ORGANIZATION OF THE GENES ENCODING COMPLEMENT RECEPTORS TYPE 1 AND 2, DECAY-ACCELERATING FACTOR, AND C4-BINDING PROTEIN IN THE RCA LOCUS ON HUMAN CHROMOSOME 1

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The most critical step in the complement reaction sequence is the formation of two enzymes, termed the classical and alternative pathway C3/C5 convertases (1). These proteases activate the third (C3) and fifth (C5) complement components, resulting in the generation of protein fragments that induce chemotactic, phagocytic, growth, and differentiation responses of leukocytes, and assembly of the membrane attack complex of complement that has cytolytic activity. Thus, it is essential that the formation and function of the C3/C5 convertases be carefully regulated by other complement proteins to prevent their action in circumstances that would be deleterious rather than beneficial to the host.

Several proteins that have regulatory activities have been identified and the following complementary DNAs have been cloned: C4-binding protein (C4bp)[1] (2, 3), factor H (4–6), complement receptors type 1 (CR1) (7, 8) and type 2 (CR2) (9–11), and decay-accelerating factor (DAF) (12–14). C4bp and factor H are plasma proteins, whereas the others are found primarily on cell membranes. All proteins bind to C4b and C3b, the noncatalytic subunits of the classical and alternative pathway C3/C5 convertases, respectively, causing either inhibition of the binding of the catalytic subunits, C2 and factor B, or susceptibility to proteolytic inactivation by factor I, or both. CR1 and CR2 also serve as receptors on myelomonocytic cells and lymphocytes and mediate phagocytic, proliferative, or differentiation responses by these cells after the binding of C4/C3 ligands. CR2 also is the receptor for EBV (15, 16).

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[1] *Abbreviations used in this paper:* bp, binding protein; CR, complement receptors; DAF, decay-accelerating factor; EtBr, ethidium bromide; OFAGE, orthogonal field alternation gel electrophoresis; PFGE, pulsed field gradient gel electrophoresis; RCA, regulator of complement activation.

In addition to sharing overlapping regulatory functions, these proteins demonstrate the common structural feature of being composed entirely of short consensus repeats of 60–70 amino acids. Linkage studies based on protein (17–19) and DNA polymorphisms (20) indicate that these proteins also are related at the genomic level. Weis et al. (21) have positioned the genes for CR1 and CR2 at or near 1q32 by in situ hybridization. This linkage group has been proposed as the regulator of complement activation (RCA) locus and contrasts with the class III region of the human major histocompatibility locus that contains the genes encoding proteins that form the C3/C5 convertases, C4A, C4B, C2, and factor B (22).

In the present study, the technique of pulsed field gradient gel electrophoresis (PFGE) (23, 24) has been used in conjunction with that of Southern (25) to demonstrate that four of the genes of the RCA locus are clustered within 750 kb in the order CR1, CR2, DAF, and C4bp.

Materials and Methods

PFGE and Southern Analysis. High molecular weight genomic DNA was prepared, digested with restriction endonucleases, and fractionated in agarose gels using an orthogonal field alternation gel electrophoresis (OFAGE) apparatus according to the procedures of Carle and Olson (24, 26) with several modifications as suggested by Van Ommen and Verkerk (27). The source of DNA was an EBV-transformed B cell line derived from human peripheral blood cells of an individual (186-2137) typed as homozygous across HLA (A2, B7, DR 2, C4A 3, B QO, Bf, S, C2 C). Blood cells were donated by Drs. A. Palsdottir and A. Arnason, Blodbankinn, (Reykjavik, Iceland). Cells were transformed with simian EBV virus by Dr. I. Yunis of the Blood Center (Boston, MA) and maintained as a permanent cell line in tissue culture. In brief, the procedures used for PFGE analysis were as follows. Cells were washed and suspended in PBS at 4×10^7 cells/ml. Cells were embedded in blocks by adding 1 vol of molten agarose (1% LMT agarose made in PBS), mixing at 37°C, and casting 100-μl aliquots in flat-bottomed microtiter wells. After solidifying, blocks were removed and treated overnight in 5 vol of 0.1% sodium lauroyl sarcosine per 0.1 mg/ml Protease K in 0.5 M EDTA at 50°C. Before digestion with restriction enzyme, 1/2 block samples (5–10 μg) of DNA were washed four times for 30 min each with 1X restriction enzyme buffer, 0.1 mM PMSF, and 50 μg/ml BSA at room temperature. For analysis, samples were digested for 5 h at 37°C (50°C for BssH II and Sfi I) in a 0.5-ml reaction containing 250 μg BSA, 1X buffer (according to New England Biolabs, Beverly, MA) and a total of 40–60 U of restriction endonuclease (enzymes were added at two intervals). After digestion, samples were washed four times for 15 min each in TE buffer (10 mM Tris/HCl/1 mM EDTA, pH 7.0) and then melted at 65°C for 5 min before loading into sample wells using a cutoff pipette tip to reduce shearing. Samples were rotated during all washing and digestion steps.

Samples were fractionated in 1% agarose gels (13.5 × 13.5 cm) using 0.5% TBE buffer (1X = 90 mM Tris, 90 mM borate, and 5 mM EDTA) for 20 h at 10°C using a switching interval of 15, 30, or 60 s. The OFAGE unit was constructed by Harvard Medical School Biological Chemistry workshop (Boston, MA) according to the specifications of Carle and Olson (24) with modifications as suggested by Van Ommen and Verkerk (27). Gels were stained in ethidium bromide (EtBr) and photographed to visualize size-markers (oligomers of phage λ and yeast chromosomes, *Saccharomyces cerevisiae*). Before Southern transfer of DNA to nitrocellulose (25), gels were exposed to UV light on a trans-illuminator for 10 min. Transfer of DNA to nitrocellulose and hybridization in a solution containing 50% formamide was performed as described by Maniatis et al. (28). Filters were hybridized overnight with DNA probe labeled with ^{32}P to $\sim 10^8$ cpm/μg by either nick translation (Amersham Corp., Arlington Heights, IL) or random primer method (29). Filters were washed four times in 0.15 M NaCl/0.15 M sodium citrate at

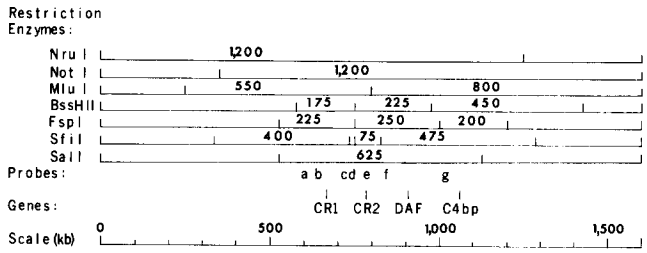

FIGURE 1. Organization of the genes within the RCA locus that encode CR1, CR2, DAF, and C4bp. The restriction map was prepared by PFGE and Southern blot analysis of genomic DNA using seven different restriction endonucleases and DNA probes specific for each of the four genes. DNA probes *a–g* represent the following regions: (*a*) CR1–15 (726 bp [nucleotide 4695–5420] of 3′-untranslated region [8]); (*b*) CR1–6 (625 bp of intron mapped between extracellular domain and transmembrane region [Klickstein, L. B., unpublished results]); (*c*) CR1–18 (253 bp of cDNA from 5′ region of long homologous repeat-A [Klickstein, L. B., unpublished results]); (*d*) CR1–35 (103 bp of cDNA containing the putative leader sequence of CR1 and overlaps probe c [Klickstein, L. B., unpublished results]); (*e*) CR2 (1.6-kb Eco R1 cDNA fragment that contains 5′ end of CR2-coding sequence); (*f*) DAF (2.5-kb full-length cDNA [13]); (*g*) C4bp (92-bp synthetic oligonucleotide based on published cDNA nucleotide #495–587 [3]). Restriction fragments were aligned on the basis of single and double digests and sequential hybridization of Southern blots with the respective DNA probes. However, small gaps could exist between apparently contigous fragments since small fragments would be missed on the PFGE gels. Fragment sizes are estimates within 10%. Some digests may not have gone to completion, in part due to methylation, and thus some restriction sites may not be designated. The position of probes and genes shown on the map indicate the region of hybridization and do not represent precise points within a restriction fragment. Abbreviations used in Figs. 2–7 for restriction endonucleases were as follows: Nru I-R; Not I-N; Mlu I-M; Fsp I-F; Sfi I-S; Sal I-L; C-Cla I; Bss HII-B.

room temperature followed by two times in 0.03 M NaCl/.003 M sodium citrate at 69°C. Dried filters were autoradiographed 1–5 d at −80°C using preflashed film (Kodak XAR) in a cassette with one Lightning Plus intensifier screen (DuPont Co., Wilmington, DE). For sequential hybridizations, filters were stripped in 0.5 M NaOH/1.5 M NaCl for 10 min, washed in 0.3 M NaCl/0.03 M sodium citrate, and autoradiographed as described to verify complete removal of the previous probe.

Results and Discussion

A map of 1,500 kb of DNA was prepared by combining the techniques of PFGE and Southern using seven different restriction endonuclease enzymes and DNA probes representing both coding and noncoding sequence (Fig. 1).

Linkage of the CR1, CR2, DAF, and C4pb Genes. Coding sequence probes representing the four genes, *CR1, CR2, DAF,* and *C4bp,* were hybridized to blots containing single and double digests with Not I, Nru I, and Mlu I. All four probes hybridized to common Nru I and Not I fragments of ~1,200 kb each (Fig. 2, *B–D,* lanes *2* and *5*; result with DAF probe not shown). Double digestion using both Nru I and Not I showed that the two fragments overlapped by ~900 kb and that all four genes were clustered within this region (Fig. 2, *B–D,* lane *6*). However, the four genes were split into two groups by Mlu I. The CR1 and CR2 probes hybridized to a Mlu I fragment of 550 kb, whereas the DAF and C4bp probes were contained in one at 800 kb (Fig. 2, *B–D,* lane *1*, Fig. 3, *B–E,* lane *2*). Thus, the *CR1* and *CR2* genes were located within 450 kb on a Not I/Mlu I fragment (Fig. 2, *B* and *C,* lane *7*), whereas the *DAF* and *C4bp* genes were positioned within 450 kb on a Nru I/Mlu I fragment (Fig. 2, *D,* lane *3*; Fig. 3, *B* and *C,* lane 1). The limits of the gene cluster were narrowed to within

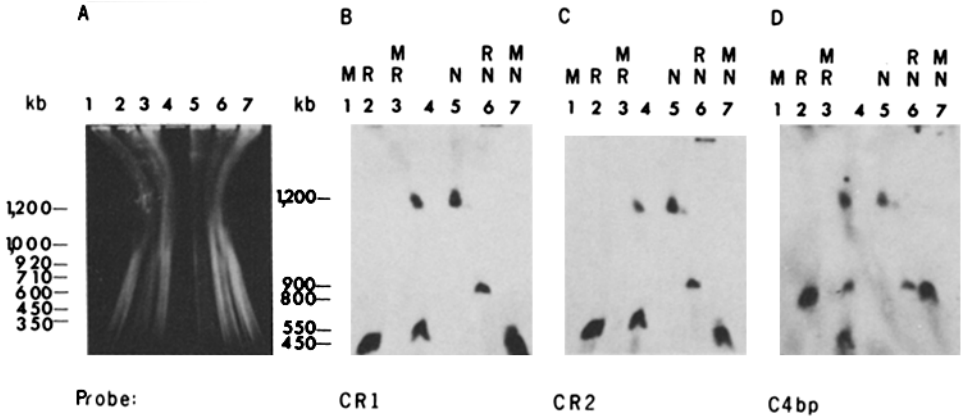

FIGURE 2. *CR1*, *CR2*, and *C4bp* genes are linked on overlapping Nru I and Not I fragments of ~1,200 kb each but *CR1* and *CR2* are on an Mlu I fragment distinct from that of C4bp by PFGE and Southern analysis using the probes b(CR1), e(CR2), and g(C4bp). Double digestion with Nru I/Not I places the three genes within 900 kb. Current was switched each 60 s for 20 h. *A* shows the EtBr-stained gel with phage λ oligomer (*M*) and yeast (*S. cerevisiae*) chromosomes (lane *4*) as markers. *B–D* represent the same filter stipped between hybridizations with the b, e, and g probes, respectively (see Fig. 1 legend for description of probes). Digests in lanes *1–3* and *5–7* are as labeled (see Fig. 1 for abbreviation of enzymes). Similar size fragments in different lanes of autoradiographs may not appear identical because of the inherent curvature of the lanes which is an artifact of the orthogonal field electrophoresis technique. The photograph of the EtBr-stained gel was not reproduced at the same scale as the relative autoradiographs.

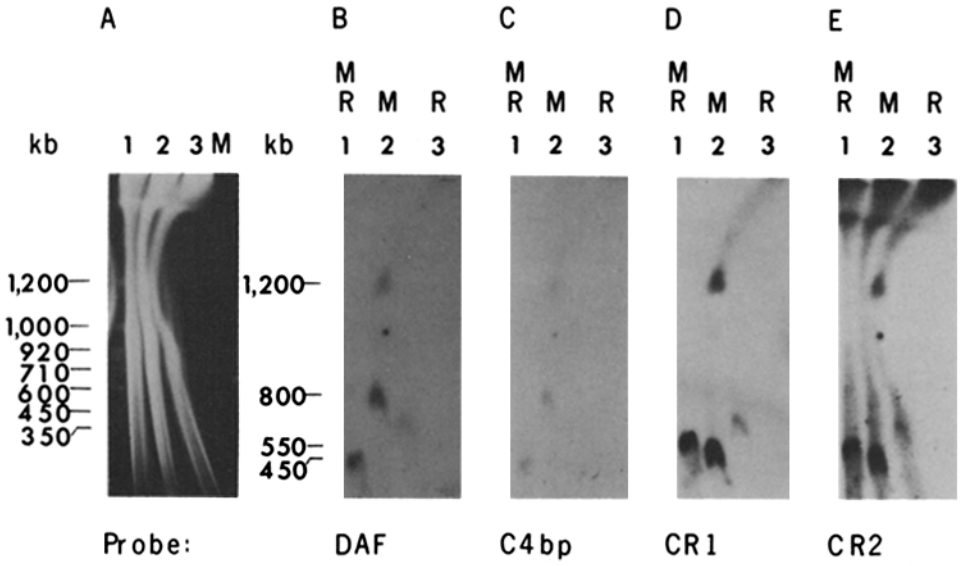

FIGURE 3. The genes encoding CR1, CR2, DAF and C4bp are linked on a 1,200-kb Nru I fragment. However, CR1 and CR2 are located on a Mlu I fragment separate from the one containing DAF and C4bp by PFGE and Southern analysis using the probes a(CR1), e(CR2), f(DAF), and g(C4bp). Current was switched each 60 s for 20 h. Panel *A* is the EtBr-stained gel. Panels *B–E* represent the same filter stripped between hybridizations with probes f, g, a and e, respectively. Digests in lanes *1–3* are as labeled.

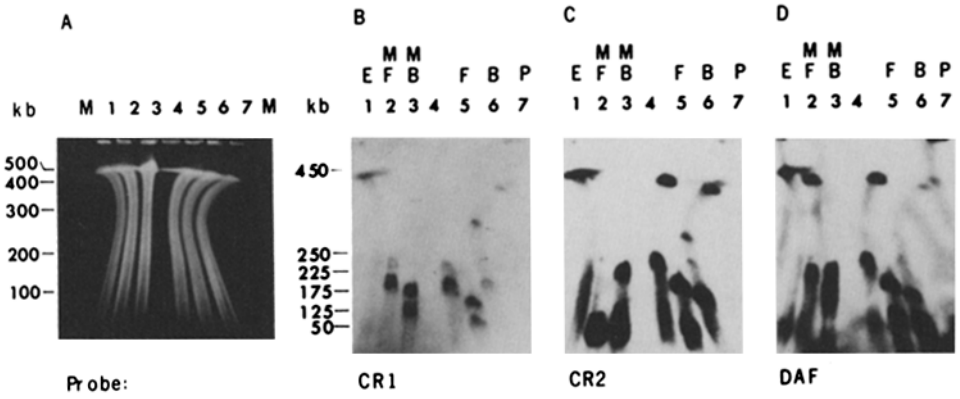

FIGURE 4. Linkage of CR2 and DAF determines the orders of genes as *CR1, CR2, DAF,* and *C4bp* by PFGE and Southern analysis using probes b(CR1), e(CR2), and f(DAF). Linkage of *CR2* and *DAF* on Bss HII and Fsp I fragments was confirmed by double digests with Mlu I/Bss HII and Mlu I/Fsp I. Current was switched each 30 s. Panel *A* is the EtBr-stained gel. Panels *B–D* represent the same filter stripped between hybridizations with the b, e, and f probes, respectively. Digests in lanes *1–3* and *5–7* are as labeled.

750 kb by further analysis with Sal I and Sal I/Mlu I (data not shown, see Fig. 1, PFGE map). The CR1, CR2, and DAF probes (C4bp was not tested) hybridized to a common Sal I fragment of 625 kb that overlapped the M1u I site. This was confirmed by a double digest that split the Sa1 I fragment into two fragments of 325 and 300 kb, and the latter was found to include both *CR1* and *CR2* genes (data not shown).

Order of the Genes. The order of the four genes was determined by linking CR2 and DAF, which had been shown (described above) to be linked on common Nru I and Not I fragments but were located on separate Mlu I fragments. The *CR2* and *DAF* genes were linked on 250-kb Fsp I and 225-kb Bss HII fragments (Fig. 4, *C* and *D*, lanes *5* and *6*) that did not include either CR1 or C4bp. This result, combined with that discussed above, suggested that CR1 and C4bp must flank CR2 and DAF, respectively, and that the order was CR1, CR2, DAF, and C4bp. The close linkage of CR2 and DAF was confirmed by a double digest with Mlu I which split the 250-kb Fsp I fragment into 200 and 50 kb with the *DAF* gene on the larger fragment and *CR2* on the smaller one (Fig. 4, *C* and *D*, lane *2*). The 500-kb Fsp I fragment represents a partial that contains the 250-kb fragment. As predicted, double digestion with Mlu I/Fsp I reduced the 500-kb partial by 50 kb. A similar result was obtained with Bss HII/Mlu I digest in which the *DAF* and *CR2* genes, which are located on a common 225-kb Bss HII fragment, were separated into two fragments of 175 and 50 kb, respectively (Fig. 4, *C* and *D*, lane *3*). The digest was not complete as some of the 225 kb remained uncut.

Analysis of the Bss HII partial digest shown in Fig. 4 suggested that the *CR2* and *DAF* genes were separated by a minimum distance of 50 kb. Probes for both *CR2* and *DAF* hybridized to a common partial fragment at 225 kb. However, the two probes also hybridized to two smaller fragments of 125 and 150 kb, respectively (Fig. 4 *C*, lane *6*). Thus, the stretch of DNA (50 kb) connecting these two fragments did not hybridize with either probe.

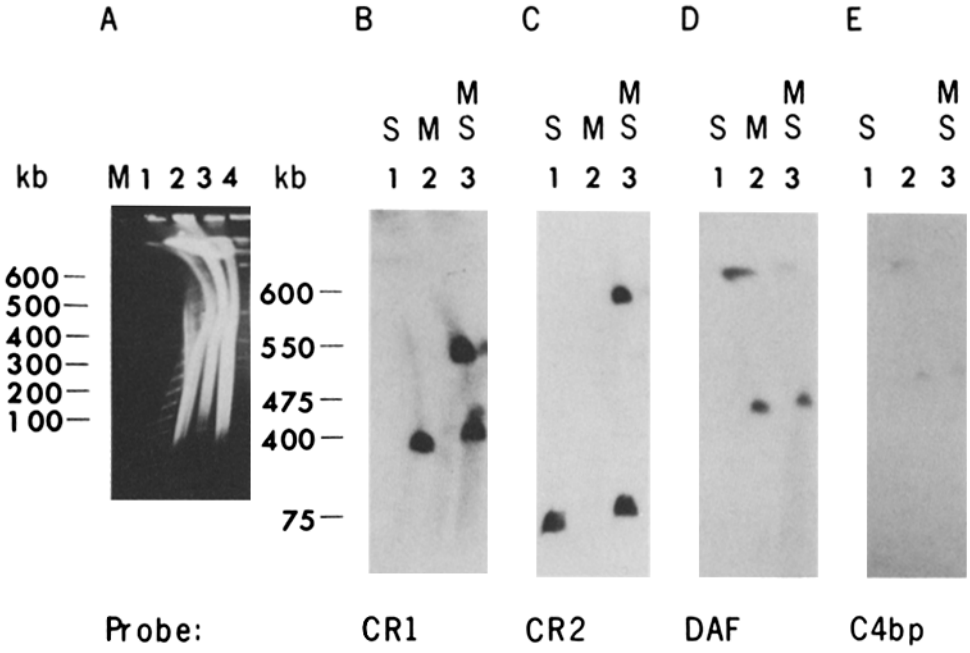

FIGURE 5. *DAF* and *C4bp* are linked on a Sfi I 475-kb fragment that is within the common 800-kb Mlu I, while *CR2* and *CR1* are each located on distinct Sfi I fragments of 75 and 400 kb, respectively, by PFGE and Southern analysis using probes a(CR1), e(CR2), f(DAF), and g(C4bp). Current was switched each 30 s for 18 h. Panel *A* is the EtBr-stained gel. Panels *B–E* represent the same filter stripped between hybridizations with the b, e, f, and g probes, respectively. Digests in lanes *1–3* are as labeled.

The *C4bp* gene was positioned adjacent to *DAF* as predicted above on a common Sfi I fragment of 475 kb (Fig. 5, *D* and *E*, lane *1*) but was found on a separate Bss HII fragment of 450 kb (Fig. 6 *C*, lane *1*). The overlap between the two fragments was confirmed by a Bss HII/Sfi I digest that yielded the predicted size fragment of 300 kb (Fig. 6, *B* and *C*, lane *6*). Similarly, the *CR1* gene was positioned adjacent to *CR2* on the opposite side of *DAF* on overlapping Mlu I and Sal I fragments of 550 and 600 kb, respectively, as described above.

Orientation of CR1 Gene. The approximate limits and 5′-3′ orientation (with respect to *CR2*) of the *CR1* gene were determined from single and double digests with Fsp I, Bss HII and Sfi I. While all *CR1* probes; i.e., a–d (see Fig. 1 legend for description of probes), hybridized to single Fsp I and Sfi I fragments of 225 and 400 kb, respectively, analysis with Bss HII gave multiple bands; i.e., 175, 125, and 50 kb, which represented a partial digest. All of the *CR1* probes hybridized to the common band at 175 kb and all except probe d hybridized to the 125-kb fragment (Fig. 4 *B*, lane *6* for probe b; Fig. 7, lane *2* for probe c; results with probes a and d not shown). However, only the most 5′ probes c and d, which overlap, hybridized to the small 50-kb fragment (Fig. 7, lane *2*; result with probe d not shown). Thus, the most 5′ probe (d) hybridized only to the 175 and 50-kb Bss HII fragments. On double digestion with Sfi I/Bss HII both the 175 and 50-kb Bss HII fragments were split, resulting in fragments of 155 and

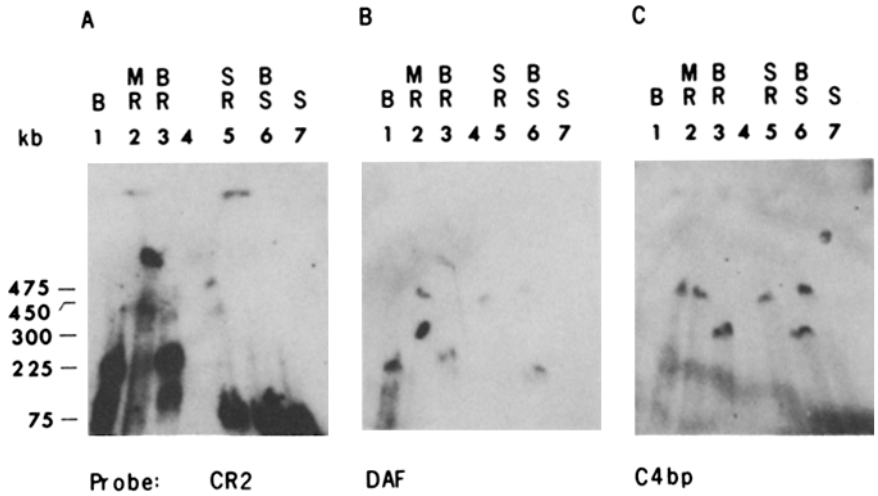

FIGURE 6. Linkage of *DAF* and *C4bp* on a 475-kb Sfi I fragment is confirmed by double digest with Sfi I/Bss HII by PFGE and Southern analysis using probes e(CR2), f(DAF), and g(C4bp). Current was switched each 30 s for 18 h. Panels A–C represent the same filter stripped between hybridizations with the e, f, and g probes, respectively.

20 kb. As predicted, the most 5′ probe d hybridized to the smaller fragment, and probe c hybridized to both (Fig. 7, lane *6*; result with probe d not shown). Thus, the double digest confirmed that the 50-kb fragment was included within the 175-kb partial and was closest to *CR2*. Since the 125-kb fragment, which hybridizes with probes a–c, was not cleaved by Sfi I, it presumably contains the 3′ end of the gene and is included within the 175-kb along with 50-kb 5′ fragment.

The Fsp I and Bss HII sites between CR1 and CR2 must be relatively close as analysis of a Sfi I/Fsp I double digest with the 5′ *CR1* probe d also showed a new fragment of ~20 kb (data not shown). Assuming that the Bss HII and Fsp I fragments that contain *CR1* are contiguous with those that contain *CR2*, then 5′ end of the *CR1* and the *CR2* gene are contained within a stretch of ~75 kb of DNA.

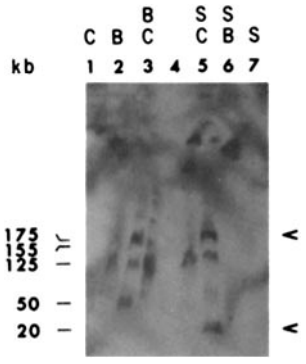

FIGURE 7. The 5′ end of the *CR1* gene is adjacent to *CR2* by PFGE and Southern analysis using single and double digests with Sfi I and Bss HII and hybridization with 5′ region–specific probe c (CR1). The current was switched each 15 s for 18 h. The size of fragments was estimated from phage λ oligomers standards (not shown). The two arrows on the right margin indicate the 155 and 20-kb Sfi I/Bss HII fragments in lane *6*. The 155-kb Sfi I/Bss HII fragment in lane *6* may appear similar in size compared to the uncut 175-kb fragment in lane *2* due to the curvature of the lanes, however, comparison of the distance of migration with the uncut 125-kb band present in the two lanes indicated the predicted shift in mobility.

It is possible that the *CR1* probes crosshybridize with other "*CR1*-like" coding sequences. For example, probes c and d hybridize to a 1.6–2.0-kb mRNA in addition to the expected *CR1* mRNA of 9 and 11 kb (30). Since these probes appear to hybridize to the ends of the 175- and 225-kb Bss HII and Fsp I fragments, respectively, which are proximal to *CR2,* this region may contain coding sequence in addition to that of the *CR1* gene.

Attempts to position the factor H gene within the PFGE map have been unsuccessful, suggesting that the gene lies outside of this region of 1,500 kb. Consistent with this finding are the recent results indicating that the H locus maps 6.9 cM away from the *C4bp/CR1* locus (31). Thus, while factor H is clearly linked to the RCA locus, the gene is separate from the other four by a greater relative distance.

The clustering of the CR1, CR2, DAF, and C4bp loci within a 750-kb region of the long arm of chromosome 1 probably relates to the similar structure and function of these proteins, each being comprised almost entirely of 60–amino acid consensus repeats and each having C3- and/or C4-binding activity. Although there are other proteins that contain short consensus repeats and that are not encoded by genes within the RCA locus, such as factor XIIIa, the IL-2 receptor, C2, and factor B, they either lack C3/C4-binding function or the repeats constitute only a small portion of the entire polypeptide sequence. Finally, it is anticipated that the RCA locus will be expanded by inclusion of additional genes, such as that for the C3b-binding membrane protein termed membrane cofactor protein in man (32) and p65 in the mouse (33) and the gene encoding the 1.6–2.0-kb B lymphocyte transcript that crosshybridizes to *CR1* cDNA (30).

Summary

The organization and physical linkage of four members of a major complement locus, the RCA locus, have been determined using the technique of pulsed field gradient gel electrophoresis in conjunction with Southern blotting. The genes encoding CR1, CR2, DAF, and C4bp were aligned in that order within a region of 750 kb. In addition, the 5′ to 3′ orientation of the *CR1* gene (5′ proximal to *CR2*) was determined using 5′- and 3′-specific DNA probes. The proximity of these genes may be related to structural and functional homologies of the protein products. Overall, a restriction map including 1,500 kb of DNA was prepared, and this map will be important for positioning of additional coding sequences within this region on the long arm of chromosome 1.

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