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Rat complement factor I: molecular cloning, sequencing and expression in tissues and isolated cells

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SUMMARY

Factor I (FI) is a regulatory serine protease of the complement system which cleaves three peptide bonds in the α-chain of C3b and two bonds in the α-chain of C4b thereby inactivating these proteins. The human protein and the recently characterized mouse factor I are heterodimers of about 88 000 MW which consist of a non-catalytic heavy chain of 50 000 MW which is linked to a catalytic light chain of 38 000 MW by a disulphide bond. For the screening of a rat liver cDNA library we used a hybridization probe produced by polymerase chain reaction (PCR) using degenerated primers which corresponded to conserved parts of the human and the murine factor I nucleotide sequences. One of the identified sequences, which had a length of 2243 base pairs (bp), contained the complete coding region and the whole 3′ untranslated region. The length of the coding region in rat consisted of 1812 bp followed by a 3′ untranslated region of 207 bp including the polyadenylation signal and the beginning of the poly A tail. Comparison of the rat cDNA-derived coding sequence revealed identities of 87% to the mouse and of 78% to the human FI nucleotide sequence. The translation product of rat FI mRNA was 604 amino acid residues (aa) in length with an identity of 85% to the mouse (603 aa) and 69% to the human protein (583 aa). The comparison of the molecular mass predicted by the primary structure and derived from rat FI isolated from rat serum as detected in immunoblot analyses suggested a glycosylation of more than 20% of the total mass of the FI protein. Expression studies using reverse transcription (RT)–PCR assays indicated that FI-specific mRNA could neither be identified in B cells, nor in T cells, monocytes or granulocytes from rat and human peripheral blood nor in rat peritoneal macrophages. These data were in agreement with the results of RT–PCR obtained with several human lymphoma cell lines (Jurkat, MOLT-4, HUT102, Wil 2-NS, Ramos, Raji, U937) all of which were devoid of FI-specific mRNA. In accord with our data from two rat hepatoma cell lines (FAO and H4IIE) and one from man (HepG2) only isolated rat hepatocytes (HC) but neither Kupffer cells (KC), hepatic stellate cells (HSC; Ito cells) nor sinusoidal endothelial cells (SEC) expressed FI-specific mRNA. FI mRNA was also detected in human umbilical vein endothelial cells (HUVEC) and in the uterus and small intestine of the rat. Spleen and lymph nodes did not contain any detectable FI-specific mRNA.

INTRODUCTION

The activity of complement factor I (FI) was first described by Lachmann and Müller-Eberhard[1] and Tamura and Nelson.[2] It is a highly specific serine protease[3,4] which cleaves the α-chain of complement components C3b and C4b. For this reason it was termed C3b inactivator (C3b INA). Co-factors which are essential for the function of FI are complement factor H, soluble and membrane-bound complement receptor 1 (CR1), membrane cofactor protein (MCP) and C4b binding protein. The affinity of human FI for C3b is 15 times higher in the presence of factor H than in the absence of any of the cofactors.[5] The biological importance of FI becomes evident in recessive genetic deficiencies. In the case of homozygosity an increased turnover of C3 and factor B with secondary deficiencies of the two proteins is noted.[6,7] An increased susceptibility for infections, especially in childhood[6,7] emphasizes the role of FI for the homeostasis of the complement system.

The first purification of human FI to homogeneity was performed by Pangburn *et al.*[8] The glycoprotein isolated from serum had a relative molecular weight of 88 000 MW. It consisted of two polypeptide chains of 50 000 and 38 000 MW, respectively, linked by a disulphide bond. The carbohydrate

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content of human FI which was determined to be 11%[8] or 27%[9] is complex and variable resulting in a heterogeneous pattern of FI in isoelectric focusing gels.[10] The gene encoding human FI is located on chromosome 4.[11] It exceeds 63 kB and contains 13 exons.[12] The amino acid sequence of the light chain (catalytic subunit) was first determined by protein sequencing.[13] The complete primary structure has been determined by nucleotide sequencing of cDNA clones of man[11,14] mouse[15] and *Xenopus*.[16] The cDNA codes for a 583 amino acid residue single chain precursor peptide in man.[11,14] The length of the mouse or the rat precursor peptide is 603 or 604 amino acid residues, respectively.[15] Intracellular processing of the precursor peptide results in the removal of the leader sequence (18 aa) and the excision of the linking peptide (4 aa) to generate the final N-glycosylated heterodimeric protein.[9,17]

Several protein domains can be identified in the non-catalytic heavy chain which contains the C3b binding site and consists of 320 amino acid residues in man[11,14] and 342 residues in the mouse[15] according to a sequence alignment. The N-terminal module of FI heavy chain is a single C6/C7 membrane attack complex protein (FIMAC) module[18,19] also present at the carboxy terminal regions of complement components C6 and C7. This module is followed by a single cysteine-rich (SRCR) module which is also present in the macrophage scavenger receptor.[20] The third (low-density lipoprotein receptor type A (LDLRA)) module is a tandem repeat of the type A domain (seven tandem copies) of the low-density lipoprotein (LDL)-receptor and of C9.[18] In contrast to the first FIMAC module in FI, the other two modules are known to be present in membrane proteins and are not found in other proteases.

The catalytic small chain which contains 244 amino acid residues in man and 243 in mouse has an organisation similar to that of chymotrypsin-like serine proteases.[11,12,14] Three potential N-linked glycosylation sites are found in each chain; all six are assumed to be occupied because of the high carbohydrate content of FI (27%).[9] FI has been reported to be synthesized in the liver[9] with only a few reports of its production in other organs. Monocytes,[21] lymphocytes[22,23] glioma[24] and endothelial cells[25] were identified as a source of FI.

We report here the complete cDNA-derived sequence of rat FI and the alignment of this sequence with the published sequences of mouse and human FI. These data are supplemented by mRNA expression data (using reverse transcription–polymerase chain reaction (RT–PCR)) in peripheral blood cells of rat and man and in several B-cell, T-cell and monocyte-derived human cell lines. In the liver only hepatocytes (HC) expressed FI. Hepatic stellate cells (HSC, Ito cells), Kupffer cells (KC) and sinusoidal endothelial cells (SEC) did not express FI-specific mRNA. In addition, the human hepatoma cell line HEP G2 as well as the two rat lines FAO and H4IIE express FI. Our data do not confirm reports of FI expression in various blood cells and blood cell-derived leukaemia cell lines but they agree with the published data on the expression of FI in human umbilical vein endothelial cells (HUVEC).[25,26]

MATERIALS AND METHODS

Reverse PCR amplification of the complement FI hybridization probe and the FI-specific mRNA signals

Messenger mRNA of rat liver tissue was prepared with the RNeasy total kit (Quiagen, Hilden, Germany). The following transcription into cDNA was performed using the Super Script Preamplification system (Gibco BRL, Eggenstein, Germany). PCR was carried out with the degenerated sense primer Is 165 (5′-GTCTTCTGCCAGCCRTGGCAGAG-3′) [R = A + G] and the degenerated antisense primer Ia 864 (5′-GTRATG-CAGTCCACCTCACCATT-3′) [R = A + G] resulting in an amplificate of 699 nucleotides. PCR was performed using Taq polymerase (Sigma, Deisenhofen, Germany) (denaturing at 94° for 1 min, annealing at 55° at 1 min, extension at 72° for 1 min, for 35 cycles). The fragment was cloned into pBluescriptII KS+/– for sequencing. As a control for RT–PCR assays the following primer pairs were used to amplify β-actin cDNA. For human β-actin control amplification sense primer (5′-GCAAGGCCAACCGCGAGAAGATGA-3′) and antisense primer (5′-ATGGA-AGAGTGCCTCAGGGCAGCG-3′) were chosen, rat β-actin control amplification was carried out using sense primer (5′-GATATCGCTGCGCTCGTC-GTC-3′) and antisense primer (5′-CCTCGGGGCATCGGA-ACC-3′).

Library screening

A rat liver TriplEx oligo (dt) primed liver cDNA library (Clontech, Heidelberg, Germany) was screened by plaque hybridization assays on nylon membranes (Amersham-Pharmacia, Freiburg, Germany). The library was plated at a density of 5000 p.f.u. per 9 cm dish and 2×10^5 plaques were screened. Positive plaques were replated three times to yield pure clones. Hybridization was performed as described[27] with the amplified probe labelled using a random primed DNA labelling kit (Amersham-Pharmacia, Freiburg, Germany). Four positive clones were PCR-analysed to identify the insert length and two recombinant λTriplEx clones containing the largest inserts were converted to the corresponding plasmid pTriplEx using *Escherichia coli* BM25.8 cells according to the manufacturer's instructions.

DNA sequencing

DNA was sequenced using the dideoxy chain termination method.[28] The complete nucleotide sequence was determined with the pTriplEx specific primer pair λTriplEx5′ (5′-CTCGGGAAGCGCGCCATTGTGTTGG-3′) and λTri-plEx3′ (5′-ATACGACTCACTATAGGGCGAATTGG-3′), which was also used for the determination of the insert length of the screened FI fragments by PCR. Both the sense and the antisense strand of the largest insert were sequenced completely. Besides the above mentioned insert flanking primers several rat FI-specific primers were synthesized to continue the sequencing procedure. Nucleotide and amino acid sequence analyses were carried out using various programs of the Genetic Computing Group (GCG) package.

Purification of human factor H

Factor H was purified from human serum,in principle, as described previously.[29] Highly purified factor H preparations were obtained by immunoaffinity chromatography using a monoclonal anti-factor H antibody (mAb C18/3) generated in our laboratory. Fifteen milligrams of this mAb were coupled to 8 ml of CNBr-activated Sepharose according to the supplier's instructions. 200 ml of human serum was diluted 1 : 4 with 50 mM Tris–HCl pH 8·0 and applied to the immunoaffinity column. Loading was carried out over night with a flow rate of

25 ml/hr. The matrix was washed with at least 15 column volumes of equilibration buffer. Captured factor H was eluted with 3·2 M $MgCl_2$ dissolved in equilibration buffer. The flow rate was 20 ml/h. Eluted Factor H was analysed by sodium dodecyl sulphate –polyacrylamide gel electrophoresis (SDS–PAGE) according to standard protocols. Only one protein band of about 150 000 MW was detected in Coomassie Blue staining.

Isolation of blood cells

Mononuclear leucocytes from three human donors and 30 Wistar rats of different weight were isolated from ethylenediamine tetra-acetic acid (EDTA)–blood according to the following procedure. The blood was diluted 1 : 3 with phosphate-buffered saline (PBS) and put on a Lymphoprep layer of a density of 1·077 g/cm^3 (Nycomed, Oslo, Norway). After a centrifugation step at 1300 ***g*** for 30 min the mononuclear leucocytes from the interphases were pooled and washed three times with PBS. During the washing steps all tubes were kept on ice to prevent the monocytes from adhering to surfaces. The interphase cells were then cultured in medium consisting of 80% RPMI/20% Medium 199/10% fetal calf serum (FCS) (called Medium 80/20/10) at room temperature (RT) for 90 min in tissue culture Petri dishes. Non adherent B- and T-cell fractions could easily be rinsed off the culture dishes, whereas monocytes with a maximal adherence after a culture time of 90 min were scraped from the Petri dishes (monocyte fraction). Both cell fractions were analysed by fluorescence-activated cell sorting (FACStar plus, Becton Dickinson, Heidelberg, Germany) using standard antibodies to characterize the cell populations (anti-CD3 for T cells (mAb SK7, Becton Dickinson), anti-CD19 for B cells (mAb 4G7, Becton Dickinson) and anti-CD64 for monocytes (mAb 10.1, Calbiochem, Bad Soden, Germany)). Rat cell fractions were also analysed using the mAb W3-13HLK (Biozol, Eching, Germany) for T cells, mAb Ox33 for B cells (Biozol) and mAb ED1 (BAM, Augst, Switzerland) for monocytes. To isolate polymorphonuclear granulocytes from human and rat blood the pellet containing erythrocytes and granulocytes was exposed to a hypertonic medium to lyse the erythrocytes. An incubation in 13 mM NH_4Cl solution (50 ml) on ice for 10 min resulted in nearly complete lysis of the erythrocytes while the granulocytes remained intact. After the lysis the cells were sedimented at 200 ***g*** for 10 min. The pellet was exposed to a second lysis step as before. After an additional centrifugation step (200 ***g*** for 10 min) the remaining granulocytes were washed twice with PBS. A contamination about 2% erythrocytes remained according to cell counting in the Neubauer chamber. For every cell fraction vitality and concentration were determined using Trypan-blue exclusion.

Isolation of rat peritoneal macrophages and different liver cell types

The peritoneum of a Wistar rat was opened and washed out with ice-cold PBS using a pasteur pipette to obtain the peritoneal cells. Because the cell suspension contained fat cells and erythrocytes from injured blood vessels peritoneal macrophages were allowed to become attached to tissue culture Petri dishes in medium 80/20/10 for 2 hr. Fat cells and erythrocytes were rinsed off the dishes and macrophages were harvested using a cell scraper.

The different liver cell types (HC, KC, HSC and SEC) were obtained, separated and, in the case of enzymatic digestion, additionally cultured for 48 hr as described in detail elsewhere.[30]

Cultivation of HUVEC and tumour cell lines. Use for RT–PCR assays

Human umbilical vein endothelial cells were obtained on dry ice from PromoCell (München, Germany) and cultured in a special medium commercially available from the supplier.

The rat hepatoma cell lines FAO and H4IIE as well as the human hepatoma line HEPG2 belonged to the cell stock of the Institute of Biochemistry and Molecular Cell Biology (Georg-August University, Göttingen, Germany) and were cultured in medium 80/20/10. All other human tumour cell lines had originally been obtained from the American Type Culture Collection (ATCC, Rockville, MD). These were the B-cell line Wil 2-NS, the Burkitt lymphoma lines Ramos (RA1) and Raji, the monocyte-like U937 as well as the T-cell leukaemia lines Jurkat, HUT102 and MOLT-4. The monocyte-like lymphoma line U937 was used for mRNA extraction before and after stimulation with 1 mM dibutyryl cAMP (Sigma, Deisenhofen, Germany) for 72 hr. Nearly 10^7 cells of each preparation were used for mRNA extraction. The reverse transcription into cDNA and PCR was performed as described above.

Preparation of rat tissues. Use for RT–PCR assays

Tissue slices of various rat organs weighing between 10 and 20 mg were subjected to treatment with the RNeasy total kit (Quiagen, Hilden, Germany) as described above. Care was taken that the slices were not entirely cut from the periphery of an organ but contained central parts of the tissue thus representing as many different cell types as possible. The procedures of reverse transcription and PCR were performed as described above.

Immunoblot analysis of FI from rat serum

SDS–PAGE and the following immunoblot analyses were performed according to standard protocols. Briefly, 4 µl of rat serum was boiled in SDS-sample buffer under reducing or not-reducing conditions for 5 min. Serum proteins were separated using an SDS-minigel system (Minigel, Biometra, Göttingen, Germany) and transferred onto a nitrocellulose sheet under a current of 150 mA for 2 hr using a semidry transfer chamber (MultiphorII-Novablot, Pharmacia, Freiburg, Germany). The nitrocellulose sheet was blocked with 2% bovine serum albumin (BSA) (30 min) and afterwards incubated with a polyclonal anti-FI antibody (originally generated against human FI) from sheep (The Binding Site, Birmingham, UK) at a dilution of 1 : 500 for 2 hr. As the secondary antibody a peroxidase-conjugated rabbit anti-sheep immunoglobulin G (IgG) antibody (Dianova, Hamburg, Germany) was used at a dilution of 1 : 1000 (2 hr). The colour reaction was developed with diaminobenzidine (Sigma, Deisenhofen, Germany) and H_2O_2 according to standard protocols.

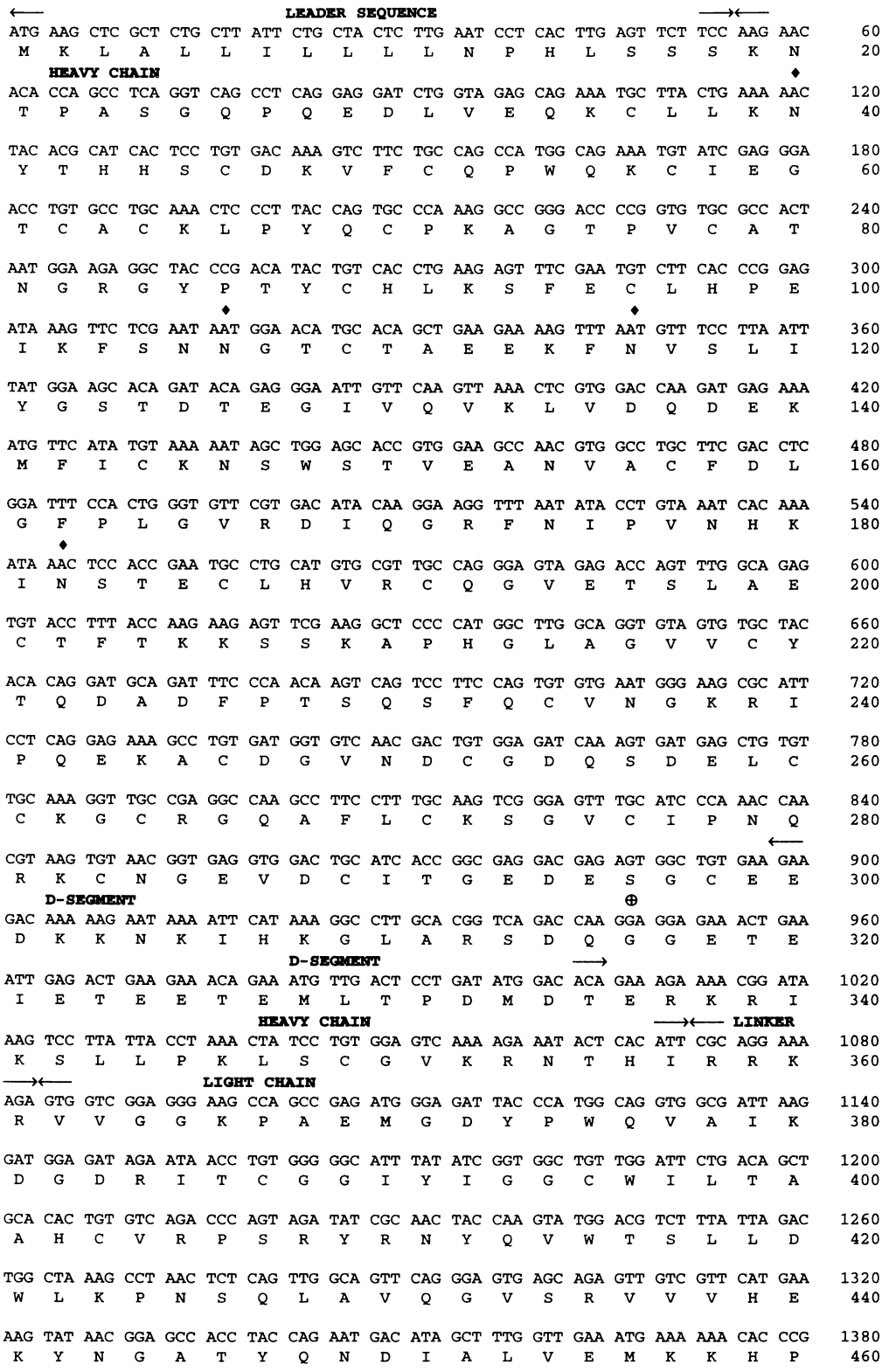
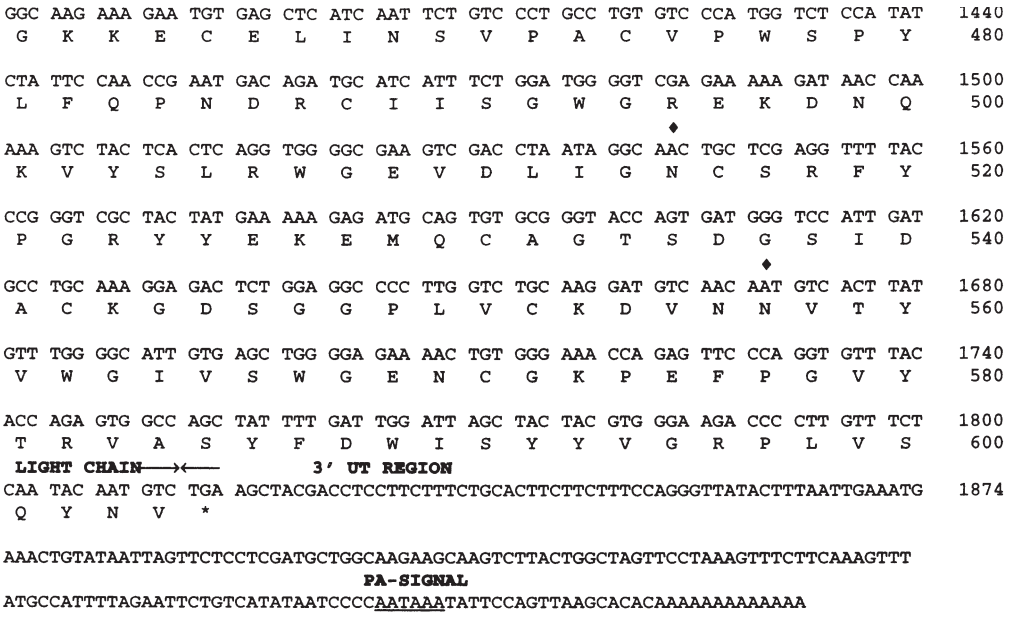


Figure 1. Nucleotide sequence and deduced primary structure for rat FI precursor protein. PA-signal: polyadenylation signal, 3′ UT region: 3′ untranslated region.



⊕ Additional glycine as the only additional aa in comparison with the mouse primary structure

♦ Potential N-glycosylation sites

Figure 1 (continued)

RESULTS

Nucleotide sequence analysis of rat FI cDNA

Degenerate primers were designed to generate a hybridization probe for library screening. The published sequences of man and mouse were investigated for conserved nucleotide sequences and wobbles were introduced to obtain best nucleotide fit. A fragment of nearly 700 bp was amplified using the primers Is 165 and Ia 864. After cloning the fragment into pBluescriptII KS+/– the nucleotide sequence was partially determined to identify the amplified fragment as the corresponding part of rat FI. The identity between the corresponding nucleotides of rat and mouse was between 80 and 89% according to the fragments' sequences. After the identification as a part of rat FI the rat liver cDNA library, which was oligo dt-primed and therefore should contain the complete 3′ end of the mRNA, was screened. Four positive clones carrying the FI inserts were selected and converted to the corresponding plasmid pTriplEx using BM25.8 cells for sequencing. The largest insert was 2234 bp in length. Sequencing revealed the whole coding region (Fig. 1) spanning 1812 bp followed by a stop codon and a 3′-untranslated region of 207 bp containing the polyadenylation signal 170 bp after the stop codon and the beginning of the poly A tail at the end (GenBank accession number Y18965). The sequence of 215 bp preceding the start codon (not documented in Fig. 1) did not represent the 5′-untranslated region and was probably part of a 3′-untranslated region of an unknown gene because it contained an AATAAA-polyadenylation signal and the beginning of the poly A tail. For this reason the linkage of this unknown sequence to the rat FI gene is most probably an artefact originated by the generation of the library. In addition two of the other clones were partially sequenced (nucleotide pos. 523–676, 662–1343, 1284–1840 according to Fig. 1). The sequences obtained by these two incomplete clones with an insert of nearly 1350 bp and 700 bp, respectively, confirmed the sequence of base pairs identified by sequencing the largest clone which contained the whole coding region.

Comparison of the rat FI amino acid sequence with the sequences of man and mouse

A comparison of the rat FI cDNA coding sequence revealed identities of 78% to the human FI[11,14] and of 87% to the mouse FI.[15] The amino acid sequences deduced from the cDNA for rat, mouse and human FI were aligned to achieve the highest sequence homologies (Fig. 2). The translation product of the open reading frame of rat FI mRNA was composed of 604 amino acid residues. The murine protein showed the highest amino acid sequence identity to the rat protein (85%), the human protein had only 69% identity. Like the primary structures of both the mouse and the human preprotein, the rat prepro-FI also possessed the domains signal peptide–heavy chain–linking peptide–light chain. A comparison of the individual domains revealed that the light chain that contains the catalytic subunit is somewhat more conserved (88·5% between rat and mouse, 73% between rat and man) than the heavy chain (82% between rat and mouse, 67% between rat and man). The so called D-segment (pos. 299–335 in Fig. 2) near the carboxy terminus of the heavy chain[15] showed much more identity between rat and mouse with 27/37 aa than between rat and man with 10/37 aa. Especially, this segment, which in man is 17 residues shorter than in the rat and 16 residues shorter than in the mouse, explained the fact that the heavy chain of rat has one more aa than the mouse heavy chain. According to the

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        1←LEADER SEQUENZ →←  HEAVY CHAIN                          50
FImouse MKLAHLSLFL LALHLSSSRS PSASDLPQEE LVDQKCLLQK YTHRSCNKVF
FIrat   MKLALLILLL LNPHLSSSKN TPASGQPQED LVEQKCLLKN YTHHSCDKVF
FIhum   MKLLHVFLLF LCFHLRFCKV TYTS...QED LVEKKCLAKK YTHLSCDKVF

        51                                                    100
FImouse CQPWQRCIEG TCICKLPYQC PRAGTPVCAM NGRSYPTYCH QKSFECLHPE
Firat   CQPWQKCIEG TCACKLPYQC PKAGTPVCAT NGRGYPTYCH LKSFECLHPE
FIhum   CQPWQRCIEG TCVCKLPYQC PKNGTAVCAT NRRSEPTYCQ QKSLECLHPG

        101                                                   150
FImouse IKFSHNGTCA AEGKFNVSLI YGRTKTEGLV QVKLVDQDER MFICKNSWSM
FIrat   IKFSNNGTCT AEEKFNVSLI YGSTDTEGIV QVKLVDQDEK MFICKNSWST
FIhum   TKFLNNGTCT AEGKFSVSLK HGNTDSEGIV EVKLVDQDKT MFICKSSWSM

        151                                                   200
FImouse AEANVACVDL GFPLGVRDIQ GSFNISGNLH INDTECLHVH CRGVETSLAE
FIrat   VEANVACFDL GFPLGVRDIQ GRFNIPVNHK INSTECLHVR CQGVETSLAE
FIhum   REANVACLDL GFQQGA.DTQ RRFKLS.DLS INSTECLHVH CRGLETSLAE

        201                                                   250
FImouse CAFTKRRTEL SNGLAGVVCY KQDADFPTSL SFQCVNGKHI PQEKACNGVN
FIrat   CTFTKKSSKA PHGLAGVVCY TQDADFPTSQ SFQCVNGKRI PQEKACDGVN
FIhum   CTFTKRRTMG YQDFADVVCY TQKADSPMDD FFQCVNGKYI SQMKACDGIN

        251                                                   300
FImouse DCGDQSDELC CKGCRGNASL CKSGVCIPDQ YKCNGEVDCI TGEDESRCEE
FIrat   DCGDQSDELC CKGCRGQAFL CKSGVCIPNQ RKCNGEVDCI TGEDESGCEE
FIhum   DCGDQSDELC CKACQGKGFH CKSGVCIPSQ YQCNGEVDCI TGEDEVGC..

        301                                HEAVY CHAIN        350
FImouse DRQQNIPKGL ARSAQ.GEAE IETEETEMLT PGMDNERKRI KSLLPKLSCG
FIrat   DKKNKIHKGL ARSDQGGETE IETEETEMLT PDMDTERKRI KSLLPKLSCG
FIhum   .......... .....AGFAS VAQEETEILT ADMDAERRRI KSLLPKLSCG
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Figure 2. Alignment of the amino acid sequences of mouse (FI mouse), rat (FI rat) and man (FI hum) FI precursor protein according to the PILEUP GCG-program. LIPEP: linker peptide (RRKR), *N*: potential N-glycosylation sites, the aa of the D-segment are underlined.

amino acid sequence alignment (Fig. 2) the glycine residue at position 316 resulted in the only additional residue in comparison with the mouse sequence (604 residues of rat prepro FI in comparison with 603 aa in mouse and 583 aa in man).

The pattern of potential N-glycosylation sites differed between the three species though the number of potential glycosylation sites is similar (six in the human, seven in the mouse and six in the rat aa sequence). Four of these potential positions were conserved in all three species (Fig. 2): N_{106}, N_{182}, N_{516} and N_{558}. N_{116} was only conserved in mouse and rat but not in the human sequence, whereas other potential glycosylation sites could only be found in one of the three species. The molecular mass predicted by the analyses of the cDNA-derived sequences (38 000 MW for the heavy chain and 28 500 MW for the light chain), in comparison with the rat FI mass derived by immunoblot analysis of whole rat serum, demonstrated that the sequence-deduced mass is much lower than the relative molecular weight of serum FI with a heavy chain of nearly 50 000 MW and a light chain of nearly 38 000 MW (Fig. 3). Thus in the rat most or all of the six potential glycosylation sites may possibly be used resulting in a glycosylation of more than 20% of the total molecular weight.

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          351  →LIPEP←  LIGHT CHAIN                              400
FImouse VKRNTHTRRK RVIGGKPANV GDYPWQVAIK DGQRITCGGI YIGGCWILTA
FIrat   VKRNTHIRRK RVVGGKPAEM GDYPWQVAIK DGDRITCGGI YIGGCWILTA
FIhum   VKNRMHIRRK RIVGGKRAQL GDLPWQVAIK DASGITCGGI YIGGCWILTA

          401                                                   450
FImouse AHCVRPSRAH SYQVWTALLD WLKPN.SQLG IQTVKRVIVH EKYNGATFQN
FIrat   AHCVRPSRYR NYQVWTSLLD WLKPN.SQLA VQGVSRVVVH EKYNGATYQN
FIhum   AHCLRASKTH RYQIWTTVVD WIHPDLKRIV IEYVDRIIFH ENYNAGTYQN

          451                                                   500
FImouse DIALIEMKMH TGKKECELPN SVPACVPWSP YLFQPNDRCI ISGWGRGKDN
FIrat   DIALVEMKKH PGKKECELIN SVPACVPWSP YLFQPNDRCI ISGWGREKDN
FIhum   DIALIEMKKD GNKKDCELPR SIPACVPWSP YLFQPNDTCI VSGWGREKDN

          501                                                   550
FImouse QKVYSLRWGE VDLIGNCSQF YPDRYYEKEM QCAGTRDGSI DACKGDSGGP
FIrat   QKVYSLRWGE VDLIGNCSRF YPGRYYEKEM QCAGTSDGSI DACKGDSGGP
FIhum   ERVFSLQWGE VKLISNCSKF YGNRFYEKEM ECAGTYDGSI DACKGDSGGP

          551                                LIGHT CHAIN        600
FImouse LVCEDTNNVT YVWGIVSWGE NCGKPEFPGV YTRVANYFDW ISYHVGRSLV
FIrat   LVCKDVNNVT YVWGIVSWGE NCGKPEFPGV YTRVASYFDW ISYYVGRPLV
FIhum   LVCMDANNVT YVWGVVSWGE NCGKPEFPGV YTKVANYFDW ISYHVGRPFI

          601→
FImouse SQHNV*
FIrat   SQYNV*
FIhum   SQYNV*
```

Figure 2 (continued)

Lack of the expression of FI in peripheral rat and human blood leucocytes and in rat peritoneal macrophages

Peripheral blood leucocytes from rat and man were isolated as described in Materials and Methods. The isolated cells were checked for their homogeneity by FACS analyses using cell-specific monoclonal antibodies. The monocytes of rat and man were found to be homogeneous for 86 and 90%, respectively. Granulocytes of both rat and man obtained from the pellet of the density gradient centrifugation step after lysis of the erythrocytes were found to be nearly homogeneous (> 98%). The B- and T-cell fractions obtained from both species after separating them from the adherent monocytes by washing the cells with ice-cold PBS had a B-cell content of 20% (man) and 16% (rat). Their T-cell content was 78% (rat) and 75% (man). All cell types were thus sufficiently enriched for the following analyses. In RT–PCR assays neither rat nor human peripheral blood cells showed FI mRNA expression (Fig. 4); the control amplificates of β-actin mRNA were clearly demonstrable in all cell types. The reliability of the RT–PCR assay was secured by demonstrating FI mRNA in the rat hepatoma cell line H4IIE and the human hepatoma cells HepG2 (Fig. 4). Thus, it appears that FI is not expressed in peripheral blood cells. In addition to blood monocytes peritoneal macrophages from rat representing a more differentiated kind of macrophages were also investigated for FI mRNA and found not to express FI-specific mRNA (Fig. 4).

Expression of FI mRNA in rat liver cells and rat and human hepatoma cell lines

After peripheral blood cells had been excluded as a site of extrahepatic production of FI isolated rat liver cells were investigated for their FI-specific mRNA content. As a result, only hepatocytes, the parenchymal cells, could be shown to generate a FI-specific amplificate (Fig. 5). The other non-parenchymal cell types (KC, HSC and SEC) were negative for FI mRNA; the control amplification of β-actin cDNA demonstrated that the RT–PCR procedure was performed

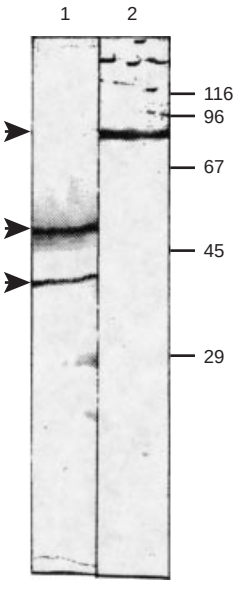

Figure 3. Immunoblot analysis of FI from rat serum under reducing (lane 1) and non-reducing (lane 2) conditions. Factor I was identified using a polyclonal antibody from sheep followed by a secondary peroxidase-conjugated anti-sheep IgG antibody. In lane 1 both aa-chains about 50 000 MW (heavy chain) and 38 000 MW (light chain) are indicated (small arrowheads) whereas in lane 2 the whole protein about 88 000 MW (large arrowhead) is visualized.

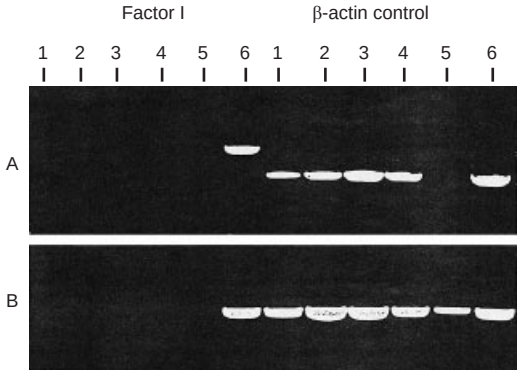

Figure 4. RT–PCR detection of Factor I mRNA and β-actin mRNA isolated from human (A) and rat (B) peripheral blood cells, from rat peritoneal macrophages and rat and human hepatoma cells. No mRNA specific for FI was present in the peripheral blood mononuclear leucocyte fraction (PBMNL) (1A, 1B), in the B/T cell fraction (2A, 2B), in monocytes (3A, 3B) or in granulocytes (4A, 4B). In lane 5A cDNA was not applied whereas in lane 5B mRNA from rat peritoneal macrophages did not show a FI-specific amplificate. Factor I mRNA was detected in human HepG2 cells (6A) and in rat H4IIE cells (6B). All corresponding human (A) and rat (B) β-actin control amplificates are distinctly visible. The rat β-actin specific amplificate (749 bp) was nearly of the same size as the FI-specific amplificate (699 bp), the human β-actin amplificate had about 450 bp.

correctly. In addition to the various primary liver cells two rat hepatoma cell lines (FAO and H4IIE) and one human hepatoma cell line (HepG2) were investigated for FI mRNA expression (Fig. 5). All three hepatocyte-derived tumour cell lines expressed FI-specific mRNA.

Messenger RNA specific for FI is not detectable in the human monocyte-derived cell line U937, the unstimulated lymphoma lines Wil2-NS, Raji, Ramos, Molt 4, Jurkat, HUT102, but is present in HUVEC

In addition to normal peripheral blood cells from rat and man various human blood cell-derived cell lines from man were probed for FI-specific mRNA expression. The monocyte derived cell line U937 was investigated with and without stimulation by dibuturyl cAMP. As shown in Fig. 6 FI specific mRNA was not detectable in any of these cell lines, whereas HUVEC displayed a distinct signal of FI-specific mRNA. In accordance with the data from wild type cells lymphoma cells derived from B cells, T cells of different stages of differentiation and monocytes did not express FI-specific mRNA either (Fig. 6).

Factor I specific mRNA expression in various rat organs

Tissue slices of various rat organs were investigated for FI-specific mRNA expression by RT–PCR assays. Because of the lack of expression in rat and human peripheral blood cells (Fig. 4), the lymphatic organs spleen and lymph nodes were of special interest. In accordance with the results obtained with blood cells there were no FI-specific signals in both lymphatic organs or in the large intestine, kidney, heart, lung, placenta, aorta and central nervous system (brain tissue containing grey and white matter) and spinal cord of the lumbar area. A positive signal could be generated with the mRNA extracted from uterus, small intestine and liver (Fig. 7).

DISCUSSION

We report here the molecular cloning of the cDNA for rat complement FI (GenBank accession number Y18965) and the alignment of the aa-sequences of the rat, mouse and human proteins. We also provide data on the expression of FI in rat and human tissues and cells. In addition, we demonstrate that the only cell type in rat liver expressing FI is the hepatocyte. FI-specific mRNA expression in several other rat organs reveals that uterus and the small intestine besides liver contain the FI-specific transcription product.

As expected, the cDNA-derived nucleotide sequence of rat prepro-FI shows more identity with the mouse (87%)[15] than with the published human nucleotide sequence (78%).[11,14] Alignment of the aa sequences of the rat, mouse and human proteins demonstrates that three additional nucleotides of rat FI result in an additional glycine at position 316, in the so called D-segment near the carboxyterminus of the heavy chain. Also, in the rat this D-segment – as in the mouse – consists of four subregions in comparison with only two subregions in the human species. The greater length of rat and mouse FI of 604 and 603 aa, respectively, as compared with the human FI of 583 aa is mainly the result of a gain of 17 or 16 aa in the less conserved D-segment (Fig. 2).

The general organization of rat FI prepro form does not differ from that of mouse and man. They all consist of the following parts: signal peptide, heavy chain, linker peptide,

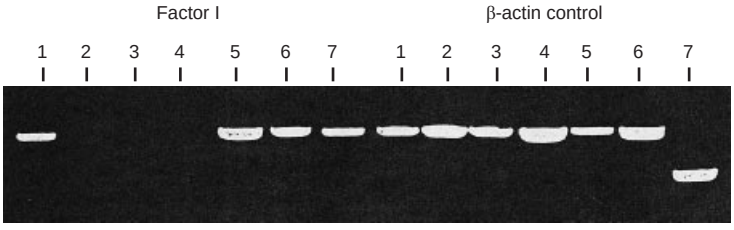

Figure 5. RT–PCR detection of FI-specific mRNA prepared from isolated rat liver cells and rat and human hepatoma cell lines. Factor I mRNA could be detected in hepatocytes (1), the rat hepatoma cell lines FAO (5) and H4IIE (6) and the human hepatoma line HEPG2 (7). No specific mRNA was detectable in Kupffer cells (2), hepatic stellate cells (3) and sinusoidal endothelial cells (4). The corresponding β-actin control amplificates (1–6 for rat β-actin, 7 for human β-actin) were clearly present.

light chain. The linker peptide with the aa RRKR motif is conserved in the three species. A detailed discussion of the aa sequence of the mouse has been presented by Minta *et al.*[15] Because of the high level of identity between rat and mouse FI, nearly all aspects mentioned in that report are applicable to both proteins. But there is a discrepancy concerning the N-linked glycosylation sites. Minta *et al.*[15] report six potential N-linked glycosylation sites for mouse prepro FI at the positions 116, 174, 182, 267, 516 and 558. Position 106, which is conserved in rat and man, represents an additional site in mouse FI not formerly recognized (all positions according to Fig. 2). The positions 106, 182, 516 and 558, i.e. four of six (in rat and man) and four of seven (in mouse) of the potential glycosylation sites are conserved in all three species (Fig. 2).

The high carbohydrate content of nearly 25% of rat FI is confirmed by the size of the rat glycoprotein identified by immunoblot analysis of rat serum (Fig. 3). The heavy chain of 50 000 MW differs by about 12 600 MW (25·2%) from the predicted molecular weight of 37 400 MW based on the aa composition. The light chain of 36 000 MW displays a difference of about 9 000 MW (25%) from the aa-deduced molecular weight of 27 300 MW. Therefore, our data on the carbohydrate content of rat FI correspond more closely with the data of Goldberger *et al.*[9] who reported a carbohydrate content of 27% for human FI than with those of Pangburn *et al.*[8] who estimated the carbohydrate content to be about 10·7%.

As demonstrated in Fig. 5 the only rat liver cell type which produces FI is the hepatocyte. The non-parenchymal cell types KC, HSC and SEC apparently do not express the gene (Fig. 5). So far investigations to identify the cellular origin of FI in normal liver cells have not been performed; only various hepatoma lines had previously been shown to synthesize FI.[9] It is interesting that the expression of the soluble cofactor for FI, i.e. factor H, in the liver differs from that of FI. Besides HC also KC express factor H-specific mRNA (data not shown). It is now obvious that FI biosynthesis in the rat liver is restricted to HC. Whether this holds true also for other species has not yet been shown.

A few reports concerning the extrahepatic production of FI have been published. Our data are in accordance with the common view that the liver is probably the main site of FI biosynthesis. Whaley[21] found FI biosynthesis in human peripheral blood monocytes. The isolation procedure and the purity of isolated monocytes were similar to those utilized in this investigation but the results concerning FI expression differ. Whaley[21] used a complex and indirect functional assay in which the lysis of erythrocytes was measured spectrophotometrically. However, as was mentioned the C3b inactivation measured in this assay could have been caused by other hydrolases/enzymes present in the supernatant of the cultured monocytes. The result of the RT–PCR assays reported here support this hypothesis of an 'unspecific' C3b inactivation because neither in peripheral blood monocytes nor in the monocyte-like cell line U937 (unstimulated or stimulated with dibuturyl cAMP) was FI-specific mRNA detectable (Fig. 6). The same negative result was obtained with rat peritoneal macrophages.

Lambris *et al.*[22] reported the synthesis of FI by B cells and the Burkitt lymphoma line Raji in response to triggering by factor H. An immunodiffusion, a rosette forming and a functional cleavage assay of ^{3}H-labelled C3b were used. All these led to the conclusion that B lymphocytes respond to

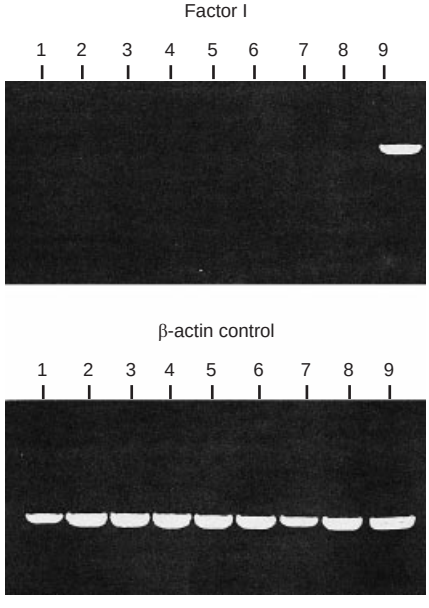

Figure 6. RT–PCR detection of Factor I mRNA isolated from several human lymphocytic cell lines and HUVEC. mRNA specific for FI could not be detected in unstimulated U937 cells (1) nor in U937 cells stimulated with db cAMP for 72 hr (2). FI specific mRNA was not found in the cell lines Wil2-NS (3), Raji (4), Ramos (5), Molt (6), Jurkat (7) and HUT102 (8) but was clearly demonstrable after amplification of HUVEC cDNA (9). The corresponding β-actin control amplificates are also shown.

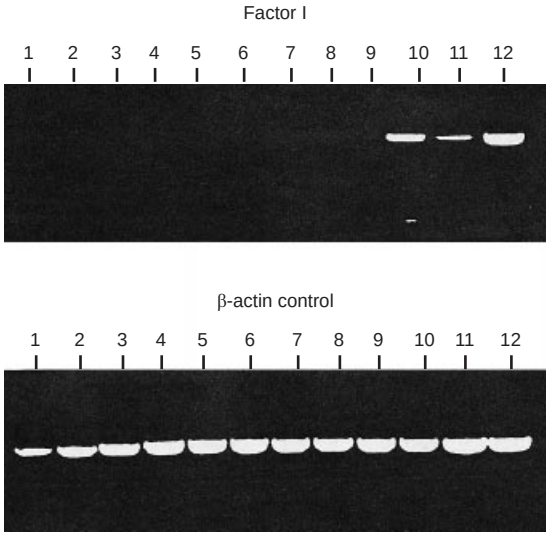

Figure 7. RT–PCR detection of FI mRNA isolated from various rat organs. Factor I specific mRNA was not found in heart (1), aorta (2), lung (3), kidney (4), large intestine (5), brain (6), spinal cord (7), lymph node (8) and spleen (9) but was detectable in liver (10), small intestine (11) and uterus (12). The corresponding control amplifications of β-actin cDNA are also shown.

factor H by releasing FI. The rate of FI release in the absence of factor H was reported to be very poor or under the detection limit which would be in line with the present finding that B/T cells do not express FI-specific mRNA. As we had used B cells without any stimulation, we repeated the RT–PCR assays using Raji cells and the B/T cell fraction from human blood after stimulation with factor H (purified by immunoaffinity chromatography). No FI-specific mRNA signals were generated by B cells or Raji cells without (Fig. 6) or with factor H being present in concentrations of 80 and 160 μg/ml for 6 hr (data not shown). Lambris and coworkers had used 30 μg factor H/ml for a stimulation period of 1 hr. As the concentration of factor H in human serum is between 200 and 600 μg/ml, B cells in the circulation would always be activated by factor H, and B cells isolated from blood would probably still be in a state of factor H-dependent activation. The discrepancy between the previous and our results cannot be explained at the present time. But according to the conventional purification method of factor H performed by Lambris *et al.*[22] the purified preparations may easily have contained some C3/C3b which might have played a role in the CR1-dependent activation of monocytes.

Vetvicka *et al.*[23] detected FI-specific mRNA in the monocyte-derived cell line U937 and the B-cell derived Raji cells using an RT–PCR assay and described FI protein secreted by these cell lines (U937, 220 ng/ml; Raji, 65 ng/ml). But they also reported the detection of FI in culture supernatants of the B-cell lines Wil-2, Daudi, U698M and the T-cell line 8402 in trace amounts. Factor I-specific mRNA in these four cell lines was not investigated. The trace amounts of FI secreted by these four cell lines may perhaps be considered as background signals because Vetvicka and coworkers defined the enzyme-linked immunosorbent assay (ELISA) range of sensitivity between 7·5 and 500 ng/ml and the calculated amounts of $\leq$ 4 ng/ml are less than half the value given as the lower detection limit.

Our data on the biosynthesis of FI by HUVEC are in accord with the results of Julen *et al.*[25] and Ripoche *et al.*[26] who reported mRNA and protein biosynthesis detected by Northern blot and Western blot analyses. A very weak signal in Northern blots found with mRNA from unstimulated HUVEC was increased 5–10-fold after stimulation with interferon-γ (IFN-γ).[25] The RT–PCR signal generated by the mRNA prepared from HUVEC was obtained in our lab without IFN-γ stimulation demonstrating the sensitivity of this assay.

The expression of FI-specific mRNA could also be demonstrated in the rat uterus and small intestine. Other methods such as *in situ* hybridization will have to define the cell types which may be responsible for FI biosynthesis in these organs. In accord with the data on rat and human lymphocytes from peripheral blood and the human lymphoma lines no FI-specific mRNA signal could be detected in slices of lymphatic tissues.

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