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MOLECULAR CLONING AND SEQUENCE ANALYSIS OF cDNA FOR HUMAN TRANSFERRIN

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A cDNA clone for human transferrin was identified from a human liver cDNA library by pre-screening with different ss-cDNA probes against length-fractionated liver mRNAs, positive hybridization-selection and nucleotide sequence analysis. The insert was of 1 kb, encoding human transferrin from aminoacid 403 through the COOH terminus, with a 3' non coding region of 166 nucleotides.

This insert hybridized with a single major mRNA species of about 2.4 kb and several genomic DNA restriction fragments. Hybridization of the Southern blots with different parts of the transferrin insert and at different stringences suggest that the various bands observed correspond to splice sites inside one gene rather than to hybridization to several related genes. Finally, a single or a low number of transferrin gene copies seem to exist in the human genome.

Transferrin is the major iron-binding protein in the plasma of vertebrate species. It is composed of a single polypeptide chain of 679 aminoacid residues, which together with the two N-linked oligosaccharide chains gives a calculated molecular weight of 79 570 (of which about 6 % is contributed by the glucidic moiety) (1,2). Transferrin belongs to a family of related proteins, e.g. lactoferrin (or lactotransferrin) found in milk and ovotransferrin which is the iron binding protein of avian egg white. Interest in these proteins has very recently been stimulated by the discovery of a onc gene expressed in chicken B cell lymphomas (3), and perhaps in human Burkitt lymphoma (4), which exhibits a homologous sequence to the amino terminus of transferrin. In addition to its role in iron binding and transport, transferrin is an essential growth factor for cells in serum free medium (3). Transferrin has also been reported to stimulate growth of lymphocytes independently of its iron transport activity (5,6). Finally a melanoma surface antigen has been identified as sharing a partial homology with transferrin (7).

In man as well as in large number of animal species a transferrin genetic polymorphism has been reported (8,9) ; about 20 human variants are known, some of

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them with a frequency of 1 % or more in the population. Linkage of some transferrin variants with chromosomal markers has enabled the probable assignment of the transferrin gene to chromosome 3 (10-14). One family with congenital atransferrinemia was reported in 1961 (15). Such a multiplicity of phenomena involving transferrin or transferrin-related proteins prompted us to clone transferrin cDNA with the goal of studying regulation of the gene(s) expression under normal and pathological conditions.

MATERIALS

All the methods used for RNA purification, construction of a human liver ds-cDNA recombinant plasmid library and RNA selection on nitrocellulose filters have been described in earlier papers (18-23).

Differential screening of the cDNA library : Each of 10 different microtitration plates (containing 96 clones per plate) was incubated on 5 different agar plates, then transfered to nitrocellulose BA 85 filters. The filters corresponding to each plate were hybridized with (^{32}P) labeled cDNA reverse transcribed from 5 different mRNA preparations : 10-14 S, 16-17 S and 18-20 S sucrose gradient fractions of human adult liver mRNAs ; 18-20 S sucrose gradient fraction of human muscle RNA (19) and, finally, very pure rat serum albumin mRNA (21). The hybridization was performed for 48 h in presence of 5×10^5 cpm of labeled probe per ml.

All the clones hybridizing with the human muscle and rat albumin cDNA probes were eliminated. Those remaining clones that displayed a more intense hybridization signal with the liver 18-20 S probe than with the probes corresponding to lighter mRNAs were selected : they represented 20 of the 960 screened clones.

cDNA sequence : The nucleotide sequences were determined by the chemical method of Maxam and Gilbert (24).

Electrophoresis of RNA in agarose-formaldehyde (25,26), transfert of RNA and DNA on nitrocellulose filters (27,28) and hybridization with nick-translated, purified cDNA inserts (29) have been previously described.

High molecular weight cellular DNA was purified from circulating leukocytes as slightly modified from reference (30).

RESULTS

Positive hybridization-selection

20 out of the 960 clones tested with the 5 ss-cDNA probes as described in methods were preselected : they corresponded to sequences which did not hybridize with albumin and muscle-specific probes and hybridized more strongly with ss-cDNA complementary to 18-20 S liver mRNAs than with probes complementary to lighter mRNA species. These 20 clones were tested by positive hybridization-selection. 3 corresponded to Aα and 1 to Bβ fibrinogene chains (Uzan et al, in preparation), 1 gave a polypeptide of 76 000-80 000-Mr and another a polypeptide of 72 000-75 000-Mr (fig. 1) which did not cross-react either with anti-fibrinogen or with anti-prothrombin antisera.

The clone encoding the 72 000-75 000-Mr polypeptide was considered a good candidate for containing transferrin cDNA sequences and was therefore sequenced. it was designated as T_f 66 G2.

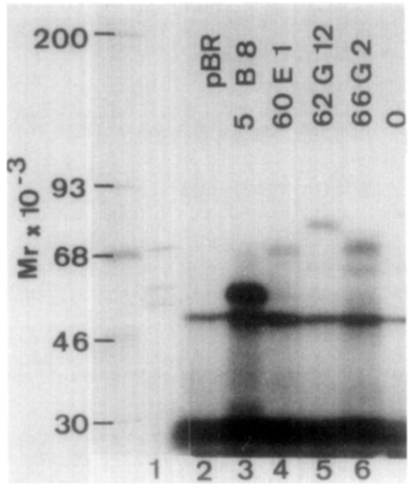

Fig 1. Positive hybridization-selection analysis.
Plasmid DNA was bound to nitrocellulose filters, then hybridized with poly A-containing human liver mRNA as indicated in "materials and Methods". Eluted mRNA was translated in 25 ul of a reticulocyte cell free system, 5 ul of the translation mixture being dissociated and analyzed by SDS-polyacrylamide gel electrophoresis.
1 : ^{14}C labeled plasma fibrinogen
2 to 6 : translation products obtained under direction of RNA eluted from filters bound to non-recombinant pBR 322 (2), plasmid 5 B8 (3), 60 E1 (4), 62 G12 (5) and 66 G2 (6). 0 : translation blank, without addition of exogenous RNA. Plasmid 5 B8 contained cDNA for the B β fibrinogen chain and 60 E1 for the A α fibrinogen chain. Plasmid 62 G12 insert has not yet been identified.

Sequence of a cDNA clone encoding part of the human transferrin structural gene (fig. 2).

Nucleotide sequence analysis of the cDNA clone T_f 66 G2 showed that this clone contains a 1.000 Kb insert in the PstI site of pBR 322. The results obtained also established that the 1001 nucleotide fragment encodes human transferrin from aminoacid 403 through the COOH terminus. The translation termination codon UAA precedes a 3'-non-coding region of 166 nucleotides. The canonical poly-adenylation signal AATAAA (31) is present in the cDNA sequence at minus 24 bp from the poly(A) tract. The predicted aminoacid sequence agrees precisely with the protein sequence results of Mac Gillivray et al (2) except for the following 6 positions : 417, an Asn instead of an Asp ; 539, a Pro instead of a Thr ; 542, a Thr instead of a Pro ; 572, a Glu instead of a Gln ; 653, the same modification, and finally a Gly instead of Glu in position 672.

Characterization of transferrin mRNA.

T_f 66 G2 plasmid was labelled by nick translation and used as a probe for characterizing specific mRNA in adult and fetal liver. In both tissues a single major species was detected, of about 2400 bases, i.e. similar to the length of Aα fibrinogen chain mRNA (fig. 3). In contrast to Aα fibrinogen mRNAs, transferrin mRNA was more abundant in fetal (4 months of intra-uterine life) than in adult liver. After a long autoradiographic exposure, a supplementary heavy mRNA species of

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                                   410
  GLY LEU VAL PRO VAL LEU ALA GLU ASN TYR ASN LYS SER ASP ASN CYS GLU ASP THR PRO
T GGT CTG GTG CCT GTC TTG GCA GAA AAC TAC AAT AAG AGC GAT AAT TGT GAG GAT ACA CCA

                                   430
  GLU ALA GLY TYR PHE ALA VAL ALA VAL VAL LYS LYS SER ALA SER ASP LEU THR TRP ASP
  GAG GCA GGG TAT TTT GCT GTA GCA GTG GTG AAG AAA TCA GCT TCT GAC CTC ACC TGG GAC

                                   450
  ASN LEU LYS GLY LYS LYS SER CYS HIS THR ALA VAL GLY ARG THR ALA GLY TRP ASN ILE
  AAT CTG AAA GGC AAG AAG TCC TGC CAT ACG GCA GTT GGC AGA ACC GCT GGC TGG AAC ATC

                                   470
  PRO MET GLY LEU LEU TYR ASN LYS ILE ASN HIS CYS ARG PHE ASP GLU PHE PHE SER GLU
  CCC ATG GGC CTG CTC TAC AAT AAG ATC AAC CAC TGC AGA TTT GAT GAA TTT TTC AGT GAA

                                   490
  GLY CYS ALA PRO GLY SER LYS LYS ASP SER SER LEU CYS LYS LEU CYS MET GLY SER GLY
  GGT TGT GCC CCT GGG TCT AAG AAA GAC TCC AGT CTC TGT AAG CTG TGT ATG GGC TCA GGC

                                   510
  LEU ASN LEU CYS GLU PRO ASN ASN LYS GLU GLY TYR TYR GLY TYR THR GLY ALA PHE ARG
  CTA AAC CTG TGT GAA CCC AAC AAC AAA GAG GGA TAC TAC GGC TAC ACA GGC GCT TTC AGG

                                   530
  CYS LEU VAL GLU LYS GLY ASP VAL ALA PHE VAL LYS HIS GLN THR VAL PRO GLN ASN THR
  TGT CTG GTT GAG AAG GGA GAT GTG GCC TTT GTG AAA CAC CAG ACT GTC CCA CAG AAC ACT

                                   550
  GLY GLY LYS ASN PRO ASP PRO TRP ALA LYS ASN LEU ASN GLU LYS ASP TYR GLU LEU LEU
  GGG GGA AAA AAC CCT GAT CCA TGG GCT AAG AAT CTG AAT GAA AAA GAC TAT GAG TTG CTG

                                   570
  CYS LEU ASP GLY THR ARG LYS PRO VAL GLU GLU TYR ALA ASN CYS HIS LEU ALA ARG ALA
  TGC CTT GAT GGT ACC AGG AAA CCT GTG GAG GAG TAT GCG AAC TGC CAC CTG GCC AGA GCC

                                   590
  PRO ASN HIS ALA VAL VAL THR ARG LYS ASP LYS GLU ALA CYS VAL HIS LYS ILE LEU ARG
  CCG AAT CAC GCT GTG GTC ACA CGG AAA GAT AAG GAA GCT TGC GTC CAC AAG ATA TTA CGT

                                   610
  GLN GLN GLN HIS LEU PHE GLY SER ASN VAL THR ASP CYS SER GLY ASN PHE CYS LEU PHE
  CAA CAG CAG CAC CTA TTT GGA AGC AAC GTA ACT GAC TGC TCG GGC AAC TTT TGT TTG TTC

                                   630
  ARG SER GLU THR LYS ASP LEU LEU PHE ARG ASP ASP THR VAL CYS LEU ALA LYS LEU HIS
  CGG TCG GAA ACC AAG GAC CTT CTG TTC AGA GAT GAC ACA GTA TGT TTG GCC AAA CTT CAT

                                   650
  ASP ARG ASN THR TYR GLU LYS TYR LEU GLY GLU GLU TYR VAL LYS ALA VAL GLY ASN LEU
  GAC AGA AAC ACA TAT GAA AAA TAC TTA GGA GAA GAA TAT GTC AAG GCT GTT GGT AAC CTG

                                   670
  ARG LYS CYS SER THR SER SER LEU LEU GLY ALA CYS THR PHE ARG ARG PRO
  AGA AAA TGC TCC ACC TCA TCA CTC CTG GGA GCC TGC ACT TTC CGT AGA CCT TAA  AAT CTC


  AGA GGT AGG GCT GCC ACC AAG GTG AAG ATG GGA ACG CAG ATG ATC CAT GAG TTT GCC CTG


  GTT TCA CTG GCC CAA GTG GTT TGT GCT AAC CAC GTC TGT CTT CAC AGC TCT GTG TTG CCA


  TGT GTG CTG AAC AAA AAA TAA AAA TTA TTA TTG ATT TTA T-poly A
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Fig. 2. Nucleotide and deduced amino acid sequences of the human transferrin 66 G2 cDNA clone.

The given nucleotide sequence is that of the noncoding strang. The deduced amino acid sequence for the correct reading frame is showed. numbers correspond to the residue number in the human serum transferrin protein.

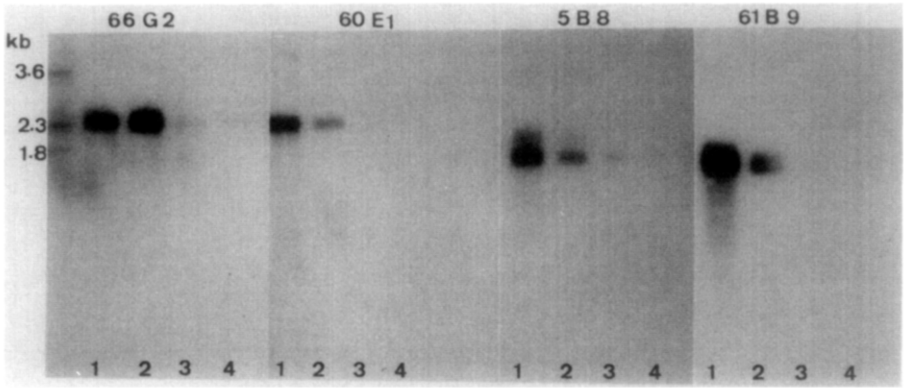

Fig. 3. Northern blot analysis of mRNA from adult and fetal, human and rat liver mRNA with different cDNA probes.
2μg poly A-containig RNA were deposited in each slot.
1 : human adult liver
2 : human fetal liver (4 months of gestational age)
3 : rat adult liver ; 4 : rat fetal liver (17 days) of gestational age). 66 G2 corresponds to the transferrin probe studied in this paper ; the three other recombinant plasmid probes are specific for the three human fibrinogen chains : Aα(60 E1, Bβ(5 B8) and γ(61 B9)

about 5000 bases could be detected. T_f 66 G2 plasmid hybridized slightly with rat liver mRNA, detecting a mRNA species with similar molecular weight to human transferrin.

Screening of human and rat liver cDNA libraries with the T_f 66 G2 insert.

Among the 1000 screened human cDNA clones, 2 hybridized strongly with the transferrin probe. This proportion can be compared to the percentage of serum albumin (about 2-3 %) and of fibrinogen cDNA clones (1-1.5 % according to the different chains) in the human cDNA library used. In rat, we found 2 positive clones out of 576 colonies screened.

Hybridization with human genomic DNA (fig. 4).

Southern blot analysis of genomic DNA restriction fragments with the T_f 66 G2 probe is shown in fig. 4. The pattern obtained with 9 different restriction enzymes was similar for 4 individual leukocyte DNA samples, and did not significantly changed when hybridization, final washing were performed in stringent (2 x SSC at 65° C for the hybridization, final washing in 0.1 x SSC at 65°) or non stringent (6 x SSC for hybridization, final washing in 2 x SSC, both at 65°) conditions. Some enzymes gave a single predominant high molecular weight fragment (such as XbaI), some other ones more complex patterns, with 9 to 10 bands with Bam HI, 4 with PstI, 3 with Eco RI.

When the insert was cut by PstI and Hind III, three fragments were generated, of 214, 364 and 423 bases. These fragments were individually purified and labeled by nick-translation, then hybridized to the Southern blots. While hybridization with the uncleaved probe gave 3 Eco RI fragments, of 8, 4 and 1.6 k bp, the 423bp 3' Hind III-

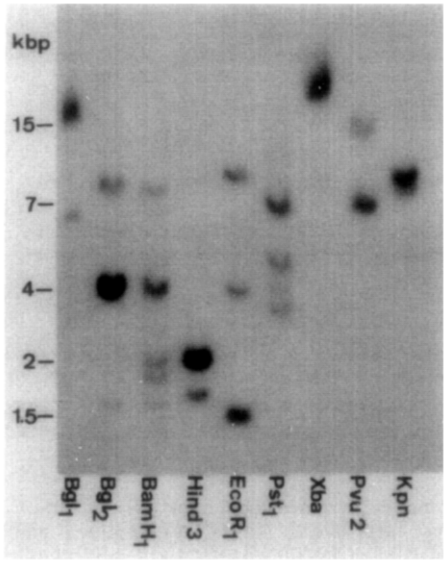

Fig. 4. Southern blot analysis of human leukocyte DNA with the T_f 66 G2 insert as a probe.

10 ug DNA were digested to completion with the various restriction enzymes indicated under the figure, then electrophoresed in a 0.7 % (w/v) agarose gel for 14 hours) at 1.5 volts/cm. After transfer to nitrocellulose, the blot was hybridized with the nick-translated insert, as indicated in Materials and Methods 10^6 cpm/ml, 18 hours hybridization). Washing was performed under stringent conditions (0.1 % SSC + 0.5 % SDS at 65° C). Exposure time : 36 hours at - 80° C with intensifying screens.

PstI fragment detected mainly the 8 kbp fragment and the 5' 214 bp PstI-PstI and 364 bp PstI-Hind III fragments detected only the 4 and 1.6 kbp fragments. in the same way, out of the 4 PstI fragments hybridizing with the uncleaved probe, the 6.8 and 3 kbp fragments were detected only with the 3' probe and the 4.2 and 3.8 fragments with the 5' probes (not shown).

DISCUSSION

To screen the human cDNA library, we selected those clones corresponding to mRNAs expressed in liver but not in muscle, with a sedimentation coefficient around 18-20 S, albumin mRNA being excluded. It is expected that liver-specific mRNAs of medium abundancy such as those encoding fibrinogen, prothrombin, antithrombin III, plasminogen, transferrin, etc... belong to this group. From a first group of 20 such clones we have been able to identify by hybridization-selection 3 clones for Aα and 1 clone for Bβ fibrinogen chains. Nucleotide sequence determination of the T_f66 G2 insert revealed unambiguously that the 72 000-75 000 Mr band synthesized under direction of mRNA hybridizing with this plasmid was pretransferrin. A fith clone, corresponding to a mRNA encoding a 76 000-80 000 Mr polypeptide, remains unidentified. The value of this approach was confirmed by studying the clones hybridizing more strongly with the 16-17 S than with the lighter or heavier liver mRNA-specific cDNA probes : among these clones we were able to identify 4 clones for Bβ and 4 clones for γ fibrinogen chains (Uzan et al, in preparation), plus clones

corresponding to five as yet unidentified liver-specific proteins (Mr from 32 000 to 44 000).

In most cases, we first characterize the preselected clones by hybridization-selection with immunological identification of the neosynthesized products (16-19). For transferrin, this approach was not successful because the antibodies raised against the seric protein poorly recognized neosynthesized transferrin (not shown), as we have previously shown for pre-prothrombin (16).

Comparison of the nucleotide sequence of clone T_f 66 G2 with the protein sequence published by Mac Gillivray et al (2) reveals 6 differences. Three, at the positions 417, 572 and 653, involves a Glu-Gln or Asp - Asn change, which could be accounted for by technical reasons. The three other differences (the deduced residue being Pro instead of Thr in position 539, Thr instead of Pro in position 542 and Gly instead of Glu in position 672) are more difficult to understand ; they could correspond either to a protein polymorphism, well described for transferrin, or to minor errors in the protein sequence determination. The sequence published by Mac Gillivray et al in 1983 (2) was already modified as compared to that reported in their 1982 paper (1).

Transferrin RNA is about 2400-2500 bases long, with a relatively short 3' non-coding sequence of 166 nucleotides and 2037 bases coding for the 679 aminoacid residues (2). Taking into account the poly A tract, the 5' non coding sequence should therefore be of the order of 150-200 bases.

Transferrin mRNA seems to be more abundant in the liver of a fetus at 4 months gestational age than in adult human liver. The most likely explanation is the known stimulation of transferrin synthesis induced by oestrogens (32-33), which are secreted at a high level during pregnancy.

The observed frequency of transferrin clones in human and rat cDNA libraries (about 3 %) is about 1:10 of that observed for albumin, which is roughly parallel to the relative synthesis rate of these proteins (34-35).

Hybridization of the genomic DNA restriction fragments with the Tf_1 66 G2 probe revealed with almost all the restriction enzymes used, more than one recognized band. The relative intensity of these bands did not change when hybridization and washing were performed either in stringent or non stringent conditions, which argues against the hypothesis that these different bands could correspond to partially cross hybridizing pseudo- or related genes. Moreover, probes constituted of the 3' or 5' part of the insert hybridized with different fragments, which seem therefore to be generated by cleavage of restriction sites inside a single gene rather than by hybridization with the different members of a gene family.

Quantitatively, the intensity of the hybridizing bands observed with the transferrin probe was similar to those observed for a same amount of DNA with probes to fibrinogen chains, aldolase B or L-type pyruvate kinase (not shown) which suggest that a single, or a low number of the transferrin gene copies exist. It appears

therefore that, at least with the cDNA probe used in this work the genes for the different members of the transferrin-related protein family are not recognized. This result does not preclude the possible utilization in the future of some subclones of genomic transferrin DNA for identifying some related genes. In addition, the availability of human and rat transferrin cDNA probes will permit to analyse the regulation mechanisms of the transferrin gene expression in response to hormones and iron.

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