The theta globin gene is the most recently discovered member of the alpha globin gene family. Its pattern of expression during development is not fully defined, and its encoded protein has not yet been detected in vivo. The detection of theta globin messenger RNA (mRNA) in embryonic and fetal erythroid tissue but not in adults has suggested that theta is an embryonic globin gene. The present study further defines the pattern of theta globin gene expression. We use a modification of the highly sensitive polymerase chain reaction (PCR) technique to assess the levels of theta globin gene expression during development. We confirm the presence of the theta globin mRNA in embryonic and fetal erythroid tissue, and, in addition, we find theta mRNA in the peripheral reticulocytes of normal adults. Furthermore, using the same analytic approach, we detect low but significant levels of the embryonic zeta and epsilon mRNAs in reticulocytes of normal adults. Both zeta and theta gene expression appears erythroid specific in that neither mRNA species is detected in RNA isolated from brain tissue, peripheral blood mononuclear cells, or three nonerythroid cell lines (B-lymphocyte, T-lymphocyte, and hepatoma cell lines). The relative levels of zeta and theta gene expression were assayed during development by a coamplification technique. The results demonstrate the expected developmental regulation of zeta globin mRNA. In contrast, the level of theta globin mRNA fails to demonstrate the significant changes of the magnitude seen in other globin genes and remains low in embryonic, fetal, and adult life. The lack of zeta and epsilon globin proteins in normal adults using highly sensitive immunologic techniques, as reported by others, stands in contrast to these mRNA results and suggests a gap between mRNA and protein expression.

For example, the inability to detect zeta protein may not be definitive proof that zeta transcription is completely turned off in adult life, since the exact correlation between the globin mRNA levels and their protein levels remains to be defined.

The present study was undertaken to characterize the pattern of theta globin gene expression and to correlate this pattern with that of the other embryonic globin genes. In this work the expression of each embryonic gene was assessed at the level of steady-state mRNA. The sensitivity of the mRNA detection was maximized through the use of polymerase chain reaction (PCR; also known as primer-dependent DNA amplification). Using this approach, we demonstrated that theta globin mRNA is expressed in embryonic, fetal, and adult erythroid tissue. This expression is relatively low and remains low throughout development. The magnitude of the developmental changes in the expression of the other globin genes is not seen in the case of the theta globin gene. Using PCR, we can detect zeta and epsilon globin mRNAs in adult peripheral blood reticulocytes at levels comparable to that of theta mRNA and approximately 5% of alpha mRNA. These results suggest that theta globin gene is expressed throughout development at levels comparable to the basal residual transcriptional activity of the embryonic globin genes.
MATERIALS AND METHODS

Source and preparation of RNA samples. Reticulocyte RNA was isolated from cord blood of three full-term newborns and from peripheral blood of four normal individuals, six individuals with hemolytic anemia (sickle cell disease), two patients with chronic myelogenic leukemia, and one patient with polycythemia vera. RNA was extracted from acid-precipitated polysomes as previously detailed. RNA from erythroid cells from 7- and 8-weeks’ gestation embryos were isolated as previously detailed. Brain tissue from the frontal lobe, obtained at autopsy five hours postmortem (cause of death: cardiovascular sudden death), was dissected from the meninges and superficial cortex and rapidly frozen in liquid nitrogen. RNA was extracted from the frozen tissue by homogenization with a polytron (Brinkman Instruments, Westbury, NY) in guanidine hydrochloride followed by differential salt precipitation. RNA from peripheral blood mononuclear cells was isolated after separating the mononuclear cells from erythrocytes. Mononuclear cells from the heparinized blood of three of the above-mentioned four normal adults were isolated over Ficoll-Paque (1.077 g/mL; Pharmacia Fine Chemicals, Piscataway, NJ) at 400 g for 30 minutes at 20°C. The mononuclear cells at the interface were collected and washed twice in cold phosphate-buffered saline (PBS) buffer before RNA extraction. RNA was isolated by the guanidine hydrochloride method mentioned above. RNA was also isolated from three human cell lines, two nonerythroid hematopoietic cell lines and one hepatoma cell line. The two hematopoietic cell lines were a B-lymphocyte (Raji) cell line and a T-lymphocyte (Jurkat, subclone J32) cell line (gift of Dr J. Kant, Department of Pathology, University of Pennsylvania). The hepatoma cell line was Hep G-2 (ATCC®HB8065). The three cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). The RNA from the three cell lines was isolated by the guanidine hydrochloride method cited above.

Oligonucleotides design and synthesis. The position and sequence of each of the oligonucleotides primers used in this study are shown in Figs 1 and 2 and Table I. Each set of primers was selected to selectively prime the reverse transcription and subsequent amplification of a single species of globin mRNA (alpha, theta, zeta, or epsilon). In addition, the zeta and alpha 5′ (sense) primers were selected to bridge two exons to prevent amplification of genomic DNA contaminating the RNA preparations. The oligonucleotides...
Fig 2. Detection of epsilon globin mRNA in adult and term cord blood. (A) The map of the beta globin gene cluster is shown in the first line. A schematic of the epsilon mRNA is shown in the second line. Positions and orientations of the primers used in the reverse transcription and the PCR reaction are shown as small arrows, and size of the amplified fragment is 526 (shown in parentheses). The 3' primer contains nine thymidine nucleotides complimentary to the first nine adenine nucleotides of the poly (A) tail. * indicate that the 5' primer is 3P end labeled. The two Rsal restriction enzyme sites on the amplified epsilon cDNA are shown, and the two end-labeled fragments (396 bp and 496) resulting from Rsal digestion are shown. (B) Autoradiograph showing the amplification products with epsilon-specific primers in term cord blood. lane [epsilon (C)]. and adult peripheral blood. lane [epsilon (A)]. Lane [epsilon (A)]. partial digestion of the amplification product using epsilon-specific primers in adult peripheral blood. Lane (M) is P end-label pBR322/Hindl size marker.

were synthesized and sequenced by the DNA Synthesis Service of the Cancer Center at the University of Pennsylvania.

Copy DNA (cDNA) synthesis. Specific primer extension with each antisense primer was carried out as previously detailed.27 Briefly, 10 μg of total cellular RNA and 400 ng of the primer were mixed, heat denatured at 92°C, and extended at 45°C in reverse transcription buffer (100 mol/L NaCl, 100 mmol/L Tris-HCl [pH 8.4], 20 mmol/L DTT, 180 μg/mL actinomycin D, 10 mmol/L MgCl2, 400 μmol/L each of the four deoxynucleotides) in the presence of 20 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). After the primer extension reaction was complete, the synthesized cDNA was extracted with an equal volume of phenol/chloroform (49:50:1 phenol:chloroform:isoamyl alcohol, pH 8.7) and precipitated from a 0.1 mol/L NaOAc solution with 2.5 vol of ethanol.

Enzymatic cDNA amplification. Polymerase chain reaction (PCR) was performed as described by Kogan et al27 with the following modification: for each reaction, either the 5' or the 3' primer was end labeled with 32P. The end labeling was carried out with gamma (32P) adenosine triphosphate (ATP) (5,000 Ci/mmol/L, Amersham Corp, Arlington Heights, IL) in the presence of polynucleotide kinase (T4 PNK, New England Biolabs, Beverly, MA). The labeled primer was separated from unincorporated nucleotide on a Sephadex G-25 spin column (Boehringer Mannheim Biochemicals, Indianapolis, IN). The cDNA was amplified in 50-μL reaction volume containing 1.5 mmol/L each of deoxynucleotides, 25 pmol each of the oligonucleotide primers and 10% dimethylsulfoxide in 1X reaction buffer. 1X reaction buffer contains 16.6 mmol/L ammonium sulfate and 67 mmol/L Tris-HCl (pH 8.8 at 25°C). 6.7 mmol/L magnesium chloride, 10 mmol/L bovine serum albumin (BSA) per milliliter. After denaturing the samples at 95°C for four minutes, the reaction was cooled to 57°C for 30 seconds for primer annealing, and 2 units of Thermus Aquaticus (Taq) polymerase (New England Biolabs) were added to the reaction. The reaction was overlaid with mineral oil, incubated at 74°C for four minutes,

<table>
<thead>
<tr>
<th>Primer</th>
<th>Length</th>
<th>Position</th>
<th>Orientation</th>
<th>Sequence</th>
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<tr>
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<td>21</td>
<td>123–133/250–261</td>
<td>+</td>
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<td>18</td>
<td>824–833 + 9T</td>
<td>–</td>
<td>TTTTTTTTTGGGCCGCCAC</td>
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<tr>
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<td>+</td>
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<tr>
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<td>–</td>
<td>GGCCCGGAGGCTTGCTGCGGTC</td>
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<tr>
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<td>19</td>
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<tr>
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<td>–</td>
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<tr>
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<td>1532–1552 + 9T</td>
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<td>–</td>
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*In each of these cases, the numbering is in reference to the initiation codon with the A of the initiation codon AUG assigned as no. 1. In all other cases, the position no. 1 is the mRNA cap site.
and then cycled at 95°C for one minute, at 57°C for 30 seconds, and at 74°C for four minutes. A total of 30 cycles was performed. The PCR reactions were carried out on a DNA thermal cycler (Perkin Elmer-Cetus Instruments, Norwalk, CT).

Analysis of amplified samples. For direct analysis of the PCR reaction, a 5-μL aliquot of each amplified sample was resolved by electrophoresis on an 8% polyacrylamide/8 mol/L urea gel. The gel was then transferred to Whatman 3M paper, covered with Saran wrap, and exposed to Kodak AR film at -70°C. The relative density of the amplification product bands was determined using Zeineh Soft Laser Scanning densitometer (Biomed Instruments Inc, Fullerton, CA). For restriction analysis of the PCR product, the amplified cDNA was phenol/chloroform extracted and ethanol precipitated prior to restriction enzyme digestion. Restriction enzyme digestion of the amplified cDNA was carried out with HindIII, PvuII, HincII, and RsaI (New England Biolabs) according to the manufacturer’s suggested conditions.

RESULTS

Theta, zeta, and epsilon mRNAs are all present in adult peripheral blood. Previous studies have detected embryonic globin (zeta) gene expression in full-term fetuses and in adult bone marrow. We wished to extend these findings by assaying for zeta as well as epsilon and theta mRNAs in the peripheral reticulocytes of normal individuals. To achieve maximal sensitivity, we applied the technique of DNA polymerase chain reaction (PCR). We primed cDNA synthesis from adult reticulocyte RNA in four separate reactions, each containing antisense oligonucleotides specific for a single species of globin mRNA: alpha, zeta, epsilon, or theta. The synthesized cDNA was then amplified between the primary oligonucleotide and a second more 5’ sense oligonucleotide, again specific for each of the globin species. Since one of the primers in each set was 5’ end labeled with 32P, the amplification reaction products could be directly autoradiographed after gel electrophoresis. The position and orientation of each of the primers are shown in Figs 1A, 2A, and Table I, and representative autoradiographs of the analytic gels are shown in Figs 1B and 2B. These results demonstrate the synthesis of cDNA fragments of the size predicted from the published sequence for each of the four globin mRNA species. The sizes of the amplified alpha, zeta, theta, and epsilon cDNAs are 465, 381, 479, and 526 bp, respectively.

To verify the identity of each of the amplified amplification products, each was mapped with a number of restriction enzymes, which should give a pattern of cleavage specific for the alpha, zeta, theta, and epsilon cDNAs. The position of each of the enzyme cleavage sites and resultant end-labeled cDNA fragments are illustrated in Figs 2A and 3A. The size of each of the amplified cDNAs and the subsequent restriction analysis confirmed the specific amplification of alpha, theta, zeta, and epsilon mRNAs (Figs 2B and 3B). The
amplified theta cDNA fragment contains the expected unique PvuII site at the appropriate position and lacks both HindIII and HincII sites. The amplified zeta cDNA fragment shows the expected unique site for HincII at the appropriate position and lacks both HindIII and PvuII sites. The amplified alpha cDNA fragment demonstrates the expected two restriction enzyme sites, HincII and HindIII, with sizes of 258 bp and 276 bp, respectively, and lacks a PvuII site. The epsilon cDNA fragment contains the two Rsal sites of the positions predicted from its sequence (Fig 2). The presence of the large end-labeled fragment results from a partial digestion. These results, therefore, confirm the detection of zeta, theta, and epsilon mRNA in normal adult reticulocytes.

In the above analysis we detected zeta, theta, and epsilon mRNAs in the peripheral blood of patients with sickle cell disease, chronic myelogenous leukemia (CML), and polycythemia vera (both without evidence of abnormal hemoglobin); the representative samples shown in Figs 1 and 2 are from a patient with sickle cell anemia. To determine whether the detected low level of theta, zeta, and epsilon in the peripheral blood of adults was in some way related to erythroid stress or abnormal maturation, we analyzed reticulocyte RNA from the blood of four normal individuals in our laboratory. In each of these individuals we were able to detect both theta and zeta globin mRNAs comparable to that in sickle cell anemia patients (data not shown). The relative level of zeta and theta mRNAs to alpha mRNA in these individuals was approximately 5% to 10%. Trace amount of epsilon globin mRNA could also be detected in these individuals after several days of exposure. Whether these faint signals are due to a lower level of epsilon basal residual mRNA or to a lower efficiency of epsilon cDNA amplification is unclear at this point.

**Erythroid-specific expression.** The low level of zeta, theta, and epsilon mRNAs in adult reticulocytes may be the result of three separate mechanisms: a general low level of expression that is tissue nonspecific, a low level of expression specific for hematopoietic cells (erythroid or nonerythroid cells), or residual "leaky" expression specific to erythroid tissue. To differentiate among these three possibilities, we attempted to detect these mRNAs in nonhematopoietic tissue (brain and hepatoma cell line) and in nonerythroid hematopoietic tissue (peripheral blood mononuclear cells and human T- and B-lymphocyte cell lines). We chose to assay deep parenchymal brain tissue due to its low level of vascularity. In addition, two nonerythroid hematopoietic cell lines were studied: B-lymphocyte (Raji) cell line and T-lymphocyte (Jurkat) cell line. A nonhematopoietic cell line, hepatoma cell line Hep G2, was also studied. We also studied RNA from peripheral blood mononuclear cells to rule out the remote possibility that mRNA from these cells was contaminating our reticulocyte RNA preparation. The peripheral blood mononuclear cells were carefully separated from the erythroid cells on a Ficoll-Paque gradient. The results of these studies are shown in Fig 1C and in Fig 4. Figure 4 shows the results of co-amplification of zeta and theta (see below for further discussion). Neither theta nor zeta globin mRNA was detectable in any of these tissues or cell lines (Fig 1C and Fig 4). As a positive control, each sample was also demonstrated to contain a very strong signal for beta actin cDNA. This confirmed that mRNA was intact and
could be reverse transcribed and amplified. Amplifying the same RNA for beta actin without prior reverse transcription was entirely negative, confirming that this signal originated from RNA.32

The relative expression of theta and zeta globin mRNA during development. The pattern of gene expression in the alpha globin cluster was further characterized by comparing the relative levels of zeta and theta mRNA during development. We analyzed RNA from the erythroid tissue of a 7- and an 8-weeks' gestation fetus from term cord blood and from adult peripheral blood. Analysis of zeta mRNA (Fig 5) shows that it is still present at a high level at 7 weeks' gestation and continues to be present, although at lower levels, in term cord blood and in adult peripheral blood. The levels of zeta and theta were directly compared during development by co-amplification of zeta and theta cDNAs in the same reaction (Fig 6). This could be done, since the amplified theta and zeta cDNA fragments can be resolved on the basis of their size difference. The relative ratio of zeta to alpha and zeta to theta was determined directly by densitometric analysis, while the relative ratio of theta to alpha was calculated from these two ratios. These results demonstrated that the level of theta in the 8-weeks' gestation fetus is a minor fraction of zeta (approximately 5%), while in cord blood and adult peripheral blood the level of theta is comparable to zeta. On the other hand, a direct comparison of zeta and alpha mRNA levels by co-amplification technique (Fig 6, right panel) demonstrated that the level of zeta mRNA in both cord and adult blood is a small but easily detectable fraction (3% to 5%) of the adult alpha mRNA. Taken together these co-amplifications demonstrate that the level of theta in cord blood and adult peripheral blood make up the same minor fraction (5% of alpha) in adult blood as it does (5% of zeta) in 8-weeks' gestation blood. The exact accuracy of these ratios should, however, be taken with caution, since there may be subtle differences in the efficiency with which each cDNA is amplified. The ratios do, however, suggest that theta is expressed at low level in embryonic, fetal, and adult life and remains low throughout development.

Our analysis of 13 different adults (four normal, six with sickle cell anemia, two with CML, and one with polycythemia vera) by co-amplification suggests that on the average zeta appears equal to theta. However, we observed some variation in the relative ratio of zeta to theta. This is demonstrated in the three different adult samples presented in Fig 6. As shown in this figure, some adults express relatively more theta than zeta, and others equal the amount of theta to zeta or slight excess of zeta. Whether the basis of this variation is due to variation in theta alone or zeta alone or to changes in both is not clear and is currently under investigation.

DISCUSSION

In this study we further characterized the pattern of theta globin gene expression and compared this pattern with that of the embryonic zeta globin gene. The application of the highly sensitive PCR technique resulted in a high level of resolution of mRNAs that have not been detected by more conventional methodologies. The results can be compared with the highly sensitive radioimmunoassay (RIA) recently established for the detection of globin proteins.33,34

We have modified the PCR technique so that each mRNA species can be specifically reverse transcribed and amplified using 32P end-labeled primers. The products of these reactions can be directly visualized on an autoradiograph of the analytic gel, thus eliminating the need for hybridization. Using this approach we have made both expected and unexpected observations. We found, as previously reported,11,12 that the theta globin gene is expressed in embryonic and fetal erythroid tissue. We confirm that the expression of the embryonic zeta globin gene is developmentally regulated: it is expressed at high level in the embryonic stage and continues to be transcribed at lower levels through term gestation. Unexpectedly we detected relatively abundant amounts of theta mRNA (3% to 5% of alpha mRNA) as well as significant levels of zeta and epsilon globin mRNAs in adult peripheral blood (5% of alpha mRNA).

The expression of zeta and theta mRNA in adult reticulocytes appears to be erythroid specific, as neither zeta nor theta mRNA was detected in nonerythroid hematopoietic tissue (peripheral blood mononuclear cells and two lymphocyte cell lines, Raji and Jurkat), nonhematopoietic tissue (brain), and a hepatoma cell line. The size of each of the amplified fragments was consistent with that predicted and was confirmed by restriction analysis. The possibility that these fragments represented amplification of genomic DNA was ruled out by the positioning of the primers so that
genomic amplification would include an intron and would be easily discernible from the amplified cDNA.

The detection of theta globin mRNA in adult erythroid tissue is intriguing and unexpected. Previous studies\textsuperscript{10-12} have detected theta globin mRNA in yolk sac, K562 cell line, and fetal liver tissue but have failed to detect theta mRNA in adults. These findings have led to the conclusion that the theta globin gene is an embryonic gene. In the present study we clearly detected theta expression in adult peripheral blood. The level of theta expression in the embryonic erythroid tissue is relatively low and remains low in fetal and adult erythroid tissue. This low level of theta expression is comparable to the low level of residual zeta mRNA in adult blood, which is approximately 5% of the alpha mRNA. However, the levels of theta mRNA do not demonstrate the marked changes in the level of expression seen with the other globin mRNAs during development. The detection of theta mRNA in the yolk sac by Proudfoot et al.\textsuperscript{11} further suggests that the theta expression is turned on very early in development. Our finding that the theta gene, which is the most 3' gene in the alpha globin cluster, is not specifically expressed as an embryonic globin gene supports the conceptualization\textsuperscript{3} that the human globin genes are expressed in the order of their arrangement 5' to 3'.

This profile of theta globin gene expression, which is distinct from the other globin genes, may relate to its location in the alpha globin cluster and/or to the unusual structure of its promoter. The theta globin gene may be located too far from the embryonic globin gene and its embryonic active chromatin domain to be fully expressed as an embryonic gene. On the other hand, the fact that the alpha 1 globin gene is located downstream of alpha 2 gene and is expressed as a minor adult gene (one third of alpha 2 gene)\textsuperscript{27,33} suggests that the location of theta gene further downstream of alpha 1 may be responsible for the lack of its full expression as an adult globin gene. The unusual structure of the theta promoter may, however, be more significant in determining its pattern of regulation than its position in the cluster. Theta globin gene differs from the other globin genes as well as from most other tissue-specific genes in its promoter structure. The CCAAT and ATA box sequences, which are highly conserved in position in all other globin genes, are displaced from the initiation codon (ATG) by the insertion of a 200-base-pair GC-rich sequence in theta gene.\textsuperscript{8,10} This unusual promoter structure is conserved in human, orangutan, and baboon, suggesting a conserved function.

The recent development of antibodies directed against epsilon\textsuperscript{35} and zeta\textsuperscript{34} globin chains has provided an extremely sensitive (0.03% to 0.06% sensitivity for zeta and epsilon chains) RIA to detect these proteins. By using this technique it has been possible to detect epsilon globin as late as 20 weeks of gestation, but not beyond, and zeta globin chains in later stages of gestation and even in full-term cord blood hemolysates,\textsuperscript{36} but not in normal adults. Moreover, zeta globin gene transcripts can be detected in adult bone marrow by nuclear run-off assay despite the absence of any detectable "zeta cells" by immunofluorescent studies on erythroid tissue.\textsuperscript{19} When compared with the results in the present report, these data suggest a significant discrepancy between the presence of zeta and epsilon globin mRNA and the absence of their translated proteins.

A similar gap between the detection of mRNA and of protein appears to occur for theta globin gene. The level of theta expression in all developmental stages appears comparable to that of the zeta globin gene in adult life. However, to date no theta protein has been detected in erythroid tissue at any stage of development. This suggests that the gap between globin mRNA and their proteins may be responsible for the lack of detectable theta protein at any stage of development. The mechanism(s) responsible for this apparent gap are at this point undefined.

The presence of embryonic globin mRNA in adults raises the possibility that its basal expression may increase in response to erythroid stress in a fashion similar to that seen in fetal globin. Early reports have detected epsilon mRNA in an adult patient with homozygous beta thalassemia treated with 5'-azacytidine without detectable epsilon globin chains.\textsuperscript{37} Other studies indicate detectable epsilon globin chains in baboons after treatment with 5'-azacytidine.\textsuperscript{38} This suggests that the level of the embryonic globin transcription increases in response to bone marrow acute expansion. Similar changes in the level of zeta mRNA in adults may be responsible for the variation in the relative expression of theta to zeta in adults demonstrated in our study. However, we observed this variation in the relative levels of zeta and theta mRNA in individuals with and without increased reticulocyte count. This suggests that the reticulocyte level is not a significant factor in this variation.

\[\theta \rightarrow \alpha \rightarrow \zeta \]

**Fig 6.** The relative expression of zeta to theta mRNAs during development. Zeta and theta mRNAs were co-reverse transcribed and co-amplified in a single tube with both sets of primers. Zeta-, alpha-, and theta-amplified cDNA bands are indicated to the left of the autographs. The RNA samples were isolated from 8 weeks' gestation embryo erythroid cells in lane (SW), from peripheral blood from three different adults in lane (A). The second (8W) lane, in the middle, is shorter exposure of the first lane. The first (A), for example, suggests that theta expression is higher than zeta, but the second and third adults show theta expression less than zeta.
In summary, epsilon and zeta globin genes are not completely turned off during development but instead continue to be expressed at low levels into adult life. The theta gene is expressed at all developmental stages at a low level that is comparable to the residual expression of the zeta gene. The inability to detect embryonic or theta protein in adult erythroid tissue suggests that there may be a block in the expression of these residual transcripts at the level of translation.

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Theta, zeta, and epsilon globin messenger RNAs are expressed in adults

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