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Human complement factor H: isolation of cDNA clones and partial cDNA sequence of the 38-kDa tryptic fragment containing the binding site for C3b*

We isolated cDNA clones coding for the functionally important tryptic N-terminal 38-kDa fragment of human complement control protein factor H using polyclonal and monoclonal antibodies to screen a human liver cDNA library cloned in a bacterial expression vector, PEX-1. By testing the reactivity of antibodies specific for the recombinant proteins produced by individual clones with proteolytic fragments of serum H the exact position of these cDNA clones within H was mapped. One clone, H-19, coding for the 38-kDa fragment of H was sequenced and found to code for 289 amino acids derived from the 38-kDa N-terminal fragment as well as for the first 108 amino acids belonging to the complementary 142-kDa tryptic fragment. The derived protein sequence could be arranged in 6 highly homologous repeats of about 60 amino acids each, the homology between the repeats being determined by the characteristic position of cysteine, proline, glycine, tyrosine and tryptophane residues. The region coding for the epitope recognized by one of our monoclonal antibodies was localized by subcloning restriction fragments of H-19 into the expression plasmid and testing for the expression of this epitope.

1 Introduction

Human factor H is a plasma glycoprotein of approximate mol. mass of 150-kDa which regulates the C3 and C5 convertases of the alternative pathway of complement activation. It accelerates the decay of the alternative pathway C3 convertase (C3bBb) [1–3] and also acts as a cofactor for the enzyme factor I which cleaves the α chain of C3b, thus converting C3b to the hemolytically inactive iC3b [4]. In this mode of action it is similar to C4-binding protein, C4-BP, which mediates the cleavage of C4b by I [5] and to complement receptor type one (CR1 or C3b receptor), which as an integral membrane molecule also mediates the cleavage of C3b by factor I [6, 7]. As in the case of the three complement components needed to form the C3 convertases of the classical and alternative pathway, *i.e.* factors C4, C2 and B (for review see [8]), there is also a polymorphism of the three control proteins factor H, C4-BP and CR1 [9–11]. Individual alleles of H, C4-BP and CR1 are linked [12] and recent work indicates that this group of genes is localized on chromosome 1 in humans [13]. Apart from being a serum protein synthesized by hepatocytes, factor H is also found in platelets [14] and monocytes [15] and small amounts of H can be found to be associated with the plasma membrane of B lymphocytes [16]. Here it contributes to the binding of C3b-coated immune complexes to these cells in addition to CR1 [16]. Furthermore, H has been shown to bind to B lymphocytes and monocytes by means of a receptor [17, 18], to stimulate the respiratory burst of and to induce the release of

factor I from human monocytes [19, 20], as well as to induce proliferation in murine B cells [21].

Using monoclonal antibodies to H and by means of limited proteolytic digestion we have recently been able to localize functionally important domains on the molecule and to devise a model of the structure of factor H [16, 22, 23]. According to these results, factor H consists of subunits of at least 15-kDa mol. mass that are linked together by disulfide bridges and which can be separated from each other by cleavage with ficin. The binding site for C3b and the domain responsible for the cofactor function for factor I are localized on a N-terminal 38-kDa tryptic fragment of H which also carries the epitopes for 6 monoclonal antibodies. The function of the complementary 142-kDa tryptic fragment is unclear at present.

In this study we set out to isolate cDNA clones coding for this functionally important 38-kDa fragment of H.

2 Materials and methods

2.1 Factor H

Factor H was purified from fresh human plasma as previously described [22]. Without reduction the purified protein showed one band of 150-kDa on silver-stained sodium dodecyl sulfate-(SDS)-polyacrylamide gels. After treatment with 1% 2-mercaptoethanol the molecular mass shifted to 160-kDa and a fragment of 38-kDa became visible (see Fig. 2, Sect. 3.2), indicating that a small proportion of the factor H preparation used in this study had been exposed to proteolysis during the purification. Treatment of factor H with trypsin (enzyme: substrate ratio 1:100 w/w in phosphate-buffered saline, PBS), pepsin (e : s 1 : 1000 in 0.1 M N sodium citrate, pH 2) and elastase (e : s 1 : 10 in PBS, pH 8.5), was performed for 5 min and 60 min at 37°C and the reaction then stopped with 2 mM DFP (trypsin, elastase) or adjusting the pH to 7.5 (pepsin) and freezing at –20°C. Five-min and 60-min fragments obtained with each enzyme were then pooled and used on Western blots.

[I 5571]

* This study was supported by FWF P5253, and Genzentrum, München.

+ Part of W. Schwäble's Ph. D. thesis.

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Abbreviations: **B:** Factor B of the alternative complement pathway **C4-BP:** C4-binding protein **CR1:** C3b receptor **H:** Regulatory complement factor, formerly β 1H **I:** C3b inactivator **MAH:** Monoclonal anti-H antibody **PBS:** Phosphate-buffered saline **SDS:** Sodium dodecyl sulfate

2.2 Antibodies

A polyclonal goat antibody to H was affinity purified on Sepharose-bound H by standard methods. Monoclonal antibodies to H, MAH 1–3, were described previously [16] and MAH-4 was obtained in a similar fashion. OX-23 and OX-24, also directed at H, were a kind gift from Dr. R. Sim, Oxford, GB. All monoclonal antibodies bind to the 38-kDa N-terminal fragment of H and MAH-1, MAH-2, MAH-4 inhibit the binding of H to C3b as well as the cofactor function of H for I [16, 22, 23].

2.3 Screening of a human liver cDNA library

A human liver cDNA library cloned in the expression plasmid PEX-1 as previously described [24] was screened using an immunoperoxidase method [24] to detect colonies that reacted with a polyclonal antibody to H. Positive colonies were recloned and screened with an affinity purified antibody and with monoclonal antibodies to H.

2.4 SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot for fusion proteins

Two hundred μ l of an overnight bulk culture of a given clone were added to 20 ml of LB-broth and incubated at 30°C with vigorous shaking to an optical density (600 nm) of 0.2–0.3. The incubation temperature was then quickly raised to 42°C and the culture incubated for a further 90 min. The bacteria were pelleted by centrifugation and lysed in 2 ml of 50 mM Tris-HCl pH, 6.8 containing 5% SDS, 2% 2-mercaptoethanol, 10% glycerol and bromophenol blue. After boiling for 5 min, 20 μ l of this extract was electrophoresed on a 7.5–15% SDS-polyacrylamide gradient gel. Transfer to nitrocellulose (Western blot) and staining with polyclonal or monoclonal antibodies, peroxidase-conjugated second antibody and diaminobenzidine as substrate (0.5 mg/ml in Tris, 50 mM pH 7.4, 0.006% H₂O₂) was performed by standard methods [25].

2.5 Affinity purification of antibodies on fusion proteins

Ten to 20 lanes of a 7.5–15% polyacrylamide gradient gel were loaded with SDS extracts from a bacterial culture that had been induced to produce recombinant proteins as described above. After electrophoresis and Western blotting the nitrocellulose filter was blocked in washing buffer (PBS containing 0.1% Triton X-100 and 0.5% gelatine) and a small strip was cut off from one side. This strip was stained with polyclonal antibody to H, peroxidase-conjugated second antibody and substrate as above to localize the position of recombinant proteins containing a part of H.

After aligning the stained strip with the rest of the nitrocellulose filter areas of the filter containing these recombinant proteins were cut out and incubated with 1 ml of goat anti-H antibody in washing buffer for 1 h at room temperature. After three washes in washing buffer bound antibody was eluted with 1 ml of 0.1 M glycine, pH 2.2 (5 min at 4°C), and the eluted antibody was neutralized with 1.5 M Tris, pH 8.8 and diluted with an equal volume of washing buffer.

2.6 Preparation and sequence analysis of cloned cDNA

Small-scale plasmid preparations were performed by the alkaline-SDS method [26]. Large-scale plasmid preparations were further purified by isopycnic centrifugation on CsCl [27]. The 1.5-kb insert of clone H-19 was digested with *Sau* 3A, *Sma* and these fragments were subcloned into M13 for sequencing by the Sanger dideoxy chain termination method [28]. In the regions indicated in Fig. 5 (see Sect. 3.4) the sequence was confirmed by additional sequencing by the Maxam-Gilbert method [29].

3 Results

3.1 Isolation and characterization of cDNA clones for factor H from a human cDNA library cloned into an expression vector

Approximately 50 000 colonies of a previously reported human cDNA library [24] cloned into PEX-1, a bacterial high-efficiency expression vector, were screened with a polyclonal antibody to H using an immunoperoxidase method to detect positive clones. Out of 8 positive clones reacting with an affinity-purified polyclonal antibody to H, two designated H-2 and H-19 also reacted with two monoclonal antibodies to H, MAH-4 and OX-24. These two clones produced an identical fusion protein of approx. 160-kDa (Fig. 1) consisting of a 117-kDa α -galactosidase part derived from PEX-1 [24] and an approx. 40-kDa fragment of factor H. In addition, H-specific bands of about 40 and 50 kDa were observed. Since both H-2 and H-19 also contained an identical insert of 1.5 kb it was assumed that they were identical and analysis was continued with H-19. Another clone, H-3, containing an insert of 700 bp, reacted with monoclonal antibody MAH-1.

Three additional distinct clones reacting only with the affinity-purified antibody to H and containing inserts of 1.4 kb, 0.95 kb and 2.2 kb were identified and designated H-40, H-46 and H-49, respectively. They have been described in detail elsewhere [30].

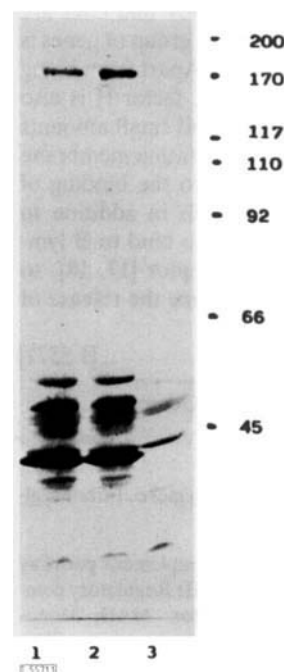


Figure 1. Western blot of recombinant proteins produced by H-2, H-19 and PEX-1. Twenty μ l of a lysate of bacteria containing plasmids H-2 (lane 1), H-19 (lane 2) or PEX-1 (lane 3) that had been induced to produce recombinant proteins as described in Sect. 2.4 was electrophoresed on a SDS-polyacrylamide gel and blotted to nitrocellulose. The blot was then stained with monoclonal antibodies MAH-4 and OX-24, peroxidase-conjugated second antibody and diaminobenzidine. Numbers on the right denote apparent mol. mass in kDa of marker proteins.

3.2 Mapping of the position of cDNA clones within H

Since the recombinant proteins of H-19 reacted with the two monoclonal antibodies MAH-4 and OX-24 known to bind to the 38-kDa tryptic N-terminal fragment of H [23], we concluded that H-19 codes for at least a part of this fragment. To map the position of H-19 more precisely with respect to the known proteolytic fragments of H, we affinity purified a polyclonal antibody to H on the H-specific proteins produced by H-19 and then tested to which of several proteolytic fragments of H this "clone-specific" antibody would bind. On Western blots of tryptic H fragments (partial digest, Fig. 2) "anti-H-19" bound to the 38-kDa fragment but not to the 142-kDa fragment or the 52-kDa and 95-kDa bands derived from the latter [22] indicating that H-19 should code for the 38-kDa fragment without overlapping with immunogenic regions within the 142-kDa fragment.

Since "anti-H-19" also reacted with pepsin fragments of 38-kDa, 42-kDa, 50-kDa, 55-kDa and 65-kDa as well as with

elastase fragments of 38-kDa and 28-kDa, these fragments (from a partial digest) must be contained in or extend into the tryptic 38-kDa fragment of H.

Similar experiments performed with H-46 and H-49 revealed that both clones code for regions within the 142-kDa tryptic fragment of H (not shown). Polyclonal antibodies affinity purified on H-3 and H-40 did not stain any of the H fragments (not shown) and could therefore not be mapped precisely.

3.3 Sequence analysis of H-19

A nucleotide sequence of 1429 base pairs was derived from H-19. The first 915 nucleotides translated into a protein sequence of 305 amino acids which could be arranged in 5 highly homologous repeats of about 60 amino acids each (Fig. 3). Likewise nucleotides 941 to 1193, if read in a different reading frame, also coded for a protein sequence with a similar internal homology pattern (Figs. 3 and 4). Therefore, H-19 must contain a shift in reading frame somewhere between nucleotides 915 and 941. With the help of a recently published partial sequence of H covering this region [31] the change in reading frame could be attributed to an additional A at position 930 present in H-19. As outlined in the discussion this is probably a cloning artefact and the complete protein sequence coded for by H-19 therefore comprised 397 amino acids forming 6 1/2 highly homologous repeats of about 60 amino acids each with the positions of cysteine, tryptophane, proline, glycine and tyrosine residues highly conserved (Fig. 3). The protein sequence contained one potential glycosylation site as asparagine residue 165 (Asn-Gly-Ser). The coding sequence of H-19 stopped at nucleotide 1193 and was followed by a 232 bp 3' untranslated region containing a putative polyadenylation signal (ATTAAA) located 15 bp upstream of a poly A tail.

3.4 Mapping of the position of the OX-24 epitope

To identify the region containing the epitopes recognized by MAH-4 and OX-24 the insert of clone H-19 was digested with *Sau* 3A. A pool of these fragments was cloned into the *Bam*HI site of PEX-1/2/3. After transformation of *E. coli* (strain POP2436) colonies were screened for the expression of MAH-4/OX-24 epitopes. Positive clones were chosen and their inserts analyzed by restriction enzyme digestion. Whereas the MAH-4 epitope seemed to be destroyed by subcloning we obtained one clone, H-19-6, reacting with OX-24, which was shown to contain an internal *Sma* site. This places the domain

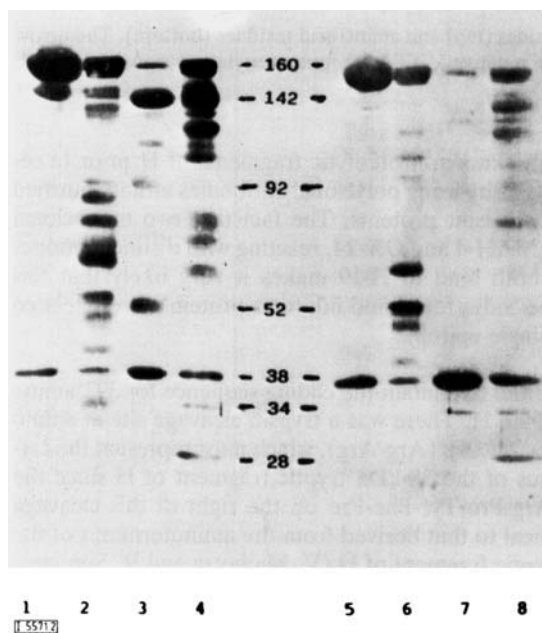


Figure 2. Mapping of the position of clone H-19 with respect to proteolytic fragments of H. A polyclonal antibody to H (lanes 1-4) and the same antibody previously affinity purified on the recombinant proteins produced by H-19 (Fig. 1) (lanes 5-8) were used to stain Western blots of purified H (lanes 1, 5) as well as peptic (lanes 2, 6), tryptic (lanes 3, 7) and elastase (lanes 4, 8) fragments from partial digests of H separated by SDS-PAGE. Numbers between the two blots denote apparent molecular mass in kDa.

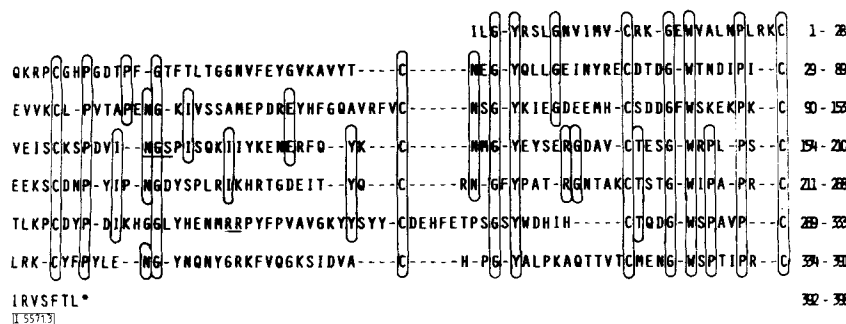


Figure 3. Assignment of the protein sequence derived from H-19 to 6 1/2 homology subunits of about 60 amino acids each. Identical amino acids present in several of the 6 homologous regions are boxed. Gaps denoted by "-" have been introduced to maximize homology and to allow space for the regions with short inserted sequences. The potential glycosylation site (residue 165; Asn-Gly-Ser) and the tryptic cleavage site (residues 289/290; Arg-Arg) marking the end of the 38-kDa tryptic N-terminal fragment are underlined.

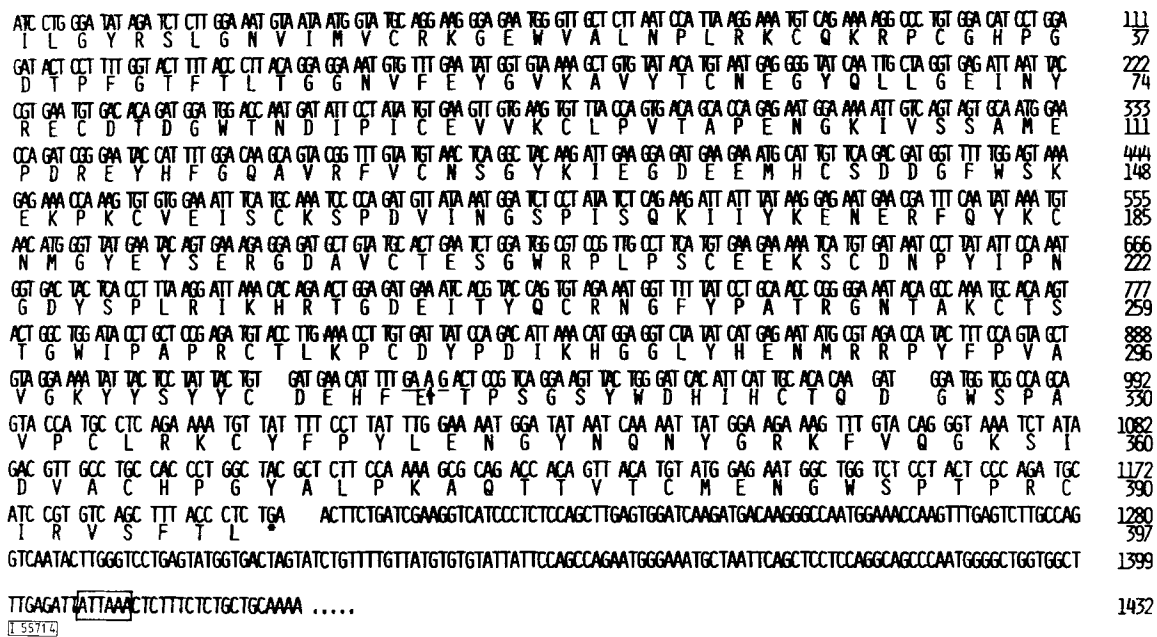


Figure 4. Nucleotide sequence derived from H-19. Numbers on the right denote nucleotides (top) and amino acid residues (bottom). The arrow denotes the additional A residue causing the observed shift in reading frame. The putative ATTTAAA polyadenylation signal is boxed. *: Stopcodon.

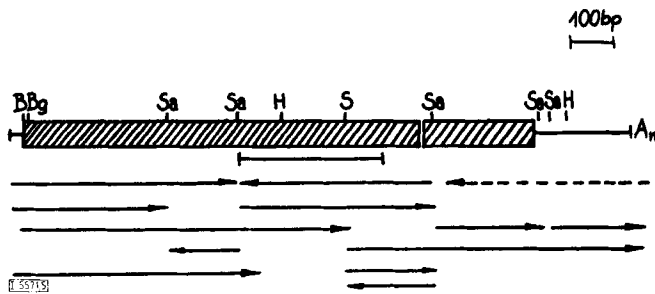


Figure 5. Insert of clone H-19: restriction map, position of the derived protein sequence and of the area containing the OX-24 epitope as well as strategy used for sequencing and site of the observed shift in reading frame. B: Bam HI, Bg: BglIII, Sa: Sau 3A, H: Hinf 1, S: Sma. The shaded areas denote the position of the protein sequence derived from H-19. The gap interrupting the two shaded areas represents the site of the observed shift in reading frame. Solid arrows represent the length of subclones and for sequencing by the sanger dideoxy chain termination method [28] and the direction of sequencing. The dashed arrow represents the piece of H-19 sequenced by the Maxam-Gilbert method [29]. The part of the sequence coding for the OX-24 epitope is designated by a bar (—).

coding for OX-24 between residues 167 and 290 of H-19 (i.e. between the Sau 3A site at nucleotide 501 and the carboxyterminus of the 38-kDa fragment, Fig. 5).

4 Discussion

The 38-kDa N-terminal tryptic fragment of H carries the binding site for C3b, and in purified form is capable of acting as a cofactor in the fluid-phase cleavage of H by I [22]. The use of monoclonal antibodies binding to this fragment [16, 23] for screening an expression library enabled us to identify two probably identical clones, H-2 and H-19, coding for this region of H. Apart from isolating cDNA clones the expression vector also permitted an exact mapping of the cDNA clones with

respect to the known proteolytic fragments of H prior to sequence analysis by using polyclonal antibodies affinity purified on the recombinant proteins. The fact that two monoclonal antibodies, MAH-4 and OX-24, reacting with distinct epitopes on H [32] both bind to H-19 makes it very likely that this cDNA clone codes for H and not for a protein merely related to H by a single epitope.

H-19 was found to contain the coding sequence for 397 amino acids of human H. There was a trypsin cleavage site at amino acid residues 289/290 (Arg-Arg), which must represent the carboxyterminus of the 38-kDa tryptic fragment of H since the sequence Arg-Pro-Tyr-Phe-Pro on the right of this cleavage site is identical to that derived from the aminoterminal of the 142-kDa tryptic fragment of H (V. Malhotra and R. Sim, personal communication). Therefore, H-19 codes for the first 108 amino acids of the 142-kDa fragment in addition to the 289 amino acids derived from the 38-kDa fragment. This was unexpected since antibodies affinity purified on the fusion protein produced by H-19 only reacted with the 38-kDa fragment of H (Fig. 2). However, as shown in Fig. 4, the cDNA sequence derived from H-19 contained an additional A residue at position 930 which was not observed in a recently published cDNA clone covering this particular part of H [31] and which caused a shift in reading frame. As a consequence only the first 20 amino acids of the 142-kDa fragment were expressed in the correct reading frame and this was probably not enough to absorb sufficient antibody that would react with the 142-kDa fragment. This also underlines that the additional A residue at position 930 was not a sequencing artefact but really present in H-19, probably due to an error that occurred during reverse transcription. The previously reported amino-terminal sequence of the 38-kDa fragment of H was not covered by the H-19 sequence. By analogy with the recently reported sequence for murine H [33] one would expect the aminoterminal of H to be about 30 amino acids from the beginning of the sequence derived from H19. Therefore, H-19 covers most of the 38-kDa amino-terminal fragment of H.

It was therefore surprising to find a poly A tail at the 3' end of H-19. However, exactly the same 3' untranslated region as well as a poly A tail were found in a recently published shorter cDNA clone for H [31]. A clone coding for the aminoterminal of the C5 α chain also contained an unexpected poly A tail [34]. Since all these as well as our clone were derived from acute phase liver cDNA libraries it is possible that this finding reflects the presence of alternative H specific mRNAs under acute phase conditions.

As shown in Fig. 3 the partial H sequence reported here revealed a marked internal homology and could be arranged in 6 1/2 homologous repeats of about 60 amino acids each with the positions of cysteine, glycine, proline, tryptophane and tyrosine residues highly conserved. Apart from human H, similar repeats have been found in murine H [33] as well as in C4BP [35] and CR1 [13], both cofactors for I in the cleavage of C4b and/or C3b [4, 5, 7] and probably encoded on the same chromosome as H [12]. It is therefore possible that these three cofactors for I evolved from a common ancestor gene by gene duplication. In addition, similar subunits have been observed in the N-terminal Ba fragment of complement factor B, in C2, in β_2 -glycoprotein I and interleukin 2 receptor [36, 37].

Subcloning restriction fragments of H-19 in PEX 1/2/3 allowed the localization of the OX24 epitope between amino acid residues 167 and 290 of H19. The fact that the MAH-4 epitope was destroyed by subcloning further underlines that OX-24 and MAH-4 recognize distinct epitopes as had been predicted on the basis of inhibition studies. The availability of a cDNA clone for human H will allow the study of the molecular organisation of the factor H gene on chromosome 1 and its relationship to the genes for CR1 and C4BP.

The excellent technical assistance of A. Sommerauer and the help of S. Wucherer with the preparation of the manuscript are gratefully acknowledged. We thank Drs. T. Kristensen, B. Tack, A. Day, J. Ripoché and R. B. Sim for helpful discussion.

Received May 9, 1986; in revised form July 28, 1986.

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