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Rat transferrin gene expression: Tissue-specific regulation by iron deficiency

(intestinal and bile transferrin/isoelectric focusing/transcription rate/SP6 hybridization)

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ABSTRACT **Rats raised on a low-iron diet were used as a model system for investigating the regulation of transferrin gene expression by iron deficiency. We quantitated transferrin mRNA in a variety of tissues from normal and iron-deficient rats and found that the level of transferrin mRNA in normal rat liver was about 6500 molecules per cell, while the level in iron-deficient animals was 2.4-fold higher. The increase of transferrin mRNA in iron deficiency was the result of a specific induction of transferrin gene transcriptional activity as measured in isolated nuclei. This increase in transferrin gene expression resulted in a corresponding increase in serum total-iron-binding capacity. Of the other tissues examined, moderate amounts of transferrin mRNA were found in brain (83 molecules per cell) and testis (114 molecules per cell), and low levels were measured in spleen and kidney. The transferrin mRNA content of brain, testis, spleen, and kidney remained unchanged in iron deficiency. The small intestine had no detectable transferrin mRNA in either normal or iron-deficient rats; however, transferrin protein was present, and its level was 2-fold higher in the iron-deficient group. We hypothesize that intestinal transferrin is synthesized in the liver and is delivered to the gut via the bile. Consistent with this idea, bile transferrin content was found to be elevated in iron deficiency and appeared to be sufficient to account for intestinal transferrin levels. In addition, treatment of plasma transferrin with bile caused an acidic shift in its isoelectric-focusing behavior so that it comigrated with intestinal transferrin.**

The serum iron-binding protein transferrin plays an essential role in transporting iron between sites of absorption, storage, and utilization. It has been proposed that most, if not all, proliferating cells utilize transferrin-bound iron. This is evidenced by the widespread distribution of transferrin receptors on many different cell types and by the requirement for transferrin that many cells demonstrate *in vitro* (1, 2). Transferrin is synthesized in several different tissues, and some of these tissues modulate the rate of transferrin synthesis quite dramatically in response to a variety of different physiological factors. The major source of serum transferrin is the liver, where steroid hormones and nutritional iron deficiency stimulate its synthesis (3–8). The chicken oviduct also synthesizes transferrin (frequently referred to as conalbumin), which becomes a major constituent of the egg white. In the oviduct, transferrin mRNA content, and consequently transferrin synthesis, increases 50-fold in response to estrogen and other steroids (3, 9, 10). Transferrin is also synthesized in and secreted from the Sertoli cells of the rat testis, and it may be involved in iron transport within the seminiferous tubules. A combination of retinol, testosterone, insulin, and follicle-stimulating hormone (FSH) regulates testicular transferrin (11). In addition, transferrin synthesis has been reported in brain, bone marrow, spleen, and submaxillary gland (5, 8, 12). No regulatory factors have been identified for transferrin synthesis in these tissues.

Iron deficiency in rats leads to an increase in transferrin levels in both the serum and the small intestine (13, 14). Intestinal transferrin is found in the lumen, on the mucosal cell surface, and inside the mucosal cells of the duodenum and jejunum (14–16). The increase in intestinal transferrin is correlated with an increase in iron absorption, and this has lead to the hypothesis that transferrin may play a role in mediating iron absorption (13, 14, 17, 18). Several lines of evidence support this idea. Early studies showed that perfusion of the intestinal lumen removed an iron-binding protein and resulted in decreased iron absorption (19). Reintroduction of the eluted factor into perfused intestine restored iron absorption. Subsequently, it was found that ^{59}Fe became rapidly associated with transferrin during absorption from the intestinal lumen (17). More recently, Huebers *et al.* (20) reported that ^{59}Fe- and ^{125}I-labeled transferrin, when added to the intestinal lumen, were both taken up into the mucosal cells lining the intestine. Furthermore ^{125}I-labeled apotransferrin reappeared in the lumen after a lag of 15 min. On the basis of these data, the authors proposed a model detailing the role of transferrin in iron absorption. They suggested that the iron–transferrin complex is taken up from the intestinal lumen into the mucosal cell, presumably via receptor-mediated endocytosis. Inside the cell, iron is released and transferred to the blood stream, and apotransferrin returns to the brush border to be recycled. Therefore, any increase in intestinal transferrin should serve to increase iron absorption. Several details of this model need further study. For example, although the amount of intestinal transferrin is clearly increased in iron deficiency, its site of synthesis is unknown. Previously reported differences in amino acid composition and isoelectric points between intestinal and plasma transferrin (15) suggested that the two transferrins could have independent sites of synthesis and could even be products of different genes.

The recent cloning of a rat transferrin cDNA (J. Huggenvik and R.L.I., unpublished data) makes it possible to directly assess transferrin gene expression in specific tissues by determining transferrin mRNA levels. We describe here a quantitative hybridization assay that uses a single-stranded [^{32}P]RNA probe specific for transferrin. We used this assay to investigate the relationship between physiological iron status and transferrin mRNA levels in rat intestine, liver, and other tissues. These results are compared with transferrin protein levels in bile, intestine, and serum. In addition, the effect of bile on the isoelectric-focusing pattern of transferrin was also examined. Our data suggest that intestinal transferrin is not synthesized locally but originates in the liver and is carried in the bile to the gut.

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Abbreviations: TIBC, total-iron-binding capacity; [^{3}H]cRNA$^+$, plus-strand [^{3}H]RNA.

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MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats were raised on a standard Purina Chow diet containing 350 mg of iron per kg of body weight. To produce iron deficiency, rats were placed on a low-iron diet at the age of 6 wk and periodically bled to hasten iron depletion (21). Normal and iron-deficient animals were studied at the age of 8–12 wk. Iron overload was produced with a high-iron diet containing 1% carbonyl iron given over several months.

Iron and Transferrin Determinations. Serum iron and liver nonheme iron were determined as described (22, 23). Total-iron-binding capacity (TIBC) was performed by the method of Cook (24). To measure bile transferrin, rats were anesthetized with ether and the abdomen was opened; the bile duct was cannulated and bile was collected for 2 hr. The flow rate was ≈1 ml/hr and was unaffected by iron status. Transferrin was quantitated by immunoelectrophoresis with transferrin-specific antiserum (25). To measure mucosal transferrin, 20 cm of duodenum and upper jejunum was removed from the animal, opened, and perfused with saline. The mucosa was scraped off with a glass slide and homogenized. A nonparticulate fraction, obtained by centrifugation of the mucosal homogenate at 100,000 × *g* for 1 hr, was assayed for transferrin as above.

Nucleic Acid Preparation and Hybridization. The levels of transferrin mRNA were measured by hybridization of total nucleic acid to a single-stranded [^{32}P]RNA probe. Total nucleic acid was prepared from tissues that were either fresh or stored at −70°C. For intestinal studies, mucosal scrapings were taken as described above, and mucosal cells were obtained by mechanical vibration (26). The tissues were disrupted with a Polytron (Brinkman), digested with proteinase K in $NaDodSO_4$, extracted with phenol/chloroform, and precipitated with ethanol to isolate total nucleic acid (3). The percentage of DNA in the samples was determined by a fluorescence assay with Hoechst 33258 dye (27). The [^{32}P]RNA probe was synthesized by using phage SP6 polymerase (Promega Biotec, Madison, WI) and the pSP64 cloning vector into which a 390-base-pair rat transferrin cDNA fragment had been inserted. Details of the cloning and sequence analysis will be published separately. The linear DNA template was transcribed as described (28) except that 15 mM NaCl was added to the reaction mixture and an incubation temperature of 37°C was used. Unincorporated NTPs were removed by Sephadex G-75 chromatography. Samples of total nucleic acid were hybridized to ≈10^4 cpm of SP6–transferrin probe (specific activity, 6 × 10^5 cpm/ng) in 20 μl of 0.6 M NaCl/20 mM Tris chloride, pH 7.5/10 mM EDTA/0.2% $NaDodSO_4$ at 70°C for 16 hr under paraffin oil. Samples were then digested with 25 μg of RNase A and 200 units of RNase T1 in 1 ml of 0.3 M NaCl/10 mM Tris chloride, pH 7.5/5 mM EDTA containing 75 μg of denatured herring sperm DNA at 37°C for 30 min. After digestion, 100 μl of 100% (wt/vol) CCl_3COOH was added to precipitate the nucleic acid, and the samples were filtered on Whatman GF/C glass fiber filters, washed with 3% CCl_3COOH containing 10 g of $Na_4P_2O_7$ per liter, followed by 95% ethanol. 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). The amount of transferrin mRNA was determined by comparison to standards. Single-stranded (+-strand) DNA standards were obtained by subcloning the transferrin cDNA fragment in the appropriate orientation into phage M13. For an additional standard, a plus-strand [^{3}H]RNA ([^{3}H]cRNA$^+$) was synthesized from an SP6–transferrin template that contained the transferrin cDNA fragment in the orientation opposite to the probe template. Molecules per cell were calculated by assuming 7 pg of DNA per rat cell.

Nuclear Transcription Assays. The relative rate of transferrin gene transcription was measured by an *in vitro* elongation assay using isolated nuclei as described (4, 10). After transcription in the presence of [^{32}P]UTP, RNA was isolated and hybridized to filters containing 0.4 μg of pF10 or pAlb. pF10 and pAlb are pBR322 plasmids into which have been cloned a 1.35-kilobase rat transferrin cDNA and a 1.3-kilobase mouse albumin cDNA, respectively. The albumin cDNA clone was provided by W. Held and N. Hastie. Hybridization efficiency was measured by including the transferrin [^{3}H]cRNA$^+$ described above. There was no difference in total UTP incorporation between the normal and iron-deficient rat groups.

Isoelectric Focusing. Bile was collected from iron-deficient rats by cannulation of the bile duct. Iron-deficient plasma was tagged with ^{59}Fe and brought to the point of saturation with additional $FeSO_4$ by a spectroscopic titration technique (29). A 100-μl aliquot of this solution was added to 1 ml of either bile or H_2O, incubated for 10 min, and applied to an isoelectric-focusing system having a pH range of 5–8 as described (30). After electrophoresis, the gel was cut into 2-mm slices, and the radioactivity was measured in a γ counter.

RESULTS

Measurement of Transferrin mRNA by SP6 RNA Hybridization. Hybridization conditions similar to those previously described for solution hybridization with a cDNA probe (31) were used. Instead of a cDNA probe, however, this assay makes use of a [^{32}P]RNA probe, synthesized by using the phage SP6 promoter and polymerase. With the current technique, similar specificity and even greater sensitivity (less than one molecule per cell) can be obtained. We chose to use single-stranded transferrin DNA as a convenient standard; however, the possibility that differences in stability of DNA·RNA and RNA·RNA hybrids might affect the calculated results was investigated. For this purpose, a single-stranded transferrin [^{3}H]cRNA$^+$ was synthesized and included in initial experiments as an additional standard. Fig. 1 shows typical curves obtained from hybridization of either the DNA standard, the [^{3}H]cRNA$^+$ standard, or a sample of total nucleic acid from rat liver. Percentage hybridization of total nucleic acid samples was converted to molecules of transferrin mRNA by comparison to the standard curves. We found that the amount of calculated transferrin mRNA in a

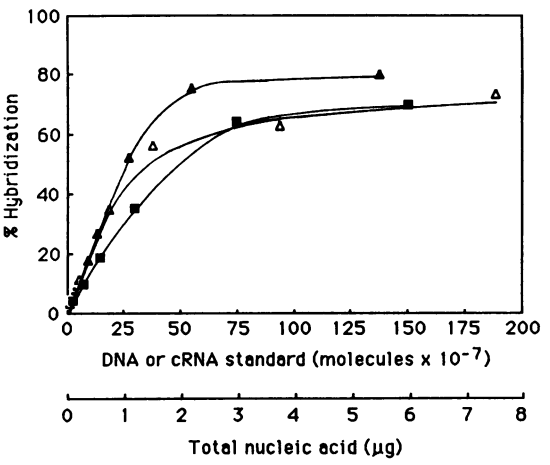

FIG. 1. Hybridization of nucleic acid to an SP6-transferrin probe. Single-stranded phage M13 DNA containing a transferrin insert (△) or transferrin cRNA$^+$ (▲) was hybridized to an SP6–transferrin probe to produce standard curves. Total nucleic acid from rat liver (■) was also hybridized.

given sample differed by <10% when either the DNA or [^{3}H]cRNA$^+$ standard curve was used, substantiating the validity of using DNA standards in this assay. In subsequent experiments, only the DNA standards were used.

Tissue Specificity of Transferrin mRNA. Total nucleic acid was isolated from six different rat tissues and assayed for transferrin mRNA as described above. The amount of transferrin mRNA in the liver (≈6500 molecules per cell) far exceeded that in any other tissue (Table 1). The brain and testis also expressed transferrin mRNA; however, they had <2% of the amount in liver. Low levels were detected in spleen and kidney. Similar relative levels of transferrin mRNA have been documented for chicken, although the absolute amounts were lower by several fold than those reported here (4, 5). In addition, we were unable to detect any transferrin mRNA in the small intestine of normal rats (Table 1). Within the intestine, transferrin protein is primarily localized in and around the mucosal cells of the duodenum, where it increases in iron deficiency (13–16). To maximize the likelihood of detecting any intestinal transferrin mRNA, we assayed samples of total nucleic acid isolated from duodenal mucosal cells from iron-deficient rats. Again, transferrin mRNA was undetectable (<2 molecules per cell). We conclude that intestinal transferrin is not locally synthesized.

Regulation of Transferrin by Iron Deficiency. Rats were raised on iron-deficient, normal, or high-iron diets in order to investigate the effects of physiological iron status on transferrin levels. Iron status of the animals was assessed by quantitating liver iron, serum iron, and serum TIBC, a measure of serum transferrin. Iron-deficient rats, in which the liver and serum iron were significantly depleted, showed an increase of about 2.5-fold in their TIBC. Iron overloading, on the other hand, resulted in large increases in liver and serum iron but had no effect on TIBC. These data are similar to those obtained in previous studies (5, 13, 26) and indicate the effectiveness with which the diets altered the iron status of the test animals.

The increase in serum TIBC seen in iron deficiency has been attributed to a stimulation of transferrin synthesis in the liver (4, 5, 8). To determine whether iron deficiency regulates transferrin synthesis by increasing the accumulation of its mRNA, we quantitated liver transferrin mRNA in iron-deficient and normal rats. Iron-deficient rat liver expressed 15,600 molecules per cell, which represents a >2-fold induction over the control level (Fig. 2*A*). This induction is similar to that previously reported in chicken liver (4, 5). Transferrin mRNA content was determined also in liver samples from iron-loaded rats; a small decrease was observed (Fig. 2*A*). Since this group of iron-loaded animals did not exhibit a change in serum TIBC (Table 2), the diminution in transferrin mRNA may not be physiologically relevant. In humans, however, iron overload is known to be associated with depressed TIBC values (32).

Table 1. Transferrin mRNA levels in normal rat tissues

Tissue	Transferrin mRNA, molecules per cell
Liver	6534 ± 376
Brain	83 ± 9
Testis	114 ± 3
Spleen	11 ± 1
Kidney	5 ± 1
Small intestine*	<3
Mucosal cells†	<2

Tissues from several groups of four untreated rats were assayed for transferrin mRNA as described. The mean ± SEM is given for a typical group.
*Mucosal scraping from the entire small intestine of normal rats.
†Obtained by mechanical vibration from the duodenum of iron-deficient rats.

Table 2. Assessment of iron status

Iron assay	Rat diet		
	Normal	Iron-deficient	Iron-loaded
Liver iron, μg/g	79 ± 5	33 ± 2	3897 ± 774
Serum iron, μg/dl	174 ± 30	30 ± 3	324 ± 38
Serum TIBC, μg/dl	388 ± 10	996 ± 25	395 ± 32

Groups of four rats were raised on normal, iron-deficient, or iron-loaded diets. The mean ± SEM for each group is given.

The possibility that nutritional iron deprivation might affect transferrin mRNA levels in other tissues was also investigated. No change in transferrin mRNA was detected in spleen, testis, brain, or kidney (Fig. 2*B*). The results in Fig. 2 show that regulation of transferrin synthesis by iron deficiency is restricted to the liver. Furthermore, a comparison of the data in Table 2 and Fig. 2 reveals that the induction of liver transferrin mRNA in iron-deficient rats is approximately equivalent to the increase in serum TIBC.

The increase in liver transferrin mRNA accumulation in response to iron deficiency could result from a change in either its rate of synthesis or degradation or a combination of the two. Therefore, we measured the relative rate of transferrin gene transcription in nuclei isolated from normal and iron-deficient rat livers. To control for the possibility that changes in iron status may nonspecifically affect RNA synthesis, we measured also albumin gene transcription. Transferrin gene activity increased from 90 ppm to 160 ppm under conditions of iron deficiency, while albumin remained unchanged (Fig. 3). Thus, the effect of iron deficiency on transcriptional activity is gene specific. Moreover, the 1.8-fold increase in transferrin gene transcription in the liver is sufficient to account for most of the observed increase in transferrin mRNA content. These results are similar to those reported in chickens (4).

The finding that mucosal cells do not synthesize transferrin mRNA was somewhat surprising in view of previous reports describing an increase in mucosal transferrin under conditions of iron deprivation (13, 14). We measured mucosal transferrin in the normal and iron-deficient rats used in this study and, as anticipated, a 2-fold increase was detected in the iron-deficient group (Fig. 4). Since intestinal transferrin is not locally synthesized, we hypothesized that it originates in the liver and is delivered to the intestine via the bile. In order

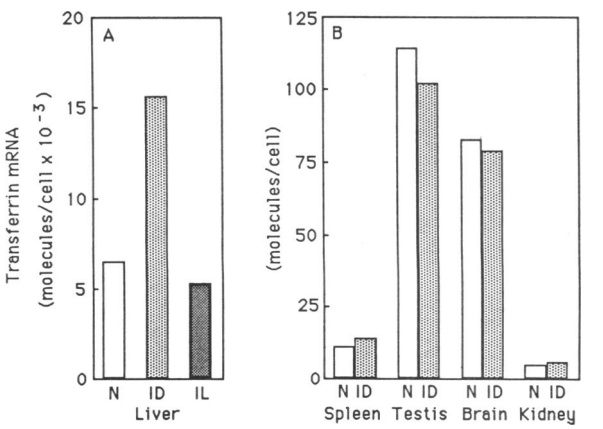

FIG. 2. Regulation of transferrin mRNA levels by iron deficiency. Transferrin mRNA was quantitated by SP6 hybridization and is expressed as molecules per cell for each tissue assayed. Four animals per group were analyzed, and the individual results were averaged. Groups: N, normal rats; ID, iron-deficient rats; and IL, iron-loaded rats.

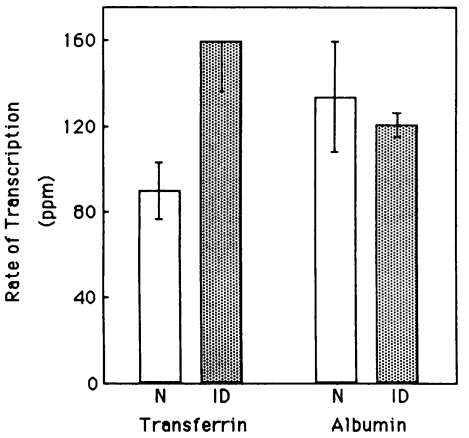

FIG. 3. Transcription rate measurements in normal (N) and iron-deficient (ID) rat livers. Nuclei were isolated from the rat livers analyzed in Fig. 2. Transcription rates for the transferrin and albumin genes were determined by *in vitro* elongation followed by filter hybridization. The results are expressed in ppm of specific gene transcription relative to total [^{32}P]UTP incorporation after correction for the hybridization efficiency, which was 20% as determined by a transferrin [^{3}H]cRNA internal standard. The error bars represent SEM. Note that the albumin cDNA used as a probe is from mouse; therefore, the reported level of albumin transcriptional activity in rat liver may be underestimated due to the highly stringent hybridization conditions used.

to test this possibility, transferrin was quantitated in bile samples from normal and iron-deficient rats. The results (Fig. 4) show that the amount of transferrin in bile increases greatly in response to iron deficiency. Over a 24-hr period, it amounts to 1.1 mg in iron-deficient rats as compared to 0.26 mg in normal rats (given an average bile flow rate of 1 ml/hr). Based on the values for mucosal transferrin in Fig. 4, it appears that intestinal transferrin could be provided solely by the bile, although a precise calculation would require knowing the rate of transferrin uptake into the mucosal cell and its half-life there. The calculation is further complicated by the apparent cycling of transferrin back into the lumen (20) and the periodic emptying of intestinal contents. These data do indicate, however, that the liver secretes transferrin into the bile and, hence, the intestine in amounts that are determined by physiological iron status.

Isoelectric Point Differences in Intestinal and Plasma Transferrin. Rat plasma transferrin has been shown to separate into two major components upon isoelectric focusing, having isoelectric points of ≈5.8 and ≈5.6 (15, 33). Transferrin obtained from the intestinal lumen exhibited a shift in isoelectric points of both isoforms to the acidic side of plasma transferrin (15). If intestinal transferrin, like plasma transferrin, were synthesized in the liver as we propose, then the shift in isoelectric points might result from exposure to bile. Indeed, the isoelectric focusing patterns in Fig. 5 reveal that the same acidic shift can be produced *in vitro* by adding bile to plasma transferrin prior to electrophoresis. We have not determined what type of modification of the plasma transferrin occurs when incubated with bile.

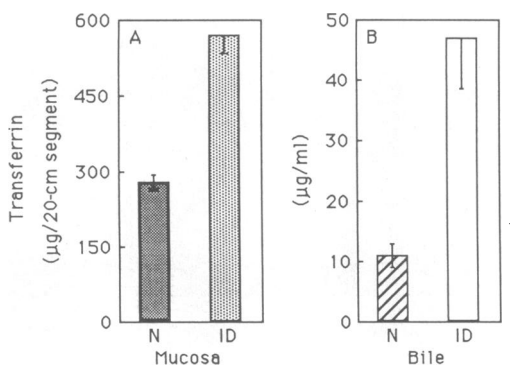

FIG. 4. Mucosal (*A*) and bile (*B*) transferrin levels in iron deficiency. The mucosa of a 20-cm segment of small intestine (starting with the duodenum and extending into the jejunum) and bile were analyzed for transferrin content in normal (N) and iron-deficient (ID) rats. Error bars represent SEM.

DISCUSSION

The transferrin gene is expressed and regulated in a highly tissue-specific manner. We report here that, although significant levels of rat transferrin mRNA are found in liver, testis, and brain, only the liver senses and responds to body stores by modulating transferrin mRNA levels. This is in contrast to the regulation of transferrin by steroid hormones, which occurs in chickens in both the oviduct and the liver (3, 4). In rats, steroids and other hormones have been shown to modulate transferrin levels in the testis as well as serum (6, 7, 11). This complex regulation of transferrin expression by various effectors in different tissues must result from specific "programming" that occurs in the tissues during differentiation. In this regard, a recent report describing the relative amounts of transferrin mRNA in rat fetal tissues is intriguing. Levin *et al.* (34) found that, whereas fetal liver expresses the highest level of transferrin mRNA, both spleen and kidney have significant amounts. Interestingly, transferrin mRNA is present in fetal mouse intestine, increasing sharply 1 day prior to birth and then plummeting within 12 hr after birth (G. Plowman and R.L.I., unpublished data). The role of extrahepatic transferrin synthesis in fetal development is unknown.

The regulation of transferrin protein levels in the upper small intestine by iron deficiency has been well documented (13, 14, 20), and this led us to assay duodenal mucosal cells from iron-deficient rats for the presence of transferrin mRNA. We were unable to detect transferrin mRNA in any region of the small intestine and conclude that intestinal transferrin must be synthesized elsewhere. The finding that the amount of transferrin in bile is regulated by iron status (Fig. 4) and is probably sufficient to account for intestinal transferrin suggests the liver as a source. It has been reported (15) that the isoelectric points and amino acid composition of the two isoforms of plasma transferrin differed from the corresponding isoforms of transferrin isolated from intestinal mucosa. The amino acid differences are minor and probably

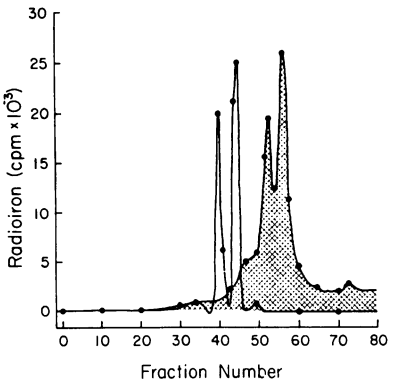

FIG. 5. Effect of bile on the isoelectric focusing pattern of transferrin. Iron-deficient plasma containing ^{59}Fe-labeled diferric transferrin was electrophoresed in an isoelectric-focusing system with (open tracing) or without (cross-hatched tracing) prior incubation with bile. The pH increases from left to right over the range of 5–8.

can be attributed to error inherent to the method of analysis. We show in Fig. 5 that treating the plasma isotransferrins with bile results in a shift in isoelectric points so that they comigrate with mucosal isotransferrins. This finding is consistent with the postulate that mucosal transferrin, like serum transferrin, originates in the liver and that exposure to bile results in the altered isoelectric points of mucosal transferrin. Furthermore, Southern blot analysis of rat genomic DNA indicates that there is a single copy of the transferrin gene per haploid genome (J. Huggenvik, personal communication), substantiating that mucosal and serum transferrin must be encoded by the same gene.

Transcription rate measurements, performed with isolated liver nuclei, demonstrate that the increase in liver transferrin synthesis in iron deficiency results from a specific induction of transferrin gene activity (Fig. 3). The molecular aspects of transferrin gene regulation by iron deficiency are as yet undefined, although other examples of transcriptional regulation by metals are known. Cadmium and zinc induce transcription of the metallothionein genes in mouse liver. Because this response has been reproduced in cell culture, it is clear that the metals act directly on the liver cell to stimulate gene expression without the involvement of other tissues (35, 36). On the molecular level, recent studies have shown that heavy-metal induction of the metallothionein I gene involves multiple "metal-response elements" that reside within 200 base pairs upstream of the transcriptional unit (37). Since it has not yet been demonstrated that isolated hepatocytes can regulate transferrin synthesis in response to iron availability, it is possible that nutritional iron deficiency triggers the release of a hormonal signal from another tissue, which then affects transferrin expression in the liver. Whether the effect of iron deficiency on the liver is direct or indirect, it almost certainly involves a liver-specific component, since the other tissues that synthesize transferrin are not affected by iron deficiency. It is also possible that transferrin-synthesizing tissues such as brain and testes are in sanctuary areas that are protected from general-body iron depletion. Further study is needed to characterize the molecular events that regulate the tissue-specific expression of the transferrin gene and its induction by iron deficiency.

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