Transferrin Receptor Gene Is Hyperexpressed and Transcriptionally Regulated in Differentiating Erythroid Cells*

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We have analyzed the developmental pattern of expression of the chicken transferrin receptor (CTR) gene in various chick embryonic tissues. Northern analyses of RNA from embryonic tissues at different stages of development and cultured chick embryonic fibroblasts (CEFs) show that CTR is hyperexpressed in differentiating erythroid cells such that the steady-state level of CTR mRNA in these cells could be 200 or more times higher than in nonerythroid cells. In vitro nuclear transcription assays using nuclei from embryonic erythroid and brain cells, as well as CEFs, demonstrate that the vast differences in CTR mRNA levels in these cells are reflected in their respective CTR gene transcriptional activities. During development, the steady-state level of CTR mRNA declines in all tissues and, in erythroid cells, this pattern is accompanied by a similar decline in β-globin mRNA levels. These changes are concurrent with the decreases in CTR and β-globin mRNA transcriptional activities during erythroid maturation. Taken together, our results indicate that the hyperexpression of the CTR gene in differentiating erythroid cells is regulated to a significant degree at the transcriptional level. We also demonstrate that, in erythroid cells, neither CTR gene transcription nor CTR mRNA stability is regulated by intracellular iron levels.

Iron is required by all living systems because it is an essential component of many important enzymes, of which some are required for oxidative respiration and DNA synthesis. The process of iron transport into cells via the transferrin (Tf)–transferrin receptor (TR) complex has been delineated (reviewed in Ref. 1). Free ferric iron binds to apotransferrin and, in turn, the iron-Tf complex binds to the cell surface TR. The iron-Tf:TR complexes are internalized in coated vesicles by means of receptor-mediated endocytosis. Iron is released from Tf within the endosomes and is translocated to its site of utilization by an unknown mechanism. The TR:TF complexes remain stably bound and are excyotacted intact, followed by release of Tf from the TRs at the cell surface. Thus, both Tf and TR are recycled and reutilized in a highly efficient transport cycle.

The number of TRs present on the cell surface regulates the amount of iron imported into cells. The transmembrane glycoproteins are expressed by almost all cell types, especially those cells that are proliferating rapidly or require high levels of iron to synthesize specialized products, such as hemoglobin (reviewed in Refs. 2 and 3). In cultured mammalian cells, intracellular iron plays an important role in modulating the expression of TRs via negative feedback. When intracellular iron levels are elevated by treatment of cells with iron salts such as hemin or ferric ammonium citrate, the number of TRs decreases (4–9). Inversely, when intracellular iron is reduced by treatment with iron chelators, such as desferrioxamine (DES) or picolinic acid, the number of TRs increases rapidly (8, 9). This up- and down-regulation of TRs by iron is due to changes in TR mRNA levels. Iron regulation of TR mRNA depends upon its 3′-noncoding region, which contains highly conserved sequences that can form stem-loop secondary structures, known as iron-responsive elements (IREs) (10–15). Cytoplasmic factors, the IRE-binding proteins (IRE-BPs), which are responsive to intracellular iron levels have been shown to interact with the IREs and rapidly alter the stability of TR mRNA (15–16). Thus, TR expression in cultured cells is sensitive to negative feedback by intracellular iron levels and is regulated at the posttranscriptional level.

The expression of TR is regulated developmentally during erythroid maturation, correlating with the changing rates of hemoglobin production (17–19). We used a developmental series of chick embryonic erythroid cells to study the mechanism of TR gene expression during erythroid differentiation. Chick embryonic erythroid cells provide an excellent system for such studies because these cells undergo maturation in the circulation so that large numbers of essentially synchronous populations of erythroid cells at various stages of maturation can be obtained with ease from embryos at different days of development (20). Our results show that the developmental changes in CTR expression are primarily due to changes in rates of CTR gene transcription which lead, in turn, to differences in steady-state levels of CTR mRNA. Correlations with developmental changes in β-globin mRNA levels are also observed.

We have reported previously that chick embryonic erythroid cells contain >100-fold more CTR mRNA than nonerythroid cells (14). TRs are hyperexpressed in immature red blood cells presumably because exceptionally high amounts of iron are required for hemoglobin synthesis. In this study, we demonstrate that immature erythroid cells contain 20 to more than 140 times higher steady-state levels of TR mRNA than other embryonic tissues, such as brain, liver, retina, lung, and limb bud.
Transferrin Receptor Gene Expression

To clarify the mechanism by which hyperexpression of the CTR gene is regulated in erythroid cells, we performed in vitro nuclear transcription (run-on) experiments to compare CTR transcriptional activity in chick embryonic erythroid cells with that in nonerythroid cells and cultured CEFs. The effects of iron depletion and iron excess on CTR gene expression in these cells were examined also. Our results show that steady-state levels of CTR mRNA in erythroid cells, as opposed to CEFs and brain cells, do not alter in response to iron manipulation. In addition, CTR transcription is not regulated by intracellular iron in either erythroid or nonerythroid cells. Taken together, these results indicate that the hyperinduced expression of CTR in erythroid cells is regulated primarily at the transcriptional level by mechanisms that are not responsive to intracellular iron levels, demonstrating that TR gene expression is regulated differently depending upon cell type and differentiation status.

MATERIALS AND METHODS

Chick Embryonic Red Blood Cells and Tissues—Fertilized White Leghorn chicken eggs were obtained from the Department of Poultry Sciences, Texas A & M University, College Station, TX. Incubation was at 40 °C in a humidified egg incubator. Circulating chick embryonic red blood cells were isolated steriley from the blood vessels and washed twice with sterile phosphate-buffered saline (PBS) before use. Different organs and tissues were dissected from chick embryos, rinsed thoroughly with PBS, and then used for isolation of total cellular RNA or for in vitro nuclear transcription assays.

Chick Embryonic Fibroblast Cultures—Primary cultures of CEFs were established by standard procedures (21). The body walls from three 9-day-old chick embryos were dissected free of internal organs, minced, dissociated mechanically by pipetting in 10 ml of 0.25% trypsin in PBS/EDTA, and then stirred at 37 °C for 7–10 min. Trypsinization was stopped by the addition of culture medium (Dulbecco's modified Eagle's medium; Ham's F-12 (1:1) with 2% chicken serum, 2% tryptose phosphate broth, 5 mM glutamine, and 150 units/ml streptomycin) to a final volume of 50 ml. Large clumps of tissues were separated from the cell suspension by gravity and cell concentration was determined with a Coulter counter. Cells were plated at 3.5 × 10⁶ cells per 85-mm (diameter) tissue culture plate and incubated at 37 °C in 5% CO₂, 3–4 h after initial plating, the medium, nonattached cells, and debris were removed, and fresh medium was added before incubation was continued. To prepare frozen stocks, confluent primary CEF cultures were harvested, and aliquots were stored in 10% Me₂SO in liquid nitrogen.

Treatment of Cells with Desferrioxamine and Hemin—Erythroid cells and brains were isolated from 12-day embryos. Embryonic brains were extracted by mincing followed by gentle pipetting. After cell clumps were allowed to settle by gravity, the remaining nonattached cells, and debris were removed, and fresh medium was added before incubation was continued. To prepare frozen stocks, confluent primary CEF cultures were harvested, and aliquots were stored in 10% Me₂SO in liquid nitrogen.

Isolation of RNA and Northern Blot Hybridization—Total cellular RNA was isolated from cells by standard procedures (22). Cells suspended in guanidium isothiocyanate buffer were homogenized mechanically by sequential passage through 18- and 22-gauge needles. The homogenates were centrifuged over a CaCl₂ cushion, and the recovered RNA pellets were purified by phenol/chloroform extractions. The concentration of RNA was determined spectrophotometrically. RNAs were separated on denaturing formaldehyde–agarose gels and stained with ethidium bromide, and Northern blots were prepared using MSI Nitroplus membranes by standard procedures (23). The 32P-labeled probes used to hybridize to the Northern blots are presented in Table I. Because most tissues are vascularized and contain some red blood cells, we used the amount of hybridization to the β-globin cDNA probe to adjust for red blood cell contamination in the nonerythroid tissues. The amount of total cellular RNA present in each sample was normalized against the corresponding amount of 18 S rRNA.

The results show that CTR mRNA is present at significantly higher levels in erythroid cells than in nonerythroid cells of the chick embryo, confirming our previous observation (14). CTR mRNA levels decline during embryonic development in all the tissues examined, reaching undetectable levels in all of the nonerythroid tissues by hatching (21 days of development). CTR mRNA appears in the brain by 9 days of development, then increases slightly to peak by 11 days, and decreases gradually thereafter. CTR mRNA is present in liver as early as 6 days and reaches a peak at 9 days, whereas it is present in the retina at the highest level in 6-day embryos. Relatively lower levels of CTR mRNA are expressed in the limb bud and lung. The 6-day embryonic lung was not studied because it is too small for dissection; the limb bud was studied instead. The relative amounts of CTR mRNA in erythroid cells as compared to nonerythroid tissues vary widely ranging from 20 times to 140 or more times (not accurately determinable because some of the mRNA levels were below the level of detection), depending upon the tissue and stage of development (Table 1). The vast difference in CTR mRNA levels between erythroid cells and cultured CEFs is evident by comparing lanes 1 and 2 in Fig. 2, which represent CTR mRNA in freshly isolated 9-day erythroid cells and in cultured CEFs, respectively. Quantitation of this data shows that 9-day chick embryonic erythroid cells contain >200 times more CTR mRNA than cultured CEFs do. This difference is even more striking when the rapidly proliferative state of the nonerythroid cells and the nonproliferative state of the erythroid cells are taken into account.
Transferrin Receptor Gene Expression

Fig. 1. Northern blot analysis of steady-state CTR and β-globin mRNA levels in various tissues of the chick embryo at different times of development. The 18 S rRNA levels were used as internal standards.

Table I
Relative steady-state amounts of CTR mRNA in various tissues of the chick embryo at different stages of development

<table>
<thead>
<tr>
<th>Days of development</th>
<th>6</th>
<th>9</th>
<th>11</th>
<th>13</th>
<th>16</th>
<th>Hatchling</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR mRNA (100)</td>
<td>3332</td>
<td>6432</td>
<td>2222</td>
<td>1222</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>Liver mRNA (100)</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Lung mRNA (100)</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Retina mRNA (100)</td>
<td>100</td>
<td>140</td>
<td>80</td>
<td>80</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Erythroid mRNA (100)</td>
<td>100</td>
<td>140</td>
<td>80</td>
<td>80</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>

* Below the limit of detection.

Fig. 2. Northern blot analysis of steady-state CTR mRNA levels in cultured CEFs and chick embryonic erythroid cells treated with DES or hemin. Lane 1, RNA from erythroid cells freshly isolated from 9-day chick embryos; lanes 2-4, RNA from cultured CEFs incubated for 16 h with no treatment (control), with 100 μM DES, and with 100 μM hemin, respectively; lane 5, RNA from erythroid cells freshly isolated from 12-day chick embryos; lanes 6-8, RNA from erythroid cells from 12-day chick embryos after incubation for 16 h with no treatment (control), with 100 μM DES, and with 100 μM hemin, respectively. The 18 S rRNA levels were used as internal standards.

Fig. 3 shows that, in erythroid cells, CTR mRNA is present at the highest level in 9-day embryos and then gradually decreases approximately 14-fold by the time of hatching. In comparison, β-globin mRNA levels decrease more slowly, by only 4-fold from 6-9-day embryos to newly hatched chicks at 21 days.

Fig. 4. In vitro nuclear transcription assays using nuclei of erythroid cells from chick embryos at 7, 12, 15, and 17 days of development and newly hatched chicks. Test DNAs immobilized on slot blots: G, chicken β-globin, p1BR15; CTR, chicken transferrin receptor, pCTReDNA; p, pT7/T3 plasmid (negative control); and 5S, hamster 5 S rDNA, pTH1 (33).
for normalization. Therefore, to make meaningful comparisons, we used equivalent numbers of nuclei for the run-on assays and used the total amount of \([^{32}P]UTP\) incorporated per reaction as the basis for determining the numbers of cpm of transcription products used to hybridize with filters containing excess amounts of immobilized test DNAs. As expected, overall transcription decreases with erythroid maturation, reflecting either a developmental decline in transcriptional rates or a decreasing proportion of transcriptionally active cells in the circulation. Incorporation of \([^{32}P]UTP\) into tricarboxylic acid-precipitable material in run-on reactions using nuclei from a developmental series of erythroid cells reflects a 7-fold decrease in total transcriptional activity between erythroid cells from 7-day embryos and newly hatched chicks. These results correlate directly with the decline in the relative amounts of total cellular RNA present per erythroid cell during the same developmental period. Circulating erythroid cells from late embryos and newly hatched chicks remain transcriptionally active, albeit at relatively lower rates.

Transcription of both \(\beta\)-globin and CTR mRNA decreases during erythroid differentiation by about 6- and 12-fold, respectively (Fig. 3). The transcription of 5 S rRNA, which utilizes a different RNA polymerase, follows a similar developmental pattern (data not shown). In erythroid cells the CTR and 5 S rRNA gene promoters, which are comparable in strength, are much weaker than the \(\beta\)-globin gene promoter, assuming that the rates of elongation for these transcripts are similar during the nuclear run-on assay. Comparison of the relative transcriptional rates of CTR and \(\beta\)-globin mRNAs with their corresponding steady-state amounts at different stages of erythroid differentiation reveals that the developmental decreases in both mRNAs can be essentially accounted for by changes in their rates of transcription (Fig. 3).

**Transcription of CTR mRNA in Erythroid and Nonerythroid Cells**—Northern blot analysis of RNA from different chick embryonic tissues indicate that the steady-state level of CTR mRNA in erythroid cells is significantly higher than that in nonerythroid tissues (Fig. 1). In order to determine whether these differences are due to hyperinduced CTR transcription in erythroid cells, we performed nuclear run-on assays using equivalent numbers of nuclei from erythroid and brain cells, both isolated from the same 12-day chick embryos. By comparing total incorporation of \([^{32}P]UTP\) into RNA per reaction, the total transcriptional activity for brain cells was estimated to be about 3 times higher than that for erythroid cells. In these experiments, equivalent cpm of transcriptional products from either cell type were used to hybridize to filters containing immobilized test DNAs. Thus, the results shown in Fig. 5 represent the different relative amounts of specific mRNA transcripts present in the total repertoire of transcriptional products of the two different cell types. In Fig. 5, the lanes marked \(F\) reveal transcripts produced by nuclei of freshly isolated erythroid or brain cells. As expected, \(\beta\)-globin mRNA is transcribed actively by the erythroid nuclei. The low levels of \(\beta\)-globin transcripts present in the brain samples are due to red blood cell contamination of the brain cell preparations and are used to adjust the estimations of CTR transcript levels in these samples determined from densitometric data. Both embryonic erythroid and brain cells actively transcribe 5 S rRNA. In erythroid cells, the transcription of CTR is less active than that of \(\beta\)-globin and is comparable to that of 5 S rRNA. In brain cells, however, transcription of CTR is barely discernible, especially after subtracting the contribution by contaminating red blood cells. These results indicate that the differences in steady-state amounts of CTR mRNA between erythroid and brain cells correlate directly with the differential levels of CTR transcription in these tissues, demonstrating that hyperexpression of CTR in erythroid cells, to a large extent, is transcriptionally regulated.

**Effects of Intracellular Iron Levels**—We treated chick embryonic erythroid cells, brain cells, and cultured CEFs with either DES or hemin to determine whether CTR mRNA stability in these cells responds to iron regulation. In addition, we performed nuclear run-on assays to determine whether iron regulation is exerted at the transcriptional level.

Northern blot hybridization results show that DES treatment of cultured CEFs induces a 4-fold increase in CTR mRNA level when compared to untreated controls, whereas hemin treatment results in a slight decrease (5-10\%) in this level (Fig. 2, lanes 2-4). Since the CTR mRNA levels in untreated control CEFs are very low, accurate measurement of the hemin effect may not be possible. For easier viewing, the autoradiograph for CTR mRNA in the CEF samples (lanes 2-4) was exposed six times longer than that for the erythroid sample (lane 1). DES- and hemin-treated embryonic brain cells show similar results (data not shown). These results are consistent with those reported for cultured K562 cells (28). In contrast, treatment of erythroid cells with DES or hemin for 16 h does not show any measurable changes in CTR mRNA levels (Fig. 2, lanes 6-8), indicating that the hyperexpression of CTR in erythroid cells is not due to negative feedback regulation of CTR mRNA level by intracellular iron. These results confirm further the conclusion that the hyperexpression of CTR in erythroid cells is due largely to highly induced CTR gene transcription, and that if posttranscriptional stabilization of CTR mRNA were also implicated, the mechanism would differ from that of cultured nonerythroid cells in being nonresponsive to intracellular iron levels. One possible basis for this lack of iron regulation is that erythroid
cells lack IRE-BPs; however, IRE-BPs are present in chick embryonic erythroid cells (data not shown), indicating that other parameters are involved.

To determine whether CTR transcription in erythroid and nonerythroid cells is regulated by intracellular iron, nuclear run-on assays were performed using nuclei from cells treated with DES for 16 h. Fig. 5 shows the results from an experiment using erythroid and brain cells obtained from the same 12-day embryos. The test DNA blots were hybridized with equivalent cpms of 32P-labeled RNA transcripts from each sample. Results from freshly isolated cells (Fig. 5, lanes F) were included to demonstrate that 40–50% of the transcriptional activity was retained after 16 h of in vitro incubation. This observation is consistent with the retention of 40% of CTR mRNA in erythroid cells after similar incubation (Fig. 2, lanes 5 and 6). Comparisons of DES-treated erythroid and brain cells with untreated controls show that chelation of intracellular iron did not affect CTR transcription in either cell type (Fig. 5, lanes C and D). In addition, cultured CEFs treated with DES or hemin gave similar results (Fig. 6). These results demonstrate that CTR transcription in cultured CEFs, chick embryonic erythroid, and brain cells is not regulated by iron.

**DISCUSSION**

Much is known about the tissue-specific regulation of gene expression. Relatively little is understood, however, about the regulation of genes that are expressed ubiquitously but are hyperexpressed in only one or a few specific cell types. The TR gene is an example of the latter category of genes because the TR is present on essentially all cells, but it is expressed at significantly higher levels on certain cell types such as immature erythroid cells.

In cultured mammalian cells, TR expression has been established clearly to be regulated predominantly, if not completely, at the posttranscriptional level by iron via a negative feedback mechanism involving IREs and IRE-BPs. Our results demonstrate, however, that hyperexpression of TR in embryonic erythroid cells is regulated transcriptionally to a significant degree. To maximize the import of high levels of iron required for hemoglobin synthesis, CTR mRNA in erythroid cells may also be stabilized in addition to being induced transcriptionally. However, if posttranscriptional regulation were also present, the lack of response in CTR mRNA levels to iron manipulation would indicate that the mechanism is different from that in nonerythroid cells. Since IRE-BPs are present in chick embryonic red blood cells, this difference may be in response of IRE-BPs to iron, in the binding of IRE-BPs to IREs, or in factors/enzymes "downstream" from IRE binding.

These differences in mechanism may not be due entirely to cell-type specificity (erythroid versus nonerythroid) since K562 cells, one of the cultured mammalian cells that was reported to show posttranscriptional iron regulation of TR expression, are of the erythroid lineage. On the other hand, unless they are induced, K562 cells are undifferentiated precursor cells that are not actively synthesizing hemoglobin. Results from studies of Friend virus transformed murine erythroleukemia cells show a "reversal" or "loss" of iron regulation of TR mRNA levels upon induction of these cells by Me2SO to undergo erythroid differentiation (29–31). Thus, the mechanism for regulating TR gene expression is determined probably by a combination of cell-type specificity and differentiation status which, in erythroid cells, is linked with hemoglobin synthesis.

Conflicting results have been reported concerning iron regulation of TR expression at the transcriptional level. In K562 cells, as well as in murine cells transfected with human TR cDNA constructs, manipulation of intracellular iron was reported to induce >20–30-fold differences in TR mRNA levels, of which 2–5-fold has been attributed to changes in rates of TR gene transcription (12, 28). In similar experiments using a deletion series of human TR promoter/cDNA constructs transfected into mouse L-cells, however, intracellular iron levels had no influence on the transcriptional activity of the TR gene (11). Our results demonstrate clearly that CTR mRNA transcription in chicken embryonic erythroid and brain cells, as well as cultured CEFs, is not regulated by intracellular iron.

During the latter part of erythroid differentiation, total cellular macromolecular synthetic activities decline gradually until mature erythrocytes become essentially inert. Whether a general mechanism "shuts down" all genes or each gene is regulated individually is unknown. Our results show that β-globin, CTR, and 5 S rRNA transcription all decline during erythroid maturation, but at different relative rates, suggesting that regulation of individual genes or groups of genes is more likely. This interpretation is consistent with our previous observation that different time courses are followed in the termination of synthesis of various plasma membrane proteins in differentiating erythroid cells (32). The presence of transcriptional activity in erythroid cells isolated from the circulation of 17-day embryos and newly hatched chicks parallels the presence of translational activity (32) indicating that even at these late stages of development, there are significant numbers of peripheral erythroid cells that are still active in macromolecular synthesis. The molecular nature of the mechanisms that regulate the hyperinduction, and subsequent suppression, of CTR gene expression during the entire course of erythroid differentiation remains to be determined.

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