Human Embryonic ζ-Globin Gene Expression in Mouse-Human Hybrid Erythroid Cell Lines

By Hong-Yuan Luo, Albert B. Deisseroth, and David H.K. Chui

The human α-globin–like embryonic ζ-globin chains are present in abundance during the first 5 to 6 weeks of gestation. Subsequently, ζ-globin chains are present in fetal blood at a very low level, which is supplanted by the expression of α-globin chains. Adult individuals who are carriers of the (--SEAI) α-thalassemia deletion, in contrast to normal adults, have low levels of embryonic ζ-globin chains in their circulating erythrocytes. In this investigation, we constructed stable mouse-human hybrid cells with murine erythroleukemia cells bearing human chromosome 16, with either the normal α-globin gene cluster (aa1) or the (--SEAI) type of α-thalassemia deletion. The results on the human ζ-globin gene expression in these hybrid cells indicate that murine adult erythroid transcription factors can induce the expression of human embryonic ζ-globin gene in cis to the (--SEAI) deletion, in parallel with the endogenous mouse α-globin gene expression. These data also show the importance of the DNA sequences within the (--SEAI) deletion in regulating the expression of ζ-globin gene in cis during normal human hemoglobin ontogeny.

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MATERIALS AND METHODS

Construction of hybrid cell lines. The tetraploid murine erythroleukemia cell line, MEL-179, which is deficient in adenine phosphoribosyl transferase (APRT), was cultured in RPMI 1640 with 10% fetal calf serum (FCS) and 100 μg/ml 2,6-diaminopurine. The parental human cells were peripheral blood mononuclear cells (MNC) from normal and α-thalassemia heterozygous adults, human fetal hepatic erythroblasts (HFHE) from homozygous α-thalassemia fetuses, and human K562 cells that express an embryonic/fetal hemoglobin synthetic program.

Cell fusion was performed with polyethylene glycol (No. 779512; Boehringer Mannheim, Indianapolis, IN) as previously described.10 Hybrids were selected for APRT activity by cultivating in medium containing adenine and alanosine with 30% FCS. The gene coding for the APRT is on the long arm of human chromosome 16. Fusion of K562 and MEL-179 cells was selected for the presence of APRT activity and resistance to 5-fluoracil.2 The hybrid cell colonies were first screened by DNA slot blot assay using 32P-labeled human Alu probe. All hybrid cell lines were later subcloned and subjected to Southern blot analysis of the human α-globin gene cluster as well as the human chromosome 16 centromere-specific repetitive sequences, D16Z1.

Globin mRNAs were determined by S1 nuclease protection assays.11 Hybrid cell lines were induced with either 1% dimethylsulfoxide (DMSO) or 5 mmol/L hexamethylene bisacetamide (HMBA). Total cellular RNA was extracted from 1 to 2 × 10⁷ cultured cells by the guanidinium/cesium chloride method. The poly A+ RNA was isolated from cellular RNA by a single cycle of oligo-dT-cellulose column chromatography.

Globin mRNAs were determined by S1 nuclease protection assays.11 The α5p3-ATP (6,000 Ci/mmole; Amersham, Arlington Heights, IL) was used for 5'-end labeling by the Klenow DNA polymerase reaction. The γ5p3-ATP (5,000 Ci/mmole; Amersham) was used for 5'-end labeling by T4 polynucleotide kinase. The antisense strand of each probe (except M6-MG probe) was isolated by

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Fig 1. a-Globin gene cluster and the extent of six natural occurring a-thalassemia deletions. The globin genes are represented as solid boxes, pseudogenes as open boxes, and hypervariable regions (HVR) as zig-zag lines. The extent of each deletion is represented as a solid box, and the uncertainty of the breakpoints is indicated by open boxes. (Adapted and reprinted with permission.)

5% polyacrylamide gel. The single-stranded cDNA probe (at 1 x 10^10 cpm) was added in excess to each S1 nuclelease protection assay. Hybridization was performed at 45°C (50°C for Mb-MG) overnight. A total of 40 to 80 U of S1 nuclease (Pharmacia 270-920-01; Pharmacia, Uppsala, Sweden) was added to each reaction and incubated at 57°C for 30 minutes. After the reaction, DNA was precipitated by ethanol, analyzed by electrophoresis on a 48% urea polyacrylamide gel, and visualized by autoradiography at -70°C for 10 to 30 days using intensifying screens.

Human ε-globin mRNA protects a 320-nt fragment of the 3'-end labeled 520-nt probe, an Ava II/Avr II-digested fragment of the pgEM-16Hε plasmid. Human a1- and a2-globin mRNAs protect 268-nt and 172-nt fragments, respectively, of the 3'-end labeled 550-nt probe, a HindIII-digested fragment of JW 101 plasmid.

Mouse ε-globin mRNA protects a 151-nt fragment of the 5'-end labeled 197-nt probe, an Ava II/Xba I-digested fragment of the pSP64-MC plasmid (gift of T. Enver, Chester Beauty Laboratories, London, UK). Mouse α-globin mRNA protects a 208-nt fragment of the 3'-end labeled 450-nt probe, a BstEII/Sac I-digested fragment of NP82 plasmid. Mouse εβ microglobulin (Mb-MG) mRNA protects a 208-nt fragment of the 5'-end labeled 1.94-kb probe, an EcoRI-digested fragment of a PBZ2mdIII.B plasmid (gift of T. Ley, Washington University, St Louis, MO).

Quantitation of human ε-globin mRNA copies. In an attempt to quantitate ε-globin mRNA in hybrid cells, the human ε-globin cDNA was subcloned into pGEM-4Z vector (Promega, Madison, WI) at the Pst I site, and a large amount of human ε-globin mRNA was synthesized using the Promega in vitro transcription kit. After treatment with RQI DNAse I (M610A; Promega), the synthesized human ε-globin mRNA was purified by Sephadex G-50 column chromatography, phenol extraction, and ethanol precipitation. The concentration of synthesized ε-globin mRNA was measured by spectrophotometry and was further confirmed by electrophoresis on 2% agarose gel and comparing with rabbit globin mRNA (8103SB; BRL, Gaithersburg, MD) as a standard. The density of the bands stained with ethidium bromide was scanned by densitometer UltroScan XL (Bio-Rad, Richmond, CA). Serial dilutions of the synthesized human ε-globin mRNA with known amount of ε-globin mRNA copies were used as standards in each S1 nuclease assay. The density of protected fragment on the radioautograph was scanned by densitometer. The numbers of ε-globin mRNA copies in the samples were then calculated by comparison with the synthesized ε-globin mRNA standards using the Minitab statistical analysis program (Minitab Inc, State College, PA). Mouse βε microglobulin mRNA was also determined in each poly-A-rich RNA sample to normalize data from different experiments to correct for variations in the efficiency of poly-A-rich RNA isolation and recovery.

RESULTS

Quantitation of human ε-globin mRNA. Serial dilutions of the synthesized human ε-globin mRNA were assayed by S1 nuclease protection analysis. A linear correlation between the signal intensities of the protected fragments and the ε-globin mRNA copies varying between 2 x 10^6 to 4 x 10^9 was observed (data not shown). In addition, RNA preparations from induced and uninduced K562 cells were similarly studied. The data indicate that the K562 cell line maintained in our laboratory has approximately 5 x 10^9 copies of ε-globin mRNA per microgram of total cellular RNA. With induction by 40 μmol/L hemin for 3 days, the ε-globin mRNA level increased to approximately 10-fold higher. In two separate determinations, the ε-globin mRNA copy number present in HFHE from midgestation homozygous α-thalassemia (--SE/SE) fetuses was found to be similar to that present in induced K562 cells, ie, 5 x 10^9 copies/μg of cellular RNA (data not shown).

The mouse βε microglobulin mRNA level remained relatively stable in mouse-human hybrid cells, with or without induction (Fig 2). Therefore, mouse βε microglobulin mRNA level was determined in each poly-A-rich RNA sample to normalize results from different experiments to correct for variations in the efficiency of poly-A-rich RNA isolation and recovery.

MEL X MNC (αα) hybrids. Seven hybrid cell lines, each containing human chromosome 16 with normal α-globin gene cluster derived from adult MNC, were studied. Four of these cell lines were constructed with MNC from two normal adult individuals (αα/αα), whereas the other three cell lines were constructed with MNC from two other individuals who were carriers of the α-thalassemia deletion (−/−αα). Ten different RNA samples were extracted from these seven cell lines (Table 1). As shown in Figs 3 and 4, human ε-globin mRNA was either undetectable in four different RNA samples or present at a very low level, with a mean of 0.8 x 10^6 copies/μg of total cellular RNA,
in six other samples. Human ζ-globin mRNA was detectable in all seven hybrid cell lines with induction by either DMSO or HMBA.

These results show that a very minute amount of human ζ-globin mRNA is present in the hybrid cells containing human chromosome 16 with the normal α-globin gene cluster. To determine if there was cellular heterogeneity in the ability to express human ζ-globin gene in these hybrid cells, we have further subcloned two of these cell lines; all five subclones tested were found to have similarly low levels of human ζ-globin gene expression (data not shown). These data are consistent with the recent finding that ζ-globin mRNA is detectable in normal adult reticulocytes by highly sensitive methods.13 Results from previous reports showing that ζ-globin mRNA was not present in similarly constructed and induced hybrid cells might be caused by the sensitivity of the assay methods used.11,14

**Table 1. Summary of ζ-Globin mRNA Levels (10^6 mRNA Copies/μg Cellular RNA) in Four Groups of MEL-Human Hybrid Cell Lines**

<table>
<thead>
<tr>
<th>Hybrid Cell Lines (human α-globin genotype)</th>
<th>Uninduced (mean ± SE)</th>
<th>Induced With HMBA (mean ± SE)</th>
<th>Induced With DMSO (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEL × MNC (aa/1) (n = 7)</td>
<td>0.48 ± 0.22 (10)</td>
<td>2.42 ± 0.59 (16)</td>
<td>3.30 ± 0.65 (8)</td>
</tr>
<tr>
<td>MEL × MNC (--SEA/1) (n = 3)</td>
<td>5.29 ± 2.84 (4)</td>
<td>9.71 ± 2.93 (3)</td>
<td>13.50 ± 2.42 (7)</td>
</tr>
<tr>
<td>MEL × K562 (aa/1) (n = 21)</td>
<td>3.69 ± 1.50 (4)</td>
<td>5.70 ± 3.24 (2)</td>
<td>5.20 ± 0.54 (5)</td>
</tr>
<tr>
<td>MEL × HFHE (--SEA/1) (n = 8)</td>
<td>7.85 ± 0.95 (9)</td>
<td>33.93 ± 9.03 (8)</td>
<td>27.53 ± 9.78 (9)</td>
</tr>
</tbody>
</table>

The P value between groups was calculated by the Kruskal-Wallis test. N is the number of independent hybrid clones. The number of RNA samples from independent experiments is in parentheses.

* P value was not calculated because only two experiments were performed with the MEL × K562 cell lines induced with HMBA.

![Fig 3. S1 nuclease protection analysis of globin gene expression in mouse-human hybrid erythroid cell lines. (A) MEL × MNC hybrid cell line bearing the normal human α-globin gene cluster. In the upper panel, 30 μg of poly-A-rich RNA from the hybrid cells was used to examine for the human ζ-globin gene expression. In the lower panel, 1 μg of poly-A-rich RNA from the same preparation as that used in the upper panel was used to examine for the human α-globin gene expression as well as for murine β2-microglobulin mRNA. Lane 1, hybrid cells without induction; lane 2, hybrid cells induced with DMSO for 3 days; lane 3, hybrid cells induced with DMSO for 3 days, followed by culture without DMSO for 5 additional days; lanes 4 and 5, same as in lanes 2 and 3, respectively, except that HMBA was used as the inducer. Hc, human ζ-globin mRNA-protected fragment; Hα1, Hα2, Hc, human α-globin mRNA protected fragments; Mβ2-MG, murine β2-microglobulin mRNA protected fragment. (B) MEL × MNC hybrid cell line bearing the human (--SEA/) deletion. The MNC were from an adult carrier of the (--SEA/) deletion. The MN cells were from an adult carrier of the (--SEA/) deletion. In the upper panel, 30 μg of poly-A-rich RNA was used to examine for the human ζ-globin gene expression. In the lower panel, 2 μg of poly-A-rich RNA from the same preparation was assayed for murine β2-microglobulin mRNA. Lane 1, hybrid cells without induction; lanes 2 and 3, hybrid cells induced with DMSO for 1 and 2 days, respectively. (C) MEL × HFHE hybrid cell line bearing the human (--SEA/) deletion. The HFHE were from a midgestation fetus with homozygous α-thalassemia. Karyotyping confirms the presence of one human chromosome 16 (data not shown). In the upper panel, 30 μg of poly-A-rich RNA was used to examine for the human ζ-globin gene expression. In the lower panel, 0.5 μg of poly-A-rich RNA from the same preparation was assayed for murine β2-microglobulin mRNA. Lane 1, hybrid cells without induction; lane 2, hybrid cells induced with HMBA for 3 days; lane 3, hybrid cells induced with DMSO for 3 days.

![Fig 4. Summary of human ζ-globin gene expression in four different sets of hybrid cell lines. These are the seven MEL × MNC (aa/1) hybrid cell lines, three MEL × MNC (--SEA/1) hybrid cell lines, eight MEL × HFHE (--SEA/1) hybrid cell lines, and two MEL × K562 (aa/1) hybrid cell lines. In each induction experiment, only the highest value of human ζ-globin mRNA level during the course of induction was included in the tabulation. The data are expressed as the mean ± SEM. The figure under each column represents the number of different experiments performed. Note that only two HMBA induction experiments were performed in MEL × K562 hybrids.](www.bloodjournal.org)
These data suggest that the human ζ-globin gene expression is higher in hybrids containing the human chromosome 16 with the (--SEA/) deletion when compared with that of hybrids with normal human chromosome 16. Furthermore, induction with either DMSO or HMBA can enhance the human ζ-globin gene expression in these hybrid cells. However, the level of human ζ-globin mRNA present in these hybrid cells, even with induction, is relatively low in comparison with the levels observed in MEL × HFHE (--SEA/) hybrids (see below and Fig 4). It is known that the human adult globin gene expression in the chromosome-mediated gene transfer experiments depends on the differentiated state of human donor cells. If the donor cells are nonerythroid hematopoietic cells, as in the aforementioned experiments, human adult globin gene expression is much less than that in hybrid cells constructed with human erythroid cells.

**MEL × K562 (aa/) hybrids.** It should be informative to determine the pattern of human embryonic ζ-globin gene expression in MEL-human hybrid erythroid cell lines constructed with normal midgestation human fetal erythroblasts. However, these human fetal cells are not readily available. Instead, we elected to use the human K562 erythroleukemic cell line as the human parental cells in constructing the mouse-human hybrid erythroid cell lines. Whereas K562 cells are erythroleukemic and not normal fetal erythroblasts, these cells are active in embryonic and fetal globin gene expression. During normal human fetal development, the embryonic ζ-globin gene expression is minimal during the second and third trimester of pregnancy. In contrast, as presented above, induced K562 cells contain similar copies of ζ-globin mRNA as in fetal hepatic erythroblasts from midgestation human fetuses with homozygous α-thalassemia (--SEA/).

Two MEL × K562 (aa/) hybrid cell lines were constructed and studied in detail. Southern blot analysis showed that they contained the normal human α-globin gene cluster. In one hybrid cell line, karyotyping was performed and one copy of normal human chromosome 16 was found to be present. In concert with previously published data, these hybrids had very few human ζ-globin mRNA, with a mean of 3.7 ± 1.5 × 10^3 copies/μg RNA in four different RNA samples. Induction with DMSO or HMBA resulted in a minimal increase in the level of human ζ-globin mRNA (Table 1). On the other hand, human α-globin gene expression in these hybrids was significantly enhanced by DMSO or HMBA, in parallel with the induction of the endogenous murine α-globin gene expression (Fig 5). These results show that the chromosomal transfer of a transcriptionally active human ζ-globin gene linked to normal α-globin genes in cis, from the embryonic erythroid milieu of the K562 cells into the adult erythroid cell environment of the MEL cells, causes suppression of the human ζ-globin gene expression.

**MEL × HFHE (--SEA/) hybrids.** To construct hybrid cell lines with human erythroid cells that express the ζ-globin gene but from which the α-globin genes have been deleted, we next obtained fetal liver tissues from two midgestation fetuses with homozygous α-thalassemia at postmortem examination. Single-cell suspension of HFHE was prepared and used to construct eight hybrid cell lines with MEL-179. All were shown by Southern blot analysis to contain human α-globin gene cluster with the (--SEA/) deletion. In two hybrid cell lines, karyotyping was performed, and each line was found to contain one copy of human chromosome 16. Human ζ-globin mRNA was present in all eight hybrid cell lines, with a mean of 7.8 ± 1.0 × 10^3 copies/μg RNA, as assessed in nine different RNA samples (Figs 3 through 5). Induction by DMSO or HMBA resulted in a significantly increased human ζ-globin mRNA level, with a mean of 27.5 ± 9.8 and 33.9 ± 9.0 × 10^3 copies/μg RNA, respectively. The presence of mouse ζ-globin mRNA was searched for in these hybrids and not found, suggesting that the hybrid cells do not have embryonic erythroid trans-acting factors. On the other hand, the induction of human ζ-globin gene expression in these hybrid cells was in parallel with the induction of the endogenous murine α-globin gene expression (Fig 5), indicating that human ζ-globin gene expression in these cells...
is directed by adult erythroid transcription factors. Thus, human δ-globin genes in cis to the (−SEA) α-thalassemia deletion are expressed in adult erythroid tissues.

DISCUSSION

The present observations, together with previously published results, suggest that MEL cells produce the adult type of erythroid trans-acting factors intracellularly, which can affect the expression of endogenous murine α-globin genes as well as the transferred human α-globin genes in the hybrid cells but not significant expression of embryonic δ-globin genes. Conversely, K562 cells produce the embryonic-fetal type of erythroid trans-acting factors, thus allowing the expression of embryonic and fetal globin genes.

The present investigation also shows that, in MEL hybrid cells containing human chromosome 16 with the (−SEA) deletion, human δ-globin gene in cis to the deletion is expressed, albeit at low level. Furthermore, the human δ-globin gene expression can be increased by induction to levels much higher than the constitutive level, in parallel with the induction of endogenous mouse α-globin gene expression. These data are consistent with the observation that low-level δ-globin chain expression is present in the circulating erythrocytes of an adult carrier of the (−SEA) deletion.

The level of human δ-globin gene expression in MEL × HFHE (−SEA) hybrid cells is very low. There are approximately 3.4 × 10^5 copies of human δ-globin mRNA per microgram of cellular RNA in these hybrids after induction with HMBA for 3 days (Table 1 and Fig 4). In contrast, there are approximately 5 × 10^6 copies of δ-globin mRNA per microgram of cellular RNA in K562 cells induced with hemin for 3 days as well as in HFHE from midgestation homozygous α-thalassemia (−SEA/−SEA) fetuses. Therefore, the major factors that allow high-level human embryonic δ-globin gene expression are lacking in the MEL cells, which could not be compensated merely by the (−SEA) type of α-thalassemia deletion in cis.

Recent studies have shown that the developmental stage-specific and tissue-specific regulation of globin gene expression is achieved through the interaction of at least three elements. These elements include both positive and negative regulatory nucleotide sequences flanking the coding sequences of the globin genes. Secondly, the distal major regulatory sequences, the locus control region of the β-globin gene cluster, and the HS-40 major regulatory region of the α-globin gene cluster are indispensable in globin gene expression. Thirdly, it is generally thought that erythroid-specific trans-acting factors are responsible for the developmental stage-specific regulation of globin gene expression.

Our present data suggest that, in the presence of the (−SEA) α-thalassemia deletion, the adult erythroid trans-acting factors produced in the MEL cells can interact, probably at a low level of efficiency, with the regulatory nucleotide elements flanking the human embryonic δ-globin gene and the HS-40 major regulatory region in cis to the α-globin gene deletion to effect the low-level expression of human δ-globin gene. These observations show the importance of the presence of the DNA sequences within the (−SEA) α-thalassemia deletion in regulating the expression of embryonic δ-globin gene in cis.

It was recently reported that, using the transgenic mouse model, human δ-globin gene expression was present only in the murine yolk sac erythroid cells and not in the fetal liver-derived erythroblasts, even when the constructs did not contain human α-globin genes in cis. These results were interpreted to show the autonomous developmental regulation of human δ-globin gene expression. However, human δ-globin genes are expressed at a very high level, as late as in the third trimester fetuses with homozygous α-thalassemia caused by the (−SEA) deletion. Cord blood of newborns who are heterozygous for the (−SEA) deletion has approximately 10 times more δ-globin chains than that of normal newborns. Adult carriers of the (−SEA) deletion have low-level δ-globin chains in their peripheral blood. Furthermore, the pattern of human embryonic ε-globin gene expression during embryonic development in transgenic mice was recently shown to be different from the normal pattern observed in human embryos. Taken together, these observations indicate that the current interpretation of the transgenic mouse experiments with regard to the δ-globin gene regulation during human development might need modifications.

Individuals who are carriers of the (−M55V) and (−SPAN) α-thalassemia deletions involving α-globin genes also have detectable δ-globin chains in their circulating erythrocytes. However, not all the natural deletions are accompanied by the low-level δ-globin gene expression in adult carriers. For example, adult carriers of (−BR7), (−SA), and [−(α) 20.5] deletions do not have detectable δ-globin chains in their peripheral blood (Fig 1). These observations suggest that the nucleotide sequences spanning approximately 5 kb between the 5' breakpoints of the (−SA) and the (−SPAN) deletions are necessary to ensure the low-level expression of embryonic δ-globin gene in cis to the (−SEA) deletion in adult carriers. These data also make it highly unlikely that there might be enhancer-like sequences present 3' to the (−SEA) deletion. Taken together, these results indicate that the human embryonic δ-globin gene expression is modulated during development by multiple positive and negative regulatory mechanisms. The data of the present investigation provide support for the hypothesis that some DNA sequences within the (−SEA) α-thalassemia deletion are essential in affecting the lack of significant δ-globin gene expression in normal adult erythroid cells. It may be speculated that this effect is mediated through the competition between δ- and α-globin genes in cis for the interaction with HS-40 major regulatory region and the stage-specific erythroid trans-acting factors.

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REFERENCES

HUMAN EMBRYONIC ζ-GLOBIN GENE EXPRESSION


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Human embryonic zeta-globin gene expression in mouse-human hybrid erythroid cell lines

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