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A Transferrinlike (Hemiferrin) mRNA Is Expressed in the Germ Cells of Rat Testis

BARBARA J. STALLARD,[1]† MICHAEL W. COLLARD,[2] AND MICHAEL D. GRISWOLD[1]*

Department of Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164-4660,[1] and Mental Health Research Institute, University of Michigan, Ann Arbor, Michigan 48109[2]

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In the testis, germ cells which are separated from the serum by the blood-testis barrier rely primarily on the Sertoli cell to obtain nutrients. For example, transferrin synthesized by the Sertoli cell is important in delivering iron from the serum to the developing germ cells. Because of its role in the testis, Sertoli cell transferrin protein and mRNA have been extensively studied. By using RNA blot analysis of rat testicular tissue, we detected a transcript of 2.6 kb which is attributed to transferrin. In addition, we detected a novel mRNA of 0.9 kb which had sequence similarity to the 3′ end of transferrin. This 0.9-kb mRNA was present in germ cells, but not Sertoli cells, liver, or brain. The primary source of this mRNA in the testis was round spermatids. Sequence analysis of a cDNA clone showed that this mRNA encoded a protein with sequence similarity to the carboxy terminus of transferrin. Polysome analysis indicated that this transcript was translated and may therefore have importance in the iron metabolism of germ cells. The evolutionary implications between the transferrinlike mRNA germ cells and the gene duplication event which resulted in the diferric binding of transferrin are discussed.

Transferrin is the iron-binding protein of vertebrates and functions to deliver iron to cells by binding to a specific cell surface receptor (1, 18). Transferrin is a major protein secreted by cultured rat Sertoli cells (20, 26) and is an important component in mediating the delivery of iron to the germ cells. Transferrin mRNA has been cloned and sequenced from a number of species (3, 6, 7, 13, 23, 27), and its expression has been localized to a discrete number of tissues including the liver, brain, mammary gland, and testis. While analyzing transferrin mRNA by Northern (RNA) blot analysis, we detected a novel transcript present in rat testicular tissue which cross-hybridized with authentic rat transferrin cDNA. This paper describes the characterization, molecular cloning, and sequencing of this novel transcript, which is made by postmeiotic (haploid) germ cells and has sequence similarity to the 3′ end of transferrin mRNA and protein. This study was undertaken in an effort to characterize this unique transcript and to determine its relationship to transferrin.

MATERIALS AND METHODS

Cell isolation and culture conditions. Sertoli cells were isolated from 20-day-old rats and cultured in serum-free Ham F-12 medium (GIBCO Laboratories, Life Technologies Inc., Grand Island, N.Y.) in the presence of follicle-stimulating hormone (0.025 μg/ml), insulin (5 μg/ml), retinol (0.1 μg/ml), and testosterone (0.7 μM) as described previously (9, 27). RNA was isolated after day 5 in primary culture. Germ cells were isolated from an adult rat and fractionated by gravity sedimentation in a Staput apparatus as described previously (4). Cell populations were monitored and purity was quantitated by light microscopy. Peritubular myoid cells were isolated as described previously (22) and cultured for 7 days in serum-free medium prior to RNA isolation.

RNA isolation and Northern blot analysis. RNA was isolated as described previously (5) except that a proteinase K (150 μg/ml) incubation step (45 min at 43°C) was substituted for the 3 M LiCl reprecipitation step. When indicated, poly(A)$^+$ RNA was isolated by chromatography over oligo(dT) cellulose columns (Collaborative Research Inc., Bedford, Mass.). The concentration of RNA was determined spectrophotometrically at 260 nm. RNA samples were fractionated on 1.2% agarose-formaldehyde gels as described previously (13) and probed with either nick-translated transferrin cDNA prepared as specified by the manufacturer (Bethesda Research Laboratories Life Technologies Inc., Gaithersburg, Md.) or in vitro-transcribed single-stranded antisense ^{32}P-labeled transferrin cRNA made from linear templates and SP6 polymerase (Promega Biotec, Madison, Wis.) or by random priming of gel-extracted fragments as described (Boehringer Mannheim, Indianapolis, Ind.). Hybridization and washing conditions were as described previously (14). All washes were under stringent conditions (55°C in 0.1× SSC–0.1% sodium dodecyl sulfate [SDS]; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.4]). The transferrin cDNA probe was a 688-bp *Pst*I-*Hin*cII restriction fragment of an 820-bp clone (13, 14).

Polysomes were isolated from rat testis or equitesticular amounts of isolated spermatocytes, round spermatids, or elongating spermatids and centrifuged in the absence or presence of EDTA as previously described (11).

cDNA synthesis, recombinant selection, and sequence analysis. Germ cell RNA (50 μg) was fractionated over a continuous sucrose gradient (10 to 25% sucrose in 10 mM Tris [pH 7.5], 10 mM EDTA, and 0.1% SDS) in an SW41 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 35,000 rpm for 24 h at 25°C. Fractions (0.5 ml) were collected and adjusted to 150 mM NaCl, and RNA was precipitated by the addition of 2.5 volumes of ethanol. The RNA concentration in each fraction was determined spectrophotometrically at 260 nm, and a volume equivalent to 2 μg was blotted onto

* Corresponding author.
† Present address: Department of Molecular Biology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080.

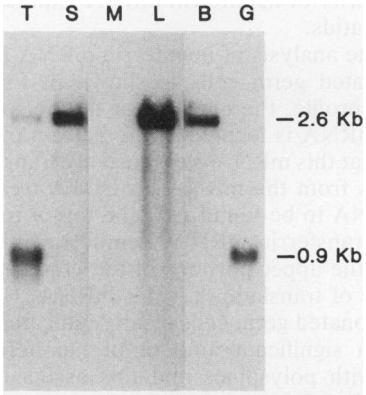

FIG. 1. Expression of transferrin mRNA in various tissues and demonstration of a cross-hybridizing 0.9-kb transcript in germ cells. RNA was isolated from the testis of an adult rat (lane T), Sertoli cell cultures (lane S), peritubular myoid cell cultures (lane M), and liver (lane L), brain (lane B), and germ cells (lane G). RNA (2 to 5 μg) was fractionated on an 1.2% agarose-formaldehyde gel, transferred to nylon membranes, and hybridized with a radiolabeled transferrin cDNA probe (13) prepared by nick translation. The transferrin probe contains the 3′ sequence of transferrin cDNA (14). The sizes of transferrin mRNA (2.6 kb) and hemiferrin (0.9 kb) are indicated on the right of the figure.

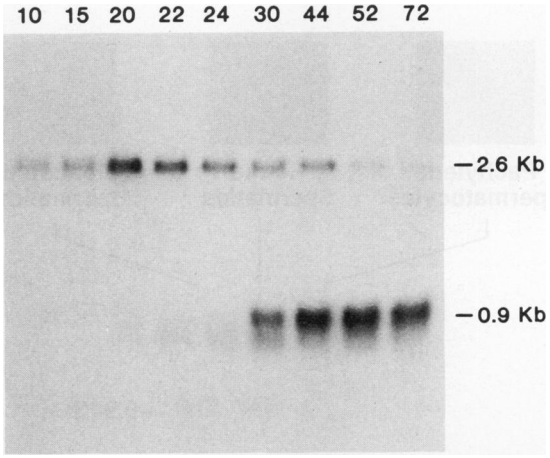

FIG. 2. Developmental expression of transferrin and hemiferrin in testicular tissue. Poly(A)$^+$ RNA was isolated from testes from 10-, 15-, 20-, 22-, 24-, 30-, 44-, 52-, and 72-day-old rats, and 2 μg of poly(A)$^+$ RNA was analyzed by Northern blot analysis as described in the legend to Fig. 1.

nylon membrane (Micron Separations, Inc., Westboro, Mass.) under suction (dot blot). The dot blot was probed with radiolabeled transferrin cDNA, and fractions positive for the cross-hybridizing transferrin transcript were pooled and applied to an oligo(dT)-cellulose column. This size-selected poly(A)$^+$ RNA highly enriched in the 0.9-kb transcript was used as a template for cDNA synthesis as described by Gubler and Hoffman (12). The cDNA was ligated with *Eco*RI linker arms (Bethesda Research Laboratories Life Technologies Inc.) and was cloned into the *Eco*RI site of PTZ18U (United States Biochemical Corp., Cleveland, Ohio) and then transfected into *Escherichia coli* JM105. Positive clones were selected by hybridization to radiolabeled transferrin cDNA. Several positive clones were analyzed further by characterization of their inserts by restriction enzyme digestion and agarose gel electrophoresis. Two of these clones were sequenced by using the Sequenase dideoxy sequencing kit (United States Biochemical Corp.) and M13-derived single-stranded templates of appropriate subclones. When clones lacked appropriate restriction enzyme sites, oligomer primers, generated by using a Gene Assembler DNA synthesizer (Pharmacia, Piscataway, N.J.), were used to obtain unambiguous sequence information.

Sequence data were analyzed on a VAX 11/785 computer (Digital Equipment Corp., Maynard, Mass.) by using the software programs of the University of Wisconsin Genetics Computer Group (8). The transferrin sequences were obtained from the GenBank database release 60.0.

RESULTS AND DISCUSSION

Expression of transferrin mRNA in a variety of tissues was analyzed by RNA blot analysis. RNA was isolated from testis, liver, brain, and germ cells of adult rats and from cultured peritubular myoid cells and Sertoli cells, subjected to RNA blot analysis, and hybridized with a rat transferrin cDNA clone (13) encompassing the 3′ end of transferrin mRNA. Figure 1 shows an autoradiograph of the blot and reveals the presence of two hybridizing mRNA species of 2.6 and 0.9 kb. The 2.6-kb mRNA species was present in testicular RNA and brain, liver, and Sertoli cells and is attributed to full-length transferrin mRNA. The 0.9-kb mRNA (hereafter called hemiferrin mRNA) was present in testicular tissue from an adult rat and in isolated spermatogenic cells, suggesting that the source of this message is from the germ cell population.

Since germ cell types arise sequentially after birth until sexual maturation, it is possible to correlate the onset of expression of a gene with the appearance of a particular germ cell type. For example, pachytene spermatocytes first appear in the rat testis at about 15 days of age and spermatids first appear at about 22 days of age and continue to develop for approximately 22 days (19). The expression of hemiferrin mRNA as a function of age was determined by RNA blot analysis (Fig. 2) to define the developmental regulation of hemiferrin mRNA in germ cells and to correlate its expression with the appearance of a particular germ cell type. Poly(A)$^+$ RNA was isolated from the testes of rats varying in age from 10 to 72 days and probed with radiolabeled transferrin cRNA. Detectable levels of transferrin mRNA were observed at all ages. At 30 days of age, high levels of expression of hemiferrin mRNA were observed. Low levels of expression of this mRNA were detected at 22 and 24 days of age. There was no detectable transcript before 22 days of age, suggesting that expression of hemiferrin mRNA is initiated during spermatid differentiation.

To further define the developmental expression of hemiferrin mRNA, we separated spermatogenic cells by unit-gravity sedimentation (4). Fractions enriched for pachytene spermatocytes, round spermatids, and elongating spermatids and residual bodies (see the legend to Fig. 3 for compositions) were collected and homogenized for RNA isolation. RNA from each cell type was analyzed by RNA blot analysis with radiolabeled transferrin cDNA as a probe (Fig. 3A). Hemiferrin mRNA was detected principally in the fractions containing round spermatids; smaller amounts of this RNA were observed in fractions containing elongating spermatids and residual bodies, and little was observed in fractions enriched for pachytene spermatocytes, further confirming

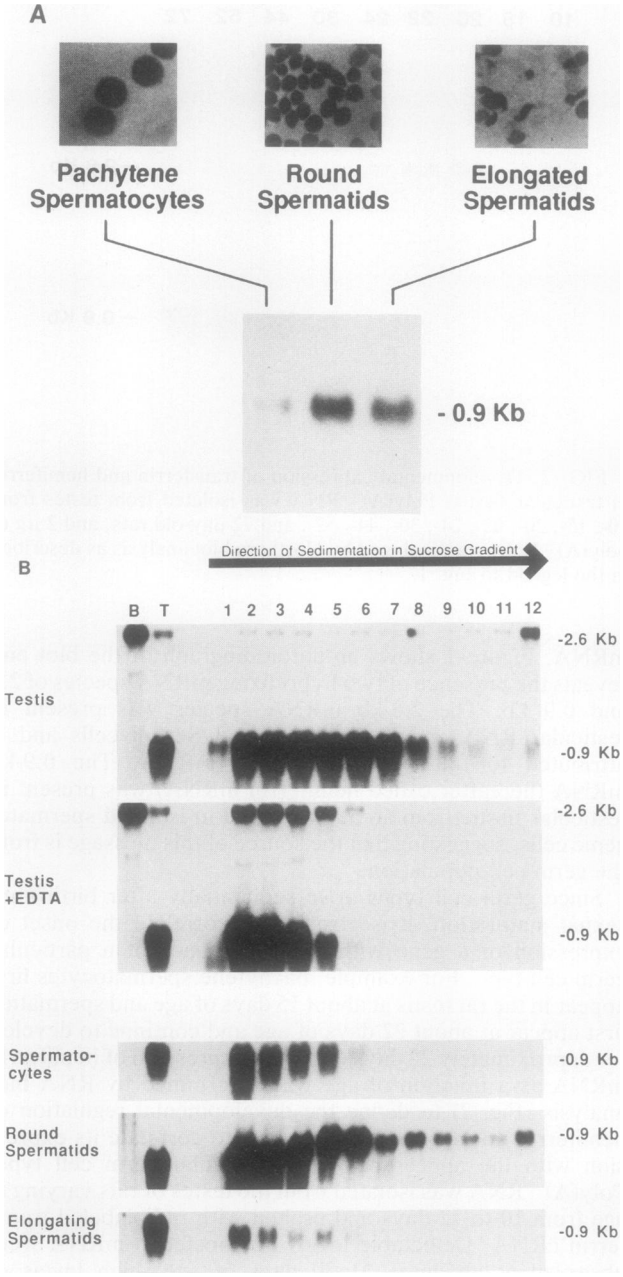

FIG. 3. Expression of hemiferrin in isolated populations of germ cells. (A) Germ cells were separated by using density gravity sedimentation, and fractions enriched in pachytene spermatocytes, round spermatids, and elongated spermatids plus residual bodies were collected and homogenized for RNA isolation. A 5-μg sample of RNA from each enriched fraction was analyzed by Northern blot analysis as described in the legend to Fig. 1. The enriched fractions are shown in the photographs (×260 magnification) at the top of the figure. The pachytene-enriched fraction contained 85% pachytene spermatocytes and 14% multinucleate cells (spermatids); the round spermatid-enriched fraction contained 87% round spermatids and 11% spermatocytes; and the elongated spermatid and residual body-enriched fraction contained a 24% contamination with round spermatids. (B) Polysomes were isolated from either rat testis or equitesticular amounts of the indicated cell types and centrifuged in the absence or presence of EDTA as previously described (11). RNA from each gradient fraction (lanes 1 to 12) was subjected to RNA blot analysis with a rat transferrin cDNA probe. Lane 1 represents the top of the gradient (dissociated polysomes); lane 12 represents the bottom of the gradient (polysomes). Brain (B) or testis (T) RNA (10 μg) was included on each blot to establish similar blot sensitivity and transcript size. The EDTA-treated testis sample represents the sedimentation rate of dissociated polysomes and was similar for the spermatogenic cell samples.

that the majority of hemiferrin mRNA expression occurs in round spermatids.

A polysome analysis of hemiferrin mRNA in whole testis and fractionated germ cells is shown in Fig. 3B. In the whole-testis profile, the majority of the Sertoli cell-derived transferrin mRNA is located at the bottom of the gradient, suggesting that this mRNA is extensively translated. Release of ribosomes from the mRNA by EDTA treatment causes the free mRNA to be found near the top of the gradient. In contrast to transferrin mRNA, hemiferrin mRNA is found primarily in the upper portions of the gradient, indicating a lower degree of translation for this mRNA. However, analysis of fractionated germ cells clearly indicates that in round spermatids a significant amount of hemiferrin mRNA is associated with polysomes and this association is EDTA sensitive. It therefore seems likely that a protein product will be synthesized from hemiferrin mRNA. Previously, other germ cell-derived mRNAs have shown a low degree of association with polysomes (11, 15). This may indicate a general property of germ cell translation rather than an inherent property of hemiferrin mRNA.

Hemiferrin mRNA was cloned and sequenced; the complete nucleotide sequence and deduced protein sequence are shown in Fig. 4. An open reading frame begins with the first ATG codon at position 27 and continues through nucleotide 674, where it terminates with a single TAA codon. The deduced protein sequence contains 216 amino acids and has a calculated molecular weight of 24,091. Alignment of the deduced amino acid sequence of hemiferrin mRNA with rat and human transferrins is shown in Fig. 5 and was generated by using the GAP program of the University of Wisconsin Genetics Computer Group package. The putative protein product of hemiferrin mRNA encodes amino acids with high similarity to the carboxy terminus of transferrin, including many of the amino acids which are thought to be involved in coordinating the iron atom (1, 18).

Transferrin contains two iron-binding domains, which are located in the N-terminal and C-terminal halves of the polypeptide chain (2, 17). The two domains have very similar sequences and very similar iron-binding properties. The final structure of transferrin is thought to have arisen through gene duplication of a precursor protein, with each domain evolving independently after the duplication event (24). The precursor protein is thought to have resembled the N-terminal domain of transferrin (24). Comparison of hemiferrin with the N-terminal domain of human transferrin showed 42% identity and 58% similarity (considering conservative changes) at the protein level. Comparison of hemiferrin mRNA with the C-terminal domain of human transferrin showed 64% identity and 75% similarity. If hemiferrin is an evolved form of the precursor protein, our data would suggest that the duplication event occurred with a protein which resembled the C-terminal domain of transferrin.

Isozyme or isotype switching occurs during germ cell development (25) and is a common mechanism for generating protein diversity by expressing new protein variants with related but nonidentical primary structures. Isozyme switching may play an integral role in germ cell development by generating proteins with different cofactor requirements or

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                                                                  10
                                  Met Leu Tyr Ser Lys Asn Asn Asn Cys Lys Phe Asp Glu
TGTGCTCGCTGCTCACAGCGCTCACG        ATG CTC TAC AGC AAG AAT AAT AAC TGT AAA TTT GAT GAA

                          20                                      30
Phe Phe Ser Ala Gly Cys Ala Pro Gly Ser Pro Arg Asn Ser Ser Ser Leu Cys Ala Leu
TTT TTC AGC GCA GGT TGT GCA CCT GGA TCT CCG CGA AAT TCC AGT AGT CTC TGT GCT CTG

                          40                                      50
Cys Ile Gly Ser Glu Lys Gly Thr Gly Lys Glu Cys Val Pro Asn Ser Asn Glu Arg Tyr
TGC ATT GGC TCA GAG AAG GGT ACA GGA AAG GAG TGT GTT CCC AAC AGC AAT GAA AGA TAC

                          60                                      70
Tyr Gly Tyr Thr Gly Ala Phe Arg Cys Leu Val Glu Lys Gly Asp Val Ala Phe Val Lys
TAT GGC TAT ACA GGG GCT TTC AGG TGT CTG GTG GAG AAG GGA GAC GTG GCC TTT GTG AAG

                          80                                      90
Asp Gln Thr Val Ile Gln Asn Thr Asp Gly Asn Asn Asn Glu Ala Trp Ala Lys Asn Met
GAC CAG ACT GTC ATA CAG AAC ACT GAC GGA AAT AAT AAT GAA GCA TGG GCA AAA AAT ATG

                          100                                     110
Lys Lys Glu Asn Phe Glu Val Leu Cys Lys Asp Gly Thr Arg Lys Pro Val Thr Asp Ala
AAG AAG GAA AAT TTT GAA GTA CTA TGC AAA GAT GGC ACC AGG AAA CCT GTG ACA GAT GCT

                          120                                     130
Glu Asn Cys His Leu Pro Glu Pro Asn His Ala Val Val Ser Arg Lys Asp Lys Ala Thr
GAG AAC TGC CAC CTG CCC GAG CCG AAT CAT GCT GTG GTC TCA CGG AAA GAT AAG GCA ACT

                          140                                     150
Cys Val Glu Lys Ile Leu Ile Lys Gln Gln Asp Asp Phe Gly Lys Ser Val Thr Asp Cys
TGT GTG GAG AAA ATA TTA ATC AAA CAG CAG GAT GAT TTT GGA AAA TCT GTA ACC GAC TGC

                          160                                     170
Thr Ser Asn Phe Cys Leu Phe Gln Ser Asn Ser Lys Asp Leu Leu Phe Arg Asp Asp Thr
ACG AGC AAT TTT TGT TTA TTC CAA TCA AAT TCC AAG GAC CTT CTG TTC AGG GAT GAC ACT

                          180                                     190
Lys Cys Leu Ala Ser Ile Ala Lys Lys Thr Tyr Asp Ser Tyr Leu Gly Asp Asp Tyr Val
AAA TGT TTG GCT TCA ATT GCG AAA AAA ACA TAT GAC TCC TAC TTA GGG GAT GAC TAC GTC

                          200                                     210
Arg Ala Met Thr Asn Leu Arg Gln Cys Ser Thr Ser Lys Leu Leu Glu Ala Cys Thr Phe
AGA GCT ATG ACC AAC CTG AGA CAA TGC TCA ACC TCA AAA CTC CTG GAA GCA TGC ACT TTC


His Lys Pro
CAC AAA CCT TAAAATCCAAGAGTGGAGCCAACACCTGATGGAGATGGGAGCTCATGGGACCCATAAGCTTCATCTGG


TTTTCTGGTTTCGCTGGTCTGAGTGATTTGGTTGCCCTCACAATTTGGTGGGTGGCGCCCTGCAGGACAAAATAAAAATA


AACATTAA
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FIG. 4. Nucleotide sequence and deduced amino acid sequence of hemiferrin. The nucleotide and derived protein sequences of the 0.9-kb cDNA insert of a representative clone are shown. The numbering corresponds to the amino acid sequence.

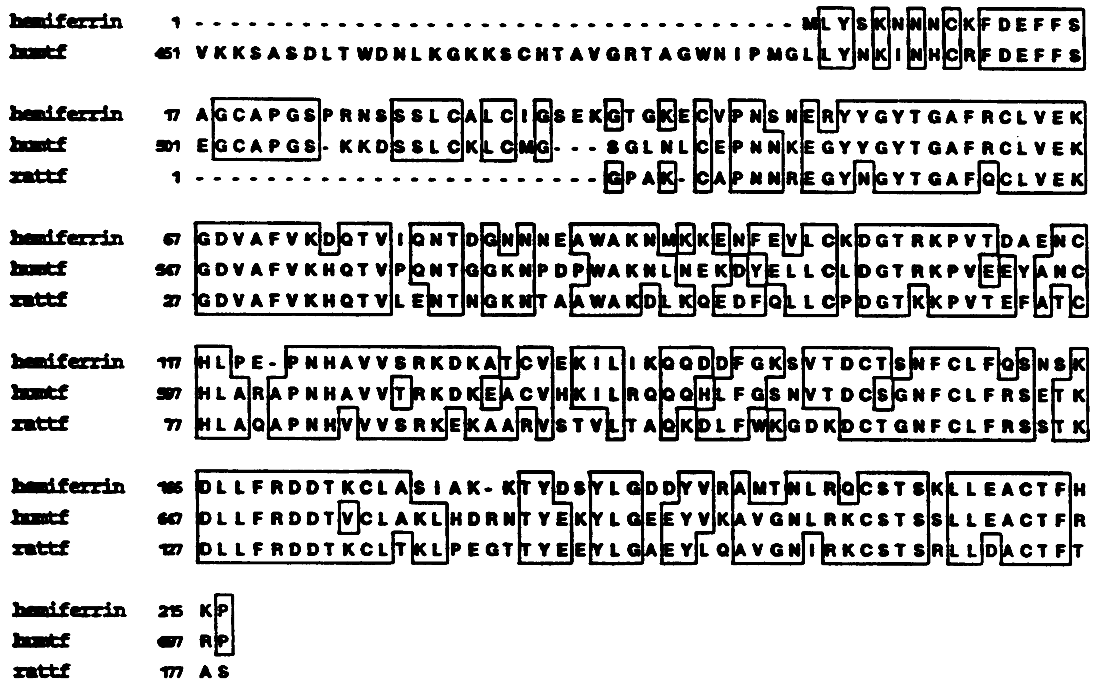

FIG. 5. Alignment of deduced protein sequence of hemiferrin with human and rat transferrin. The multiple sequence alignment was generated by using the GAP program of the University of Wisconsin Genetics Computer Group package. The sequences used in the gapping program were hemiferrin, human transferrin (humtf [23, 27]), and rat transferrin (rattf [13]). The transferrin sequences were obtained from the GenBank database release 60.0. The numbering of the rat transferrin sequence is arbitrary, as a full-length clone was not available. The sequence presented represents the deduced amino acid sequence of a partial cDNA clone encompassing the C terminus of rat transferrin.

different regulatory mechanisms which are important for maturation of the germ cell. Hemiferrin mRNA appears to be a special subset of this class of proteins made by germ cells in that it encodes a protein homologous to approximately one-half of the transferrin protein. The significance of this structure is unknown. Of considerable bearing are the recent reports on the structure of the testicular isozyme for angiotensin-converting enzyme (ACE) (10, 16, 21). The testicular form of ACE is identical to the C-terminal half of endothelial ACE. The structure of the endothelial enzyme is similar to that of transferrin in that it consists of two homologous domains with each half of the molecule containing a putative metal-binding site (10, 16, 21). The duplicate structure of ACE is also thought to have arisen through a gene duplication event. The appearance of two spermatogenic isotypes (hemiferrin and ACE) which are similar to the C terminus of their somatic counterpart is an interesting finding and may provide further information on the mechanism of duplication events. In addition, the translational regulation of hemiferrin and its structural similarity to transferrin may provide clues to its role during spermatogenesis.

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