lead to better understanding of the regulation and coordination of globin synthesis as well as cellular proliferation during normal mammalian erythroid cell differentiation.

Sideroblastic anemia in man is characterized by hypochromic and microcytic red blood cells with excessive non-heme iron accumulation in mitochondria, similar to the mutant f/f fetal reticulocytes.\textsuperscript{29,31} In some patients with either the acquired or the hereditary forms of the disease, heme deficiency in erythroid cells as well as unbalanced globin-chain synthesis with decreased α-chain formation have been described.\textsuperscript{15,21} The present study on f/f fetal erythropoiesis demonstrates that sideroblastic anemia recognized by identical morphologic criteria may be a heterogeneous group of disorders with different basic biochemical abnormalities.

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Hemoglobin synthesis in siderocytes of flexed-tailed mutant (f/f) fetal mice

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