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Expression from the Transferrin Gene Promoter in Transgenic Mice

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Transferrin is an iron-binding protein that is expressed as a major product in liver and secreted into the plasma. To study the tissue-specific regulatory regions of this gene, the genomic mouse transferrin (mTf) gene was cloned and characterized by partial sequence analysis and S1 nuclease mapping of the transcriptional start site. Fusion genes containing the transferrin gene promoter and 5′-flanking sequences were ligated to the human growth hormone (hGH) gene and used to produce transgenic mice. A deletion construct containing the −581 to +50 region of the transferrin gene was sufficient to direct a high level of liver-specific expression resembling endogenous transferrin gene expression. Deletion to −139 base pairs of 5′-flanking sequence gave a construct which retained liver specificity, but the magnitude of expression decreased severalfold. These results demonstrate the presence of a liver-specific transcriptional element between −139 and +50 and suggest the presence of a distal element between −581 and −139 that can further increase expression. Surprisingly, fusion constructs containing −3 kilobase pairs (kb) of 5′-flanking sequence gave higher levels of mRNA in nonhepatic tissues than did either the −581 or −139 construct. Further studies indicated that the high levels of circulating hGH in these transgenic mice specifically induced the endogenous transferrin and albumin genes in liver and also stimulated the normally low levels of expression of the endogenous transferrin gene in brain, heart, kidney, and muscle. A mutated hGH gene that does not produce active growth hormone was fused to the −3- to +50-kb transferrin sequences to produce the −3-kb mTf-hGX construct. A liver-specific pattern of expression was observed in transgenic mice harboring the −3-kb mTf-hGX construct, and this mutated transgene was shown to be induced four- to sevenfold by either bovine or human growth hormone. These results demonstrate the presence of a growth hormone-responsive element between −3 and +50 kb in the 5′-flanking region of the mTf gene promoter.

Transferrin is an essential iron-binding protein that is responsible for transporting iron throughout the body. The major site of transferrin synthesis and the principal source of serum transferrin is the liver, although other tissues also produce transferrin in significant quantities. The tissue specificity of transferrin gene expression has been quantified in several species. In rats, for example, transferrin mRNA accumulates to a level of 6,500 molecules per cell in liver and roughly 100 molecules per cell in brain and testis, whereas most other tissues have less than 12 molecules per cell (18). Transferrin gene expression has also been studied during fetal development, revealing a pattern of expression that deviates dramatically from the adult pattern. Significant levels of transferrin mRNA are found in fetal rat lung, kidney, and intestine in addition to liver, whereas the fetal brain transferrin mRNA level is much lower than in the adult (22).

The transferrin gene is induced by a diversity of factors, again in a tissue-specific fashion. In rats (18) and chickens (25), nutritional iron deficiency has been shown to cause a specific induction of transferrin gene transcription in liver, resulting in a severalfold increase in transferrin synthesis. Glucocorticoids and estrogen also stimulate hepatic transferrin synthesis. In chickens, these hormones are known to act at the transcriptional level to produce a roughly twofold increase in transferrin mRNA. Furthermore, estrogen and iron deficiency have an additive effect on hepatic transferrin gene expression. The transferrin gene is also highly inducible in mouse mammary gland during lactation, although the factors regulating this response have not been identified (7, 21).

One of the most powerful techniques for studying tissue-specific and developmental gene regulation relies on transgenic mice. Successful expression of foreign genes in mice has provided an in vivo method for identifying *cis*-acting DNA-regulatory elements. Correct tissue-specific and developmental expression has been obtained with a number of transgenes (5), and for some of these the critical DNA sequences have been localized. For example, multiple enhancerlike elements found within 7 kilobase pairs (kb) upstream of the promoter direct the expression of the α-fetoprotein gene to fetal liver and yolk sac (12), while an enhancerlike element located between 8.5 and 10.4 kb upstream of the albumin gene promoter appears to be important for liver-specific expression of the gene (33). One of the best-characterized tissue-specific regulatory elements is the 134-base-pair (bp) enhancer, located 5′ of the rat elastase I gene, that directs correct cell-specific expression in the adult as well as appropriate temporal expression in the developing exocrine pancreas (13). Transgenic mice have also been used to study inducible promoters such as the metallothionein gene promoter, which responds to heavy metals (31), the immunoglobulin gene promoter, which is induced by bacterial lipopolysaccharide (40), and the chicken transferrin gene

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promoter, which can be induced by estrogen in transgenic mice (11).

Since transferrin plays a central role in iron metabolism and is regulated by multiple factors in several different tissues, it represents an excellent model for studying genes that exhibit complicated patterns of expression. In previous experiments on the expression of the chicken transferrin gene in transgenic mice, we found that whereas chicken transferrin mRNA was preferentially expressed in mouse liver and was correctly induced by estrogen, the quantitative expression of the chicken gene in mouse liver was about 10-fold lower than expected (11, 24). Thus, to characterize the gene control elements that contribute to the timing, cell type specificity, and inducibility of transferrin expression in transgenic mice, we have cloned and characterized the genomic mouse transferrin (mTf) gene. Using the human growth hormone (hGH) structural gene as a reporter sequence, we have constructed fusion genes to examine the regulatory properties of the mTf gene promoter in transgenic mice, focusing on tissue-specific expression in liver.

MATERIALS AND METHODS

Screening of a mouse genomic library. An amplified λ Charon 4A library containing partial *Hae*III-*Alu*I digestion fragments of BALB/c mouse genomic DNA flanked by artificial *Eco*RI sites (a gift of U. Storb, University of Washington; 27) was plated to yield confluent plaques. Plaques were lifted and fixed to nitrocellulose filters by standard techniques and then hybridized sequentially to ^{32}P-labeled nick-translated probes to identify transferrin-containing clones. The probes used were a 0.55-kb *Pst*I-*Eco*RI rat transferrin cDNA fragment (17) and a 0.35-kb *Pvu*II-*Pst*I mTf cDNA fragment (from p6mTf, a 0.55-kb 3′-terminal clone that was identified by cross-hybridization to the rat transferrin cDNA and was a generous gift from J. Darnell, Rockefeller University). To obtain the 5′ end of the gene, another screening was performed, using a nick-translated 0.68-kb *Eco*RI-*Xba*I mTf genomic fragment spanning exon 2 that was isolated from a clone identified in the initial screenings.

Characterization of transferrin clones. Restriction maps were constructed of the bacteriophage clones containing transferrin gene sequences. To orient the 5′ and 3′ termini of the gene, the three probes described above were individually hybridized to Southern blots (37) of restriction enzyme digests of the genomic clones. The specific restriction fragments encompassing the first two exons of the gene were identified by using two different 18-nucleotide oligomers as hybridization probes. The sequences of the oligomers were based on published 5′-terminal rat transferrin cDNA sequence data (1). The probes, labeled with T4 polynucleotide kinase and [^{32}P]ATP, were hybridized to the filters at 30°C in 0.8 M NaCl–80 mM sodium citrate (pH 7)–4.5× Denhardt reagent–0.05% sodium pyrophosphate–100 μg of herring sperm DNA per ml–20 μg of yeast RNA per ml–2 × 10^6 cpm of probe per ml. Filters were washed at 39°C in 0.9 M NaCl–90 mM sodium citrate (pH 7)–0.1% sodium pyrophosphate and subjected to autoradiography. Selected regions of the transferrin clones were subcloned into M13 and sequenced by the dideoxy-chain termination method (26, 35), using [^{35}S]dATP (Dupont, NEN Research Products).

S1 nuclease mapping. S1 nuclease mapping was used to identify the transcriptional start site of the transferrin gene essentially as described previously (2). Nuclear RNA was isolated from BALB/c mouse liver nuclei prepared with citric acid (34), and a 5-μg sample was hybridized to a 0.8-kb *Hin*dIII-*Bam*HI transferrin genomic fragment that spans exon 1, with the *Bam*HI site lying in the first intron. The fragment was labeled at the *Bam*HI site with [^{32}P]ATP and T4 polynucleotide kinase. After hybridization, samples were treated with S1 nuclease, ethanol precipitated, suspended in 5 μl of formamide, and then electrophoresed through a 6% polyacrylamide sequencing gel. M13mp10 dideoxy-sequencing reactions were run alongside for size standards.

Construction of the mTf-hGH fusion genes and production of transgenic mice. A 3.55-kb *Eco*RI fragment containing exon 1, part of intron A, and 3 kb of 5′-flanking sequence of the mTf gene was subcloned into pUC13 and then cleaved at the *Bam*HI site located within intron A. The resulting linear plasmid was treated with exonuclease III and S1 nuclease (15) to produce a cluster of deletion breakpoints situated just 5′ of the ATG initiating methionine codon within exon 1. *Bam*HI linkers were ligated to generate an artificial *Bam*HI restriction site, which was used to fuse the shortened *Eco*RI-*Bam*HI mTf gene fragment to a 2.2-kb *Bam*HI-*Eco*RI fragment of the hGH gene (36), making −3-kb mTf-hGH. The fusion occurred at +50 in exon 1 of the transferrin gene as ascertained by sequence analysis. Truncated fusion genes were prepared by cleaving the transferrin gene promoter at a *Hin*dIII site (−581 mTf-hGH) or at a *Bcl*I site (−139 mTf-hGH) (see Fig. 3). A mutated version of the hGH gene in which the *Bgl*II site in exon 5 had been filled in to cause a frameshift affecting the carboxyl-terminal 55 amino acids was used to produce −3-kb mTf-hGX. The polypeptide synthesized from this construct should have no growth hormone activity, and as expected, none of the animals expressing this construct grew larger than nontransgenic littermates. For production of transgenic mice, fusion genes were isolated by restriction digestion and separation on low-melting-point agarose gels to remove all vector sequences. DNA fragments were extracted from agarose, precipitated from 2 M ammonium acetate with isopropanol, and then microinjected into C57 × SJL/F2 mouse eggs to produce transgenic mice (3, 4). Transgene copy number was ascertained by dot blot analysis, using a nick-translated 2.2-kb *Bam*HI-*Eco*RI hGH gene fragment to probe samples of DNA isolated from mouse tail (31).

Quantitation of mRNA levels. The assay method for quantitating mRNA levels has been described in detail previously (18). Briefly, total nucleic acid was isolated from the specified tissues and then hybridized in solution to a single-stranded ^{32}P-labeled RNA probe. After hybridization, samples were treated with RNase to digest unhybridized probe; the nucleic acid was precipitated with trichloroacetic acid and collected on Whatman GF/C glass fiber filters, and radioactivity was determined. RNA probes were synthesized from DNA templates obtained by subcloning cDNA or genomic fragments into appropriate SP6 vectors. The fragments used include a 1.3-kb mouse albumin cDNA (from W. Held and N. Hastie), the 0.35-kb *Pvu*II-*Pst*I mTf cDNA fragment described above, and a 0.3-kb *Ssp*I-*Bgl*II hGH gene fragment. The hGH probe did not cross-hybridize to any normal mouse transcripts (data not shown). Probes for protein kinase RIα and Cα subunits are described elsewhere (39). The mRNA content in molecules per cell was calculated from a standard concentration curve.

Hybridization with a 5′-end-labeled oligonucleotide (5′-AGATCGATCTGCCCAGTCCGG-3′) specific for hGX (which encodes a mutant hGH peptide) was used to detect the mutant hGX mRNA in crosses between hGX and hGH animals. The oligonucleotide was hybridized with liver total

nucleic acid at 45°C for 16 h, followed by S1 nuclease digestion as described previously (30). Standards of M13 DNA complementary to the oligonucleotide were used to allow quantitation of the hybridization data as molecules per cell.

Northern (RNA) blot analysis. Total nucleic acid was isolated from specified tissues of −581 mTf-hGH transgenic mouse 76-6, using sodium dodecyl sulfate, proteinase K, and phenol-chloroform extraction (18), followed by precipitation of RNA with an equal volume of 4 M LiCl. A 10-μg sample of each was heated at 60°C for 5 min in running buffer (6.6% formaldehyde, 20 mM morpholinepropanesulfonic acid [MOPS; pH 7], 1 mM EDTA, 5 mM sodium acetate) with 50% formamide and then electrophoresed through a 1% agarose gel in running buffer (16). The RNA was transferred to nitrocellulose and hybridized to a nick-translated 2.2-kb hGH gene fragment. After autoradiography, the filter was rehybridized to the nick-translated 0.35-kb mTf cDNA fragment.

Primer extension. The total nucleic acid samples used for mRNA quantitation were treated with DNase I to obtain RNA and then hybridized to a ^{32}P-end-labeled 26-nucleotide oligomer that is complementary to a sequence in exon 1 of the hGH gene (positions 29 to 54). After extension with avian myeloblastosis virus reverse transcriptase, the samples were electrophoresed under denaturing conditions on a 10% polyacrylamide gel alongside ^{32}P-labeled size markers (1-kb ladder from Bethesda Research Laboratories, Inc.) and then subjected to autoradiography (38).

RESULTS

Isolation and characterization of the mTf gene. An amplified mouse genomic library cloned in λ Charon 4A was screened with two different probes: a 3′-terminal 0.35-kb fragment of mTf cDNA and a 0.55-kb cDNA fragment corresponding to the central region of rat transferrin mRNA. Of approximately 10^6 recombinant plaques, eight distinct clones were detected, purified, and restriction mapped. To obtain the 5′ terminus of the transferrin gene, it was necessary to rescreen the library with a DNA fragment isolated from the 5′-most genomic clone. This screening produced clone 24 (Fig. 1A), which was found to contain exon 1 plus 3 kb of upstream DNA. In all, 36 kb of genomic DNA was restriction mapped; three representative clones spanning this distance are shown in Fig. 1A.

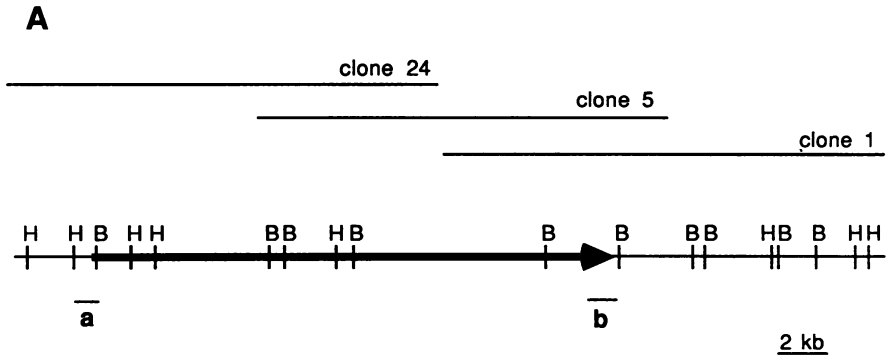
By using cDNA and oligonucleotide probes, the transferrin gene was oriented on the map, and the restriction fragments containing the first and last exons were identified and sequenced. The sequences of these two exons, their intron boundaries, and 581 bp of 5′-flanking sequence are shown in Fig. 1B. The intron-exon boundaries correspond to those determined for the chicken transferrin gene (8, 19) and conform to the published consensus sequences of splice junctions (28). Clones from the human transferrin gene have been obtained and sequenced (23, 32); of the intron positions reported, all are identical to those of the chicken gene and to the 5′ and 3′ introns described here for the mouse gene.

Inspection of the sequence presented in Fig. 1B reveals the presence of a TATA box at position −31, several potential CCAAT-box homologies, and a remarkable 45-bp span of alternating GT starting at −241. A computer-assisted comparison of the 5′-flanking region with the published human and chicken transferrin genomic sequences revealed several regions of high homology centered around −100. The potential significance of these homologous regions is discussed later.

The transcriptional initiation site of the transferrin gene was directly mapped by using an S1 nuclease protection method. Nuclear RNA from mouse liver was hybridized to a *Hin*dIII-*Bam*HI fragment (fragment a in Fig. 1A) that was labeled at the *Bam*HI site in intron A. This approach ensures that only nuclear precursors containing an unprocessed intron A will be detected in the assay. After S1 nuclease treatment, the sample was electrophoresed alongside M13 sequencing reactions in a polyacrylamide gel in order to obtain the size of the protected fragment. A single band of 231 nucleotides was detected (Fig. 2, lane 2). The intact probe was loaded in lane 1. This result indicates that the initiation site of transferrin gene transcription is 54 bp upstream of the start site of translation. The 3′ end of the mTf gene is not precisely known, although a putative polyadenylation signal sequence is located 144 bp downstream of the termination codon. Thus, the total mTf gene transcription unit is approximately 22 kb in length.

Construction of mTf-hGH fusion genes. To study the expression and regulation of the mTf gene promoter in transgenic mice, mTf-hGH fusion genes (diagrammed in Fig. 3) were prepared. The construct −3-kb mTf-hGH contains 3 kb of mouse genomic sequence 5′ of and including the transcriptional start site of the mTf gene and extending to an artificial *Bam*HI site placed at position +50 in exon 1. This sequence is fused to the hGH gene at a unique *Bam*HI site 62 nucleotides upstream of the initiator methionine. The truncated construct, −581 mTf-hGH, was produced by removing all mouse sequences upstream of the *Hin*dIII site at position −581, and −139 mTf-hGH was constructed by deletion to a *Bcl*I site at position −139.

Transcripts of the mTf-hGH transgenes are correctly initiated and processed in transgenic mice. Transcription of the mTf-hGH transgenes results in production of hybrid mRNAs that encode normal human growth hormone protein; as a result of the growth hormone production, expressing animals grew larger than normal littermates. To routinely monitor expression, transgene mRNA levels were quantified in a variety of tissues by using an hGH riboprobe that does not cross-hybridize with mouse growth hormone. However, quantitation of mRNA with riboprobes, although sensitive and accurate, does not ensure that the sequences measured are correctly initiated and processed. Possible problems with random initiation or readthrough from tandem arrays of transgenes could lead to a misinterpretation of specific promoter effects. We therefore analyzed several transgenic mice by primer extension and Northern blot analyses to demonstrate that the transgene mRNA sequences quantified were correctly initiated and processed. Primer extension with 5 μg of liver RNA or 50 μg of RNA from six other tissues obtained from a transgenic mouse carrying the −3-kb mTf-hGH construct all yielded the expected 102-nucleotide fragment (Fig. 4A), indicating correct initiation within the transferrin gene promoter. A representative Northern blot of one of the −581 mTf-hGH transgenic lines (Fig. 4B) demonstrated that the mRNA sequences were of the predicted size (1.2 kb), indicating that the transgene was accurately processed and expressed in liver. As a control, the endogenous transferrin mRNA was assayed on the same Northern blot and gave a 2.4-kb mRNA in liver and mammary gland, with undetectable expression in other tissues. Although the endogenous transferrin gene was highly expressed in the transgenic mammary gland, the transgene was not active in this tissue (Fig. 4B). The endogenous transferrin gene in virgin female mice expressing the hGH transgene was induced approximately 50-fold, and the mammary gland un-



B

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-581
   AAGCTTCCAG AGCAGGCCAG GCCTGGCAGG GATAAGAGGG AGGGAGAGCT GCCCCCGGAC TGTTGCAGGG ATCATGGCGT

   GCTGCCTCTC TGACCTGAGC CAAGTTTCCC AGTTAGGTTC CTTCTCCGGT TCTAGTCTGC CGTCCTAGGC AGTTTCCCTT

   CCGCTCAAGC CTCAGTCTCT CCCCCGTATA AGGTTGATGA AGCTGTTGGC AAGTAGAAGC CCTGGCACCC GGGAGCAAGC

   CACTCCCGCC CAAGCAGCTG TACCATGCTG CTCCAGGCAG GTGATAACAA AGAGTGGGTT CTGCTTTTAG CTTCCTTCTA

   AACACAGGCT TTTTGAGTGA GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGATTGT CGTGGTAGTG

   GATTCCATGC GCAGTTCTGT CCCACAGCTC AGGAAATGAG GTGATCATCA GGGCAAGAAA GGAAGGGGGT GGATGCGGGG

   TGATTGGGCA ATTGGACTGC GCAAACACAA GAGGTCAAAG ATTGCGCCCA GCCGGCCCCC TCCCTCCGTG CATAAAGGCG
                         +1
   AGCGGCGCAC GGGCAGCGTG CGCAGAAGCG GGTCGGTCTG TACTCCCCGC TCCTCGCCAC ACACACACCG AGAGG ATG
                                                                                    Met

   AGG CTC ACC GTG GGT GCC CTG CTG GCC TGC GCT GCC CTG G GTGAGT-Intron A.....
   Arg Leu Thr Val Gly Ala Leu Leu Ala Cys Ala Ala Leu .

   ........Final Intron-TCCCAG GA CTC CTG GAA GCC TGC ACT TTC CAC AAA CAT TAA   AATCCAAGAG
                               .. Leu Leu Glu Ala Cys Thr Phe His Lys His term

   GTGGGTTGCC ACTGTGGTGG AGACAGATGC TCCCTCCCGT GCCCATGGGC TTCTCTTGGT CTTCATGCCT GAGGGGTTGG

   GGCTAACTGG TGTAGTCTTC GCTGCTGTGC CTTACCACAT ACACAGAGCA CAAAATAAAA ACGACTGCTG ACTTTATATT

   TCCCAAACTC CATTCTTTTC TAGA
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FIG. 1. (A) Composite restriction map of genomic clones comprising the mTf gene. *Hin*dIII (H) and *Bam*HI (B) restriction sites were mapped on transferrin-containing recombinant λ clones. Three representative clone inserts, 24, 5, and 1, are shown. The bold arrow denotes the mTf gene transcription unit. (B) Partial sequence data. The nucleic acid sequences of regions a and b (panel A), encompassing the first and last exons and flanking DNA, are shown. +1 marks the transcriptional start site. The TATA box and putative polyadenylation signal are underlined. The amino acid sequence was derived from the nucleotide sequence.

derwent morphologic changes characteristic of lactation (data not shown), presumably because of the known lactogenic effects of hGH (10). The −581 mTf-hGH construct appears to be missing the mammary gland-specific signals, since this construct was not expressed at a high level in the hGH-stimulated mammary gland (Fig. 4B).

Tissue specificity of mTf-hGH expression. The results of quantitative riboprobe hybridizations on the various lines of transgenic mice are depicted in Fig. 5, in which the mRNA levels in brain, kidney, heart, and other tissues are compared as a percentage of the liver value for each animal. Either the founder or an F_1 offspring from each line was used to determine tissue specificity. Although the absolute level of expression in liver varied greatly between animals, all animals containing at least one copy of the transgene per cell expressed growth hormone mRNA. The −3-kb mTf-hGH construct gave the highest levels of expression in liver, with an average of 12,200 molecules per cell, and also showed considerable expression in brain, kidney, and heart (Fig. 5A). The −3-kb mTf-hGH construct was truncated at a *Hin*dIII site to yield the −581 mTf-hGH construct, and this transgene exhibited high expression in liver and much less nonhepatic expression than did the −3-kb mTf-hGH construct (Fig. 5B). A further truncation to a *Bcl*I site at −139 was tested in transgenic mice, and the results for the three animals obtained (Fig. 5D) demonstrated expression only in liver. In each panel of Fig. 5 the average value for liver expression is shown, and the range is given in the figure legend. It is apparent that the shorter −581 mTf-hGH construct was not expressed as highly in liver as was −3-kb mTf-hGH. The −139 mTf-hGH construct was expressed at a lower level in liver (average of 535 molecules per cell) but gave essentially no expression in nonhepatic tissues. These results indicate that regulatory elements within 139 bp of the transcription initiation site on the transferrin gene are sufficient to confer liver-specific expression and suggest that sequences upstream of −139 can further enhance expression. Since we did not observe a direct correspondence

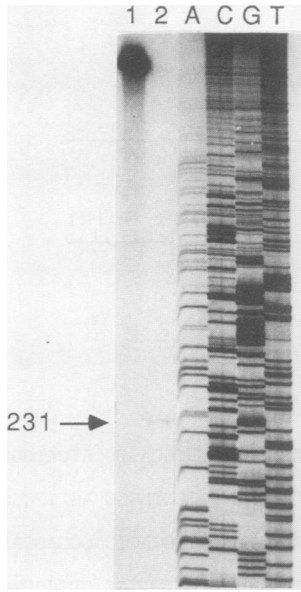

FIG. 2. S1 nuclease analysis of the mTf gene. S1 nuclease mapping of the transcriptional start site was performed with mouse liver nuclear RNA and a 0.8-kb *Hin*dIII-*Bam*HI genomic fragment (fragment a in Fig. 1A) that was end labeled at the *Bam*HI site in intron A. After S1 nuclease digestion, the sample was electrophoresed on a sequencing acrylamide gel along with M13 sequencing reactions for size standards. Lanes: 1, undigested probe; 2, protected fragment; A, C, G, and T, M13 sequencing reactions. Arrow points to protected band in lane 2 at 231 nucleotides.

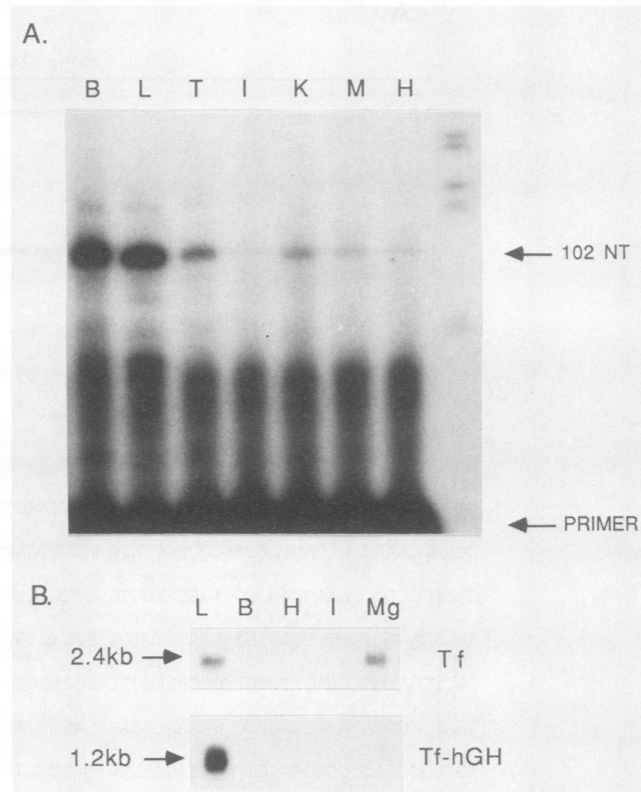

FIG. 4. (A) Primer extension analysis of −3-kb mTf-hGH transgene expression in mouse 69-8. Total RNA was prepared from brain (B), liver (L), testis (T), intestine (I), kidney (K), skeletal muscle (M), and heart (H), and 5 μg of liver RNA or 50 μg of RNA from other tissues was hybridized to a primer as described in Materials and Methods. After extension, samples were electrophoresed alongside ^{32}P-labeled 1-kb ladder size markers (right-most lane) on a polyacrylamide gel. Arrows point to free oligonucleotide primer and the expected 102-nucleotide (NT) fragment. (B) Northern blot analysis of tissues from −581 mTf-hGH transgenic mouse 76-6. RNA was isolated from liver (L), brain (B), heart (H), intestine (I), and mammary gland (Mg), electrophoresed on a formaldehyde–1% agarose gel, transferred to nitrocellulose, and hybridized sequentially to probes for mTf mRNA (Tf) and mTf-hGH transgene mRNA (Tf-hGH).

between transgene copy number and liver expression (see legend to Fig. 5 for data) for any of the constructs, it is likely that expression was being influenced by neighboring sequences at the site of each independent insertion into the genome.

Effects of hGH on mTf gene expression. The endogenous mTf gene was also monitored in both transgenic and control littermates. Surprisingly, we found that the endogenous mTf gene was induced in transgenic liver from about 5,000 molecules per cell in controls to 12,000 molecules per cell in transgenic hGH-producing animals (Table 1). Another liver-specific mRNA, albumin, was induced about 2.5-fold in liver, whereas the constitutively active RIα and Cα genes of cyclic AMP-dependent protein kinase remained constant

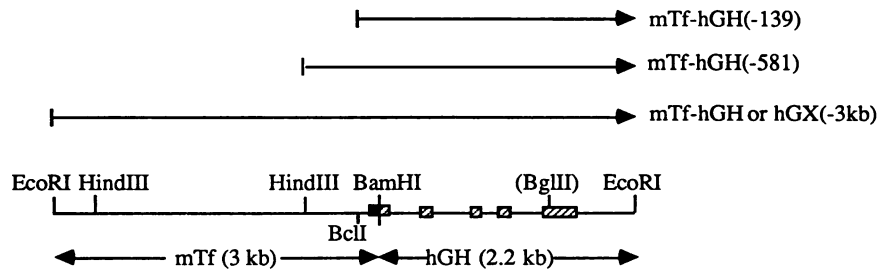

FIG. 3. mTf-hGH fusion gene constructs. A *Bam*HI site was introduced at position +50 in the first exon of the mTf gene by using exonuclease III, followed by ligation of *Bam*HI linkers. The 5′ end of the mTf gene was joined to the hGH gene by ligating this synthetic *Bam*HI site to the *Bam*HI site contained within exon 1 of the hGH gene. The initiating methionine is encoded 3′ to the site of fusion. The fusion gene, −3-kb mTf-hGH, is contained within a 5.2-kb *Eco*RI fragment. The 2.8-kb *Hin*dIII-*Eco*RI fragment comprises a truncated fusion gene, −581 mTf-hGH; it contains only 581 bp of 5′-flanking mTf gene sequence. The −139 mTf-hGH construct was truncated to a *Bcl*I site at position −139 in the transferrin gene promoter. The -3-kb mTf-hGX construct was produced by using an hGH gene in which the *Bgl*II site in the last exon (shown in parentheses) had been filled in to create a frameshift mutation disrupting the synthesis of active growth hormone.

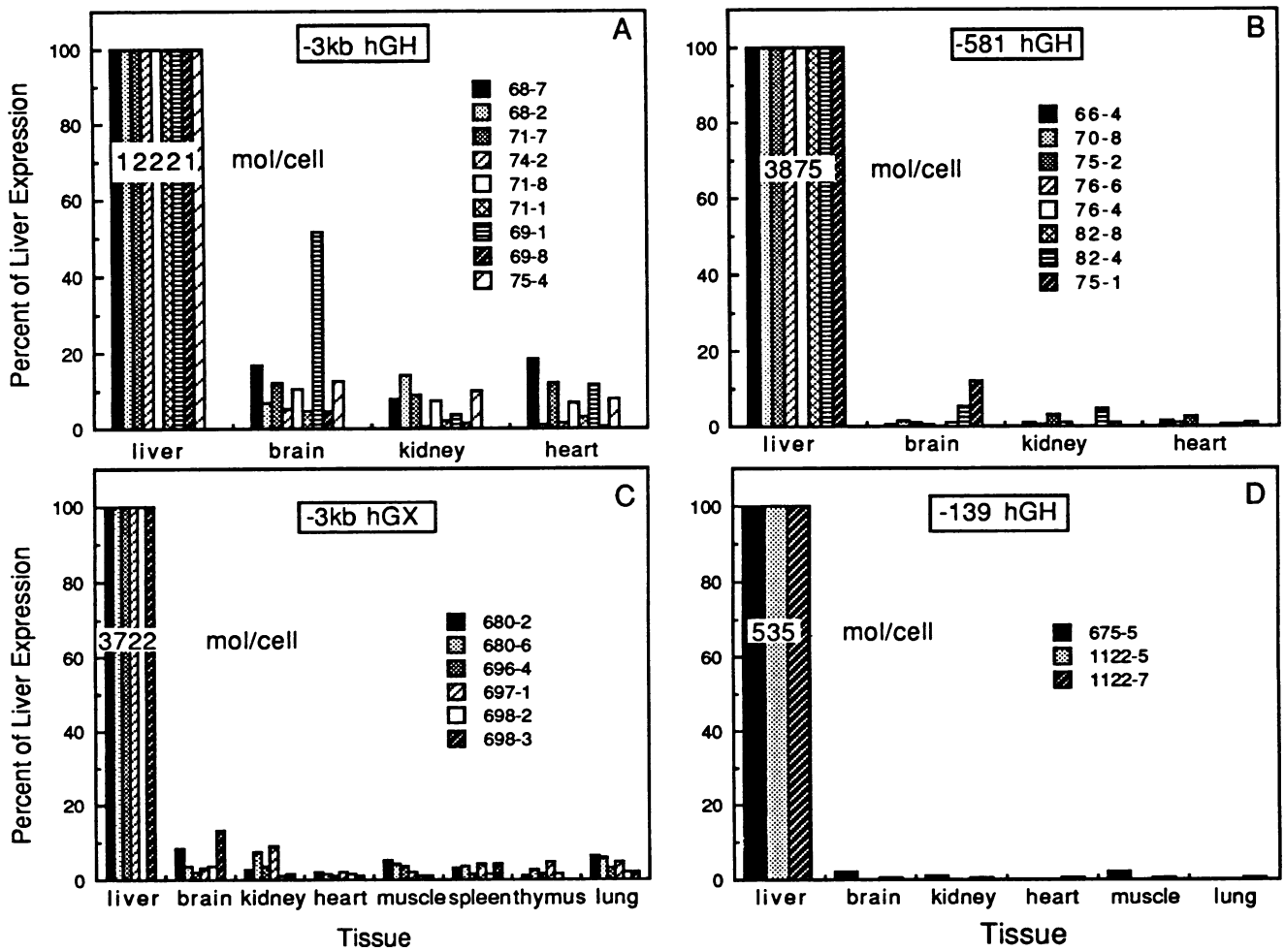

FIG. 5. Relative levels of transgene mRNA expression. Tissues from individual founder or offspring mice were analyzed for transgene mRNA expression, using a riboprobe specific for 3' hGH sequences in solution hybridizations. The results were calculated as molecules per cell and then plotted as a percentage of the level found in the liver for each animal. The average level of expression is indicated on the liver bar graph for each construct. For each animal, the number of transgenes per cell and the level of transgene mRNA (molecules per cell) in liver are given below in the following format: animal number, gene copy, transgene mRNA. (A) −3-kb mTf-hGH. Information for individual animals: 75-4, 2, 1646; 68-7, 4, 1503; 68-2, 8, 10563; 71-7, 10, 1460; 74-2, 14, 21331; 71-8, 18, 6088; 71-1, 25, 19290; 69-1, 31, 3305; 69-8, 34, 44811. (B) −581 mTf-hGH. Information for individual animals: 66-4, 1, 880; 70-8, 2, 1457; 75-2, 2, 659; 76-6, 2, 4137; 76-4, 3, 4207; 82-8, 3, 14167; 82-4, 6, 4007; 75-1, 16, 1492. (C) −3-kb mTf-hGX. Information for individual animals: 697-1, 1, 1771; 680-6, 2, 6187; 696-4, 2, 1766; 680-2, 3, 1385; 698-3, 5, 1472; 698-2, 8, 10751. (D) −139 mTf-hGH. Information for individual animals: 675-5, 3, 400; 1122-7, 2, 320; 1122-5, 18, 885.

(Table 1). The expression of endogenous transferrin in extrahepatic tissues also increased from 1 to 5 molecules per cell to 10 to 50 molecules per cell in transgenic animals. Further analysis of other transgenic mice expressing hGH from the metallothionein or albumin gene promoter demonstrated that the increase in transferrin mRNA in liver was a growth hormone effect and not related to the use of the transferrin promoter (data not shown). Histological analysis of liver tissue from human or bovine growth hormone-producing transgenic mice revealed a combined hypertrophic and hyperplastic response leading to a threefold increase in liver wet weight (C. J. Quaife, personal communication). Thus, excess growth hormone production has a significant effect on liver function and appears to preferentially stimulate the accumulation of transferrin and albumin mRNAs.

TABLE 1. Expression of albumin and protein kinase subunit genes in transgenic and control mouse livers

Type of mouse	Mean mRNA level (molecules/cell) ± SD			
	Transferrin	Albumin	RIα subunit	Cα subunit
Transgenic[a]	12,157 ± 2,336	60,225 ± 5,634	112 ± 12	65 ± 23
Control[b]	4,913 ± 1,213	25,648 ± 8,054	138 ± 5	52 ± 2

[a] Four each of representative −3-kb mTf-hGH and −581 mTf-hGH mice listed in Fig. 5 were analyzed.
[b] Six mice were analyzed, and the results were averaged.

To test whether the hGH produced from the reporter gene was altering tissue-specific expression of the transgene, we constructed −3-kb mTf-hGX by using an hGH gene that had been mutated at the unique *Bgl*II site in exon 5 (Fig. 3) to give an inactive hGH peptide (L. Matthews and R. Palmiter, unpublished data). The average level of transgene expres-

TABLE 2. Induction of the −3-kb hGX transgene by active bovine or human growth hormone in livers of double-transgenic mice

Animal	Transgene(s)	mTf-hGX (mTf) mRNA[a]	Fold induction[b]
698-2-29-1	hGX-bGH	13,296 (14,910)	3.7 (3.9)
698-2-29-3	hGX	3,625 (3,770)	1.0 (1.0)
696-4-12-5	hGX-hGH	17,053	5.4
696-4-12-6	hGX-hGH	22,529	7.2
696-4-12-7	hGX-hGH	11,890	3.8
696-4-12-10	hGX	3,129	1.0
696-4-12-11	hGH	0	0.0

[a] Molecules per cell determined by solution hybridization. mTf mRNA values represent endogenous control levels.

[b] The value for hGX single-transgenic littermates was set at 1.0. Numbers in parentheses represent fold induction of endogenous mTf mRNA.

sion in liver was only 3,722 molecules per cell, compared with 12,221 molecules per cell for the −3-kb mTf-hGH construct, and expression in extrahepatic tissues was very low. As expected, expression of endogenous transferrin mRNA was not induced in the hGX animals, and they showed no growth response. The similar levels and specificities of expression between the −3-kb hGX and −581 hGH animals (Fig. 5C and B) suggest that the −581 construct has lost the DNA sequences that respond to growth hormone, although the endogenous transferrin gene is still induced in these animals.

To clearly demonstrate that high circulating growth hormone was affecting the transgenes, we crossed lines of −3-kb hGX mice with other transgenic mice expressing either bovine growth hormone (transcribed from the mouse metallothionein 1 gene promoter) or hGH (−581 mTf-hGH). Double-transgenic offspring were obtained that harbored the −3-kb hGX construct and also expressed an active form of either bovine growth hormone or hGH. The levels of hGX-specific mRNA were measured by solution hybridization and compared with the basal expression of hGX in littermate controls that did not overproduce active growth hormone. The −3-kb hGX transgene responded to growth hormone with a four- to sevenfold induction of mRNA levels, similar to that seen for the endogenous transferrin mRNA (Table 2). This result suggests the presence of a growth hormone-responsive element in the 5′-flanking region of the mTf gene.

DISCUSSION

Genomic clones have now been obtained for the mouse, human (23, 32), and chicken (8) transferrin genes, allowing a comparison of gene structure and nucleic acid sequence. In all three species, a mature transcript size of approximately 2.4 kb has been observed, and intron positions are highly conserved. Regions of sequence homology were found in the 5′-flanking regions of all three genes, which may be of functional significance. A region between −160 and −45 is particularly well conserved between mouse and human genes and shows considerable similarity to the same region of the chicken promoter. Upstream from this conserved region, the mouse transferrin gene also contains a 45-bp stretch of alternating GT starting at position −241; it has been postulated that such alternating purine-pyrimidine tracts play a role in transcriptional activity (29). However, neither the chicken nor the human transferrin gene contains a GT stretch in the corresponding position, suggesting that this region is not essential for liver-specific expression.

Fusion genes containing either 3 kb, 581 bp, or 139 bp of upstream mTf genomic sequence linked to the hGH structural gene region were used to produce transgenic mice. All of the animals that incorporated at least one complete copy of the transgene expressed substantial levels of growth hormone mRNA in liver; expression in nonhepatic tissues was lower and more variable. Transgene expression was initiated at the correct cap site in the transferrin promoter, as shown by primer extension, and the transcripts were processed correctly to the expected 1.2-kb mRNA in all animals tested.

Since the construct containing only 139 bp of 5′-flanking sequence from the transferrin gene promoter directed expression of mRNA to liver, we can conclude that a major liver-specific element is located very close to the transferrin promoter. The addition of sequences between −139 and −581 did not substantially change the tissue specificity, but the levels of expression in liver increased about fivefold, suggesting the presence of an enhancerlike element in this distal promoter region. Further extension of the transgene to contain the −3-kb transferrin promoter resulted in an unexpected increase in nonhepatic expression and a threefold increase in expression in liver. Since all of these constructs are producing active hGH, we examined the possibility that the hGH was affecting both the endogenous transferrin gene and the exogenous transgene constructs. Our results indicated that hGH caused a threefold induction of transferrin mRNA in liver and also stimulated low but detectable levels of endogenous transferrin expression in other tissues. Another liver-specific gene, albumin, was also induced by hGH, but the levels of constitutively active genes such as the regulatory and catalytic subunits of protein kinase A were unaffected. These results suggested that the −3-kb mTf-hGH construct contained the hGH-responsive element of the transferrin gene, causing the transgene to be autoregulated by its hGH product, stimulating both hepatic and nonhepatic expression.

To test this hypothesis, we constructed a −3-kb mTf-hGX fusion gene that contained a 4-base insert in the coding region of the hGH structural gene. The resulting mRNA should translate to an inactive peptide because of the frameshift mutation. As expected, transgenic mice carrying this mutated gene did not exhibit abnormal growth, although high levels of transgene mRNA were expressed in liver. The expression of −3-kb mTf-hGX was significantly lower in nonhepatic tissues than was −3-kb mTf-hGH expression, and the overall pattern and magnitude of expression closely resembled that of −581 mTf-hGH. To demonstrate the presence of a growth hormone-responsive element in the 5′-flanking sequences of the transferrin gene, −3-kb mTf-hGX animals were crossed with other transgenic mice expressing either bovine growth hormone or hGH. The excess growth hormone induced the hGX transgene by four- to sevenfold in the double-transgenic animals as compared with values for littermate controls. This induction of hGX is comparable to the three- to fourfold induction of endogenous transferrin mRNA seen in the same experiments and demonstrates the presence of a growth hormone-responsive element in the 5′-flanking region of the transferrin gene.

The nature of the liver-specific control elements within the −139- to +50-bp transferrin promoter is unknown. Preliminary analysis by gel retardation and DNase footprinting has demonstrated a high affinity C/EBP-binding site between −99 and −90 (data not shown; Barbara Graves, personal communication). The sequence of this site is 5′-ATTGGGCAAT-3′ and is homologous to the sequence of

the C/EBP-binding site of herpes simplex virus thymidine kinase (20). Deletion of this site renders the transferrin gene promoter inactive in rat hepatoma cells (data not shown), but the regulatory role of this binding site in transgenic mice has not been delineated. Recently, Brunel et al. (6) analyzed the protein-binding sites within the human transferrin gene promoter and also reported a C/EBP-binding site between −103 and −83. It is possible that the C/EBP protein participates in liver-specific expression, since binding sites for this factor have been found on other liver-specific genes, including the albumin (14), α1-antitrypsin, and transthyretin (9) genes.

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