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Estrogen Regulation of the Avian Transferrin Gene in Transgenic Mice

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The intact chicken transferrin gene was microinjected into fertilized mouse eggs, and the resulting transgenic animals were used to produce lines of mice containing integrated copies of the chicken gene. The levels of expression of the chicken gene were quantitated in various tissues, and the response of the gene to estrogen stimulation was measured after chronic or acute estrogen exposure. Two of the three mouse lines studied maintained stable levels of expression in successive generations of offspring, and the third line had two- to threefold-higher levels in offspring than in the original parent. In the third line, the original transgenic parent was found to be a mosaic. The chicken transferrin gene was expressed at 10- to 20-fold-higher levels in liver than in any other tissue; however, the levels of chicken transferrin mRNA in kidney were higher than expected, indicating that the tissue specificity was only partial. In all three lines, the foreign gene was induced by estrogen administration. After 10 days of estrogen administration, there was a twofold increase in both transferrin mRNA and transcription of the chicken transferrin gene. A single injection of estradiol led to a fourfold increase in transferrin mRNA synthesis at 4 h. As a control the levels of mouse albumin were measured, and both the level of albumin mRNA and its rate of transcription declined about twofold after estrogen administration. Our results indicate that the intact chicken gene with 2.2 kilobases of 5′ flanking sequence contains signals for both tissue specificity and steroid regulation that can be recognized in mice.

When foreign DNA is microinjected into the pronucleus of a fertilized mouse egg, it becomes stably integrated into the mouse genome at a high frequency (3). Analysis of the expression of these foreign DNA sequences in offspring can provide insight into the tissue-specific and developmental aspects of gene regulation as well as the response to characterized inducers such as steroid hormones. Although relatively few genes have been studied by this technique, the available results suggest that tissue-specific DNA sequences exist within or near the genes they regulate and that these signals are sufficient to confer a high degree of specific expression in appropriate tissues, despite the random nature of integration into the mouse genome. Tissue-specific expression in transgenic mice has been reported for genes coding for rat elastase I (25), mouse immunoglobulin κ light chain (4), myosin light chain (24), β-globin (5, 27), α-fetoprotein (12), a proinsulin-T antigen fusion gene (10), and immunoglobulin heavy chain (9). In addition, it has been found that fusion genes constructed with the metallothionein promoter continue to respond to heavy-metal inducers (21, 22) as well as bacterial lipopolysaccharide inducers (8). Other genes, such as β-globin and α-fetoprotein, show appropriate developmental regulation in transgenic mice (12, 15, 27).

We used transgenic mice to study the tissue-specific and steroid-regulated expression of the chicken transferrin gene. In chickens, the transferrin gene is expressed in a highly tissue-specific manner in the liver and oviduct (14). Transcription of the gene is induced by estrogen in both tissues and by iron deficiency in the liver (17, 18). Our previous studies demonstrated that the chicken transferrin gene is also preferentially expressed in transgenic mouse liver, indicating at least partial recognition of tissue-specific signals between species (16). This report examines the stability of tissue-specific expression in three pedigrees of transgenic mice and demonstrates that transcription of the chicken gene is induced by estrogen in mouse liver.

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MATERIALS AND METHODS

Transgenic mice. The transgenic mouse lines carrying the chicken transferrin gene were the same as those previously described (16). They were produced by microinjection of a linear 17-kilobase (kb) *Kpn*I-*Sal*I DNA fragment containing the entire structural transferrin gene and 2.2 and 3.7 kb of 5′ and 3′ flanking sequences, respectively. Chronic estrogen treatment involved the implantation of 15-mg diethylstilbestrol pellets subcutaneously in the back. Short-term estrogen stimulation was done by direct injection of 100 μg of estradiol benzoate in corn oil. In some experiments, animals were subjected to partial hepatectomy before being given the steroid treatment.

Nucleic acid analysis. Identification of transgenic mice carrying the chicken transferrin gene was done by dot-blot analysis on samples of DNA isolated from the tail, as described previously (21). Total nucleic acid was isolated by protease K digestion followed by phenol-chloroform extraction and ethanol precipitation (17). RNA was obtained from liver by extraction with guanidine isothiocyanate and centrifugation through a CsCl gradient (7). Northern blot analysis of RNA was performed on agarose gels by using glyoxal denaturation, as described by Thomas (26). S1 nuclease mapping of the 5′ end of transferrin mRNA was performed essentially as described previously (2), with a 1.6-kb *Hin*dIII-*Eco*RI fragment spanning the initiation site previously determined with chicken oviduct RNA (6). The *Eco*RI site is in the first intron, 509 nucleotides downstream of the transcription start site, and was labeled with ^{32}P by using T4 polynucleotide kinase. Nuclear RNA was isolated from nuclei prepared with citric acid as described previously (23).

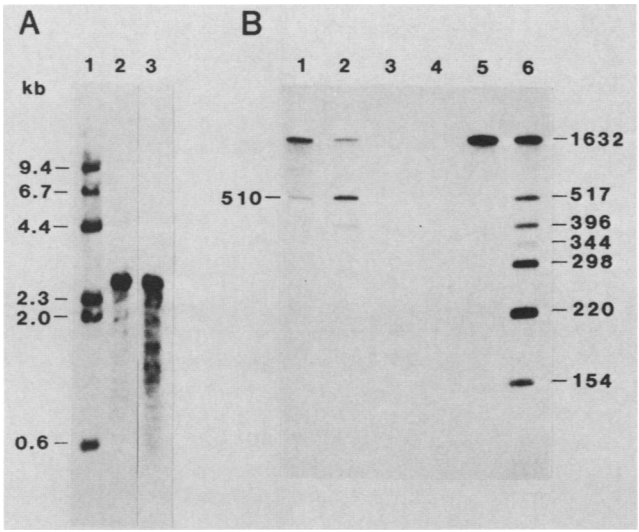

FIG. 1. Northern blot and S1 nuclease analysis of chicken transferrin mRNA in mouse liver. (A) Total RNA was isolated from the liver of a transgenic mouse from the 20-2 line, treated with glyoxal, and electrophoresed on a 1.5% agarose gel. The RNAs were transferred to nitrocellulose as described by Thomas (26) and hybridized to nick-translated cDNA sequences isolated from pCON after digestion with *Taq*I (16). Lanes: 1, ^{32}P-end-labeled *Hin*dIII fragments of λ DNA; 2, 2.4 μg of mouse liver RNA from a 20-2 mouse; 3, 300 ng of hen polysomal RNA. (B) S1 nuclease analysis of transferrin transcripts was performed as described in Materials and Methods, with a 1.6-kb fragment of the chicken transferrin gene, end labeled at an *Eco*RI site in intron A. The RNAs tested for protection of specific fragments were 100 μg of 20-2 liver nuclear RNA (lane 1); 50 μg of chicken liver nuclear RNA (lane 2); 100 μg of control mouse liver nuclear RNA (lane 3); no RNA (lane 4); undigested ^{32}P-labeled fragment (lane 5); end-labeled *Hin*fI cut pBR322 markers (lane 6). The band marked in lane 1 at 510 nucleotides is the predicted size for protection by transcripts initiated at the correct in vivo site (6).

The levels of chicken transferrin mRNA were measured by hybridization to a [^{3}H]cDNA probe as described previously (14). Levels of mouse transferrin and mouse albumin mRNAs were quantitated with a single-stranded [^{32}P]RNA probe synthesized by using an SP6 promoter and SP6 polymerase (Promega Biotec) as described previously (20). Samples of total nucleic acid were incubated with the SP6-transferrin or SP6-albumin probes in 0.6 M NaCl–20 mM Tris hydrochloride (pH 7.5)–10 mM EDTA–0.2% sodium dodecyl sulfate at 70°C for 16 h under paraffin oil. Samples were then digested with 25 μg of RNase A per ml and 200 U of RNase T1 per ml (Sigma Chemical Co., St. Louis, Mo.) in 1 ml of 0.3 M NaCl–10 mM Tris hydrochloride (pH 7.5)–5 mM EDTA–75 μg of denatured herring sperm DNA per ml at 37°C for 30 min. After digestion, the samples were precipitated by the addition of 100 μl of 100% (wt/vol) trichloroacetic acid and filtered on Whatman GF/C glass fiber filters (Whatman, Inc., Clifton, N.J.). Filters were treated with 250 μl of Soluene 350 (Packard), and the radioactivity was determined after the addition of 4 ml of Omnifluor (New England Nuclear Corp., Boston, Mass.). The number of molecules per cell was calculated by comparison to M13 standards that contained the sense strand complementary to the SP6 probe. DNA was determined by a fluorescent dye-binding assay (13) or, for single-stranded M13 DNA, by A_{260} assuming that 1 A_{260} unit is equal to 40 μg/ml.

Transcription rates. The rates of transcription were measured by using isolated nuclei in an elongation assay as described previously (19). The filters contained about 0.4 μg of either p17, which contains the entire gene for chicken transferrin (16), or p-alb, a 1.3-kb cDNA insert specific for mouse albumin in pBR322 (1). The chicken transferrin clone was generously provided by the laboratory of P. Chambon, Strasbourg, France, and the albumin cDNA clone was provided by W. Held and N. Hastie.

RESULTS

Characterization of the transcript from the chicken gene. Transgenic mice harboring the chicken transferring gene were produced as described previously by microinjecting fertilized eggs with a linear 17-kb fragment of chicken genomic DNA containing the entire transferring gene and about 2 kb of 5′ flanking sequence (16). Three of the founder mice were bred, and the transgenic offspring were used to determine the degree of tissue-specific expression in liver and other tissues. To demonstrate that the mRNA sequences being measured represented faithful transcripts of the transferrin gene, we isolated RNA from the liver of an offspring from the 20-2 line, electrophoresed the RNA on a 1.5% agarose gel, and transferred it to nitrocellulose. Figure 1A shows an autoradiogram of this Northern blot, demonstrating that the chicken transferrin mRNA synthesized in mouse liver (lane 2) is the same size (ca. 2.7 kb) as authentic chicken oviduct transferrin mRNA (lane 3). The smaller bands visible in lane 3 are degradation products present in this sample of polysomal RNA and are not seen in undegraded total RNA. Normal mouse liver RNA shows no hybridization under these conditions, indicating that the chicken and mouse transferrin RNA sequences do not cross-hybridize (unpublished data). Correct initiation at the chicken transferrin promoter was demonstrated by S1 nuclease mapping with a 5′-end-labeled 1.6-kb fragment from the chicken genomic transferrin gene and nuclear RNA isolated from 20-2 mouse liver, chicken liver, or control mouse liver. The ^{32}P-labeled *Eco*RI site is in the first intron, 509 nucleotides from the initiation site. Nuclear RNA from either chicken liver (Fig. 1B, lane 2) or 20-2 mouse liver (Fig. 1B, lane 1) protected a major fragment at 510 nucleotides, whereas no hybridization was seen with control mouse liver (lane 3) or no RNA (lane 4). This confirms that the chicken transferrin gene is correctly initiated in transgenic mice. The upper band at 1.6 kb comigrates with undigested probe (lane 5) and appears in variable amounts in protection experiments with chicken liver, 20-2 mouse liver, and chicken oviduct (data not shown); it is preferentially digested when higher levels of S1 nuclease are used and probably represents hybrid molecules containing both the transferrin transcript and the reannealed probe.

Tissue-specific expression. The levels of chicken transferrin mRNA in liver, kidney, brain, testis, and skeletal muscle from three transgenic founder mice and four of their offspring are shown in Fig. 2. The insert in panel A shows the levels of transferrin mRNA in the respective chicken tissues for comparison. The original 20-2 transgenic mouse had about 100 molecules of transferrin mRNA per cell in the liver and less than 10 molecules per cell in all other tissues examined. Offspring from this line expressed threefold-higher levels of mRNA in all tissues (Fig. 2A). This increased expression in offspring, together with our previous observation that only 22% of the 20-2 offspring carried the foreign gene (16), suggested that the original animal was a mosaic. We examined both the number of genes per cell and

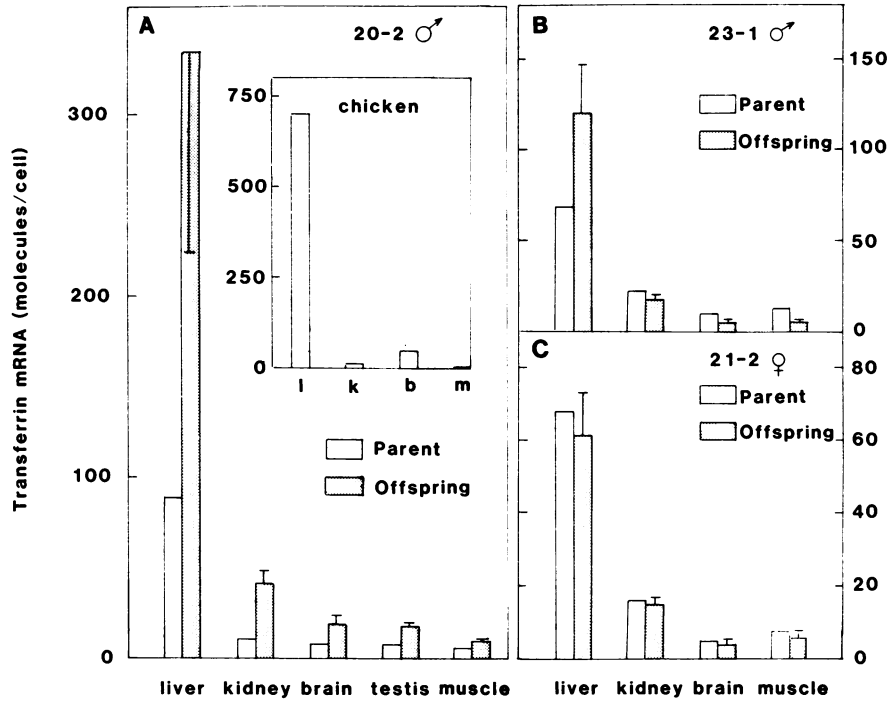

FIG. 2. Tissue specificity of transferrin expression. The levels of chicken transferrin mRNA were determined by cDNA hybridization to total nucleic acid and expressed as molecules per cell for each tissue analyzed. Four offspring from each line were assayed, and the error bar represents the standard deviation. (A) Data for the 20-2 line, with an inset showing comparable data on the expression of transferrin mRNA in chicken tissues. (B) Results with the 23-1 line. (C) Results with the 21-2 line. Only females were analyzed for the 21-2 line, since the foreign gene is X linked (unpublished data), and transgenic males were bred to obtain the offspring. Open histograms represent the data for the parent, and filled histograms represent the data for offspring. Other tissues analyzed (lung, heart, and skin) contained less chicken transferrin mRNA than did muscle.

the organization of the chicken genes in 20-2 and its offspring by Southern blotting and found that, consistent with the suggested mosaicism, the offspring had approximately 30 copies of the chicken gene per cell as compared with 14 copies for the original 20-2. The pattern of restriction fragments specific for the chicken gene did not change, although the intensity increased twofold (unpublished data).

The average level of chicken transferrin mRNA in liver from 20-2 offspring was 340 molecules per cell, compared with 10 to 15 molecules per cell in skeletal muscle and brain (Fig. 2A). We also measured chicken mRNA sequences in heart, lung, intestine, and skin tissue and found low levels similar to or less than that in muscle (unpublished data). Fig. 2B and C show the same analysis for the 23-1 and 21-2 lines, respectively. The results were similar, with approximately a 10- to 20-fold preferential expression in liver compared with brain and skeletal muscle. In all three mouse lines the kidney expressed significantly higher levels of chicken mRNA than expected on the basis of the observed levels in chicken kidney. This may indicate that the tissue-specific signals on the chicken gene are not completely recognized by mouse cells; alternatively, the kidney may normally express endogenous and hence exogenous transferrin genes in mice. To rule out the latter possibility, we examined the tissue specificity of mouse transferrin expression by using a mouse transferrin SP6 probe.The results were qualitatively similar to those shown for the endogenous chicken gene (Fig. 2A, insert). Mouse transferrin mRNA levels were 6,000 molecules per cell in liver, 20 molecules per cell in brain, and below detection in muscle and kidney. From a comparison of these data, it is clear that the expression of the chicken gene deviates from the expected tissue-specific pattern, especially in kidney.

Estrogen regulation. The stable expression of the chicken gene in offspring allowed us to test whether the signals required for estrogen regulation were present within the 17-kb DNA fragment that had been transferred into mice and whether these signals might be recognized by mouse liver cells. The induction of transferrin synthesis in liver by estrogen has been documented in rats (11) and chickens (14). We previously demonstrated that estrogen in chickens acts to produce a two- to threefold increase in the rate of transferrin mRNA synthesis and consequently the level of transferrin mRNA per cell (18).

To test the responsiveness to steroids of the chicken gene in mice, offspring from all three lines were subjected to partial hepatectomy to obtain an untreated value for chicken transferrin mRNA sequences. Half these animals were then estrogen treated by implantation with diethylstilbestrol pellets for 10 days before they were killed and the transferrin mRNA levels and transcription rates in liver were measured; the group not treated with estrogen served as controls for the surgery. The results are given in Table 1 as the ratio of treated to untreated mRNA levels or rates of transcription. The control group gave a ratio of approximately 1:1 in all three lines (data not shown), indicating little change in transferrin mRNA levels as a result of partial hepatectomy. The estrogen-treated mice showed a twofold increase in transferrin mRNA levels after the 10-day estrogen treatment in all three lines. Consistent with this increase in mRNA levels, we have also observed a twofold increase in the concentration of chicken transferrin in serum (unpublished

TABLE 1. Induction of the transferrin gene in transgenic mice after 10 days of chronic treatment with estrogen

Tissue	Transferrin mRNA (-fold increase with estrogen)	Transcription rate of transferrin mRNA (-fold increase with estrogen)
20-2 liver	2.1 ± 0.5[a]	2.1 ± 0.3
23-1 liver	2.2 ± 0.4[a]	ND[c]
21-2 liver	1.9 ± 0.5[a]	ND
Chicken liver	2.1[b]	2.0

[a] Average of eight animals ± standard deviation.
[b] Determination on tissues pooled from four animals.
[c] ND, Not determined.

data). The rate of chicken transferrin mRNA transcription was measured on liver samples from 20-2 mice by using an elongation assay in isolated nuclei (see Materials and Methods). The rate of transferrin mRNA synthesis increased about twofold in response to chronic estrogen treatment, an increase similar to that observed in estrogen-treated chickens (18; Table 1). The response of the endogenous mouse genes coding for transferrin and serum albumin were also measured by using appropriate SP6 probes. The levels of mouse transferrin mRNA were 4,000 molecules per cell in the untreated liver and induced slightly (20 to 30%) after chronic estrogen treatment. Chronic estrogen treatment caused the levels of albumin mRNA to decrease from a control value of 3×10^4 molecules per cell to 1×10^4 molecules per cell. The rate of mouse albumin mRNA transcription also decreased two- to threefold during chronic estrogen treatment (unpublished data).

Acute response to estrogen. To substantiate that the gene induction we measured after 10 days of estrogen treatment was mediated directly by the steroid, we measured the early response of the chicken transferrin gene in mice after a 4-h treatment with estradiol. In this experiment, groups of four animals were injected with either corn oil alone or 100 μg of estradiol benzoate in corn oil. The average rate of transcription from the chicken transferrin gene is shown in Fig. 3A for the control and estrogen-treated mice. We observed a fourfold increase in the rate of transcription after 4 h. In contrast, the rate of mouse albumin gene transcription decreased twofold in the same samples (Fig. 3B). Previous studies with chickens have documented an estrogen-induced decline in albumin synthesis in liver (14). Our results with the mouse albumin gene indicate that it is rapidly repressed by estrogen and that both transcription and mRNA levels decline twofold after chronic steroid treatment.

DISCUSSION

Transgenic mice provide a vehicle for studying the regulation of gene expression in normal differentiated tissues under physiological conditions. In this report we examined the tissue-specific expression and regulation of the 17-kb chicken transferrin gene in three lines of transgenic mice. It is important to point out that these three lines represent independent integrations of the foreign gene. For the 21-2 line we know from transmission data that the genes are inserted on the X chromosome (unpublished data); the other two lines have autosomal integration sites. In two of the lines, the levels of expression in selected tissues remained constant in successive generations of heterozygous offspring. Offspring from the third line (20-2) showed a twofold increase in expression compared with that of the original parent, and we determined that the 20-2 parent was a mosaic. In all three lines, the expression of chicken transferrin mRNA was 10- to 20-fold higher in liver than in either brain or skeletal muscle, indicating that tissue specificity was partially retained. However, we found a low-level expression of the chicken transferrin gene in tissues (especially kidney) that do not express their endogenous mouse transferrin sequences.

The absolute levels of expression vary somewhat in the three lines of transgenic mice analyzed in Fig. 2, and several factors may be affecting the absolute expression level. Offspring from the 20-2 line contain 30 copies of the chicken gene and have a constitutive level of about 340 molecules of mRNA per cell, whereas offspring from the 23-1 and 21-2 lines contain about 12 copies of the gene per cell and express 125 and 60 molecules of mRNA per cell, respectively. Expression in the 21-2 line may be diminished as a result of X inactivation of the chicken gene, since only female offspring were analyzed in this line. Variations in the level of expression of foreign genes in transgenic mice that cannot be explained by differences in gene copy number have also been frequently reported in other studies (12, 27).

The stable expression of the chicken gene in transgenic mice allowed us to examine whether the response of this gene to estrogen would be retained. All three lines showed a similar twofold induction of chicken transferrin mRNA after chronic estrogen stimulation, and this increase correlated with a twofold increase in the rate of transferrin gene

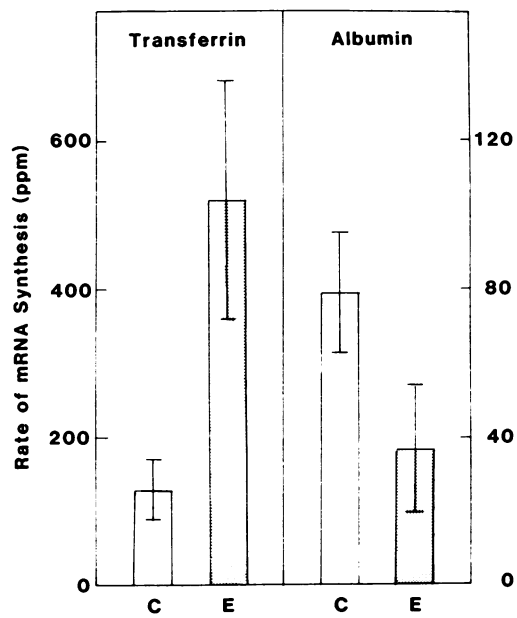

FIG. 3. Acute induction of transferrin by estrogen. Transgenic mice from the 20-2 line were injected subcutaneously with 100 μg of estradiol benzoate in corn oil. After 4 h, the liver was excised and frozen on dry ice. Transcription rates were determined by using isolated nuclei in an elongation assay, as described in Materials and Methods. The rates of both chicken transferrin and mouse albumin mRNA transcription are shown for estradiol-treated mice (E, shaded bars) and control mice injected with oil alone (C, open bars). In comparing the rates of albumin and transferrin transcription, we have not corrected for the fraction of the transcription unit that is represented in the filter-bound plasmid DNA. The chicken transferrin genomic clone contains at least 11 kb of transcribed sequence, whereas the mouse albumin clone contains only 1.3 kb of transcribed sequences.

transcription. This increase was not due to a general change in total mRNA transcription following estrogen treatment, since we could detect less than a 10% increase in α-amanitin-sensitive transcription after estrogen administration (unpublished data). In addition, the mouse albumin gene was examined as a control, and its rate of transcription decreased twofold after estrogen treatment. The inhibition of albumin mRNA synthesis was not unexpected, since estrogen has been shown to diminish the synthesis of albumin in both frogs (28) and chickens (14), although we are unaware of any data showing a decline in albumin production in mammals.

To show that estrogen was acting directly to regulate the transcription of the chicken transferrin gene, we examined the acute response by measuring transcription rates in mouse liver 4 h after a single estradiol injection. A fourfold induction of the chicken transferrin gene and a twofold decline in mouse albumin mRNA synthesis was observed. It is unclear why the acute response of the transferrin gene is greater than that seen after chronic stimulation, although it is possible that the level of hormone produced by the slow-release diethylstilbestrol pellet is suboptimal.

From the limited evidence available, we suggest that DNA control elements that determine tissue specificity, developmental regulation, and the response to inducers can function independently of the chromosomal environment when introduced into whole animals. The maintenance of both tissue specificity and estrogen responsiveness of the chicken transferrin gene in transgenic mice indicates that these control elements are highly conserved between species that diverged more than 150 million years ago.

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