

# IDENTIFICATION OF A RESTRICTION FRAGMENT LENGTH POLYMORPHISM BY A CR1 cDNA THAT CORRELATES WITH THE NUMBER OF CR1 ON ERYTHROCYTES

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The human C3b/C4b receptor (CR1) is present on the surface of E, polymorphonuclear leukocytes, monocytes/macrophages, B lymphocytes, some T lymphocytes, follicular dendritic cells, and glomerular podocytes (1, 2). CR1 mediates the binding to these cells of particles or immune complexes on which C3b has been deposited by complement activation (1). ~90% of the CR1 that is present in blood resides on E (3), and this CR1 is considered to have a role in the clearance and processing of circulating immune complexes (4, 5).

CR1 is a large glycoprotein composed of a single polypeptide chain that is polymorphic with respect to molecular weight (6-9). Three of the allotypes, S, F, and F', have apparent molecular weights in the reduced state of 260,000, 240,000, and 210,000, respectively, as determined by SDS-PAGE. The molecular weight difference between the S and F allotypes is not abolished by removal of N-linked oligosaccharides, suggesting that the polymorphism is caused by differences in the length of the polypeptide chain (7).

A quantitative polymorphism of CR1 that is independent of the structural polymorphism has also been described (10). The number of CR1 expressed by different cell types ranges from an average of 500 sites per cell for E to 40,000 sites per cell for neutrophils, monocytes, and B lymphocytes (3, 10, 11). Whereas the number of CR1 on leukocytes varies little between individuals (12), E from different normal individuals may show up to 10-fold variation in their number of CR1 per cell (10). The possibility that these differences were genetically determined was initially suggested by their persistence over time and by pedigree analyses of normal families (10). This possibility is of particular importance, as studies in two populations have suggested that inheritance plays a role in the decreased numbers of CR1 on E of patients with systemic lupus erythematosus (10, 13, 14). A mechanism for the genetic determination of CR1 number on E

This work was supported by Grants AI22833, AM36797, AM35907, AM36308, RR01996, AM35907, and AI23401 from the National Institutes of Health, IM-412 from the American Cancer Society, and a grant-in-aid from the American Heart Association. W. W. Wong is a postdoctoral fellow of the Arthritis Foundation. L. B. Klickstein is supported in part by National Institutes of Health Training Grant GM07753-06. Address correspondence to Douglas T. Fearon, Seeley G. Mudd Building, Rm. 607, 250 Longwood Avenue, Boston, MA 02115.

was suggested by the observation that the frequency distribution of this number in the normal population was not unimodal (10, 14); rather, two groups of individuals were found to have numbers of CR1 in the high and intermediate range of the population, respectively, with a third, less distinct group having numbers in the lower range. It was proposed that this distribution reflected polymorphism of a regulatory element having at least two autosomal codominant alleles. Additional evidence for the existence of such a regulatory element has now been obtained by the identification of a CR1 genomic polymorphism that correlates with the level of CR1 expression on E.

### Materials and Methods

**Antibodies.** Rabbit IgG anti-CR1 was prepared as described previously (3). The murine monoclonal IgG1 anti-CR1 YZ-1 (15) was purified from ascites by precipitation with 50% saturated ammonium sulfate and chromatography on DEAE Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ), and portions were radiolabeled with  $^{125}\text{I}$  (Amersham Corp., Arlington Heights, IL) to sp act of  $0.8\text{--}1.4 \times 10^6$  cpm/ $\mu\text{g}$  (16). Fab fragments of the purified YZ-1 were produced by papain digestion (17) and were radiolabeled to a sp act of  $1.4 \times 10^6$  cpm/ $\mu\text{g}$ . Murine IgG1 from the tumor line MOPC 21, which has no known antigen-binding activity (Litton Bionetics, Kensington, MD); biotin-conjugated monoclonal anti-B1 (B1-biotin;<sup>1</sup> Coulter Electronics Inc., Hialeah, FL), which recognizes a 35,000  $M_r$  antigen present on the surface of B lymphocytes (18), and FITC-conjugated goat IgG anti-mouse IgG (FITC-GaM) (Southern Biotechnology Associates, Birmingham, AL) were purchased.

**Enumeration of CR1 of Erythrocytes, Neutrophils, and B Lymphocytes.** The number of CR1 on erythrocytes was determined by incubation of washed erythrocytes for 1 h at  $22^\circ\text{C}$  with  $1 \mu\text{g/ml}$   $^{125}\text{I}$ -YZ-1 in the absence or presence of excess, unlabeled YZ-1 followed by centrifugation of the erythrocytes through cushions of dibutylphthalate (Eastman Kodak Co., Rochester, NY) (10). Total cellular CR1 of purified neutrophils (19) was measured by a modification of the sandwich protocol of Unkeless and Healy (20, 21) in which the detergent-soluble fraction of lysates of neutrophils was incubated with  $1.25 \mu\text{g/ml}$   $^{125}\text{I}$ -YZ-1 Fab, and the CR1- $^{125}\text{I}$ -Fab complexes were immunoprecipitated by *Staphylococcus aureus* particles (Bethesda Research Laboratories, Gaithersburg, MD) charged with polyclonal rabbit IgG anti-CR1. CR1 on B lymphocytes was quantified by two-color immunofluorescent flow cytometry (12); B cells were identified by staining with B1-biotin and rhodamine 600 (XRITC)-conjugated avidin (Vector Laboratories, Inc., Burlingame, CA), and the amount of CR1 on the cells was measured by staining with YZ-1 and FITC-GaM. Immunofluorescent staining was analyzed with a Cytofluorograf System 50-H (Ortho Diagnostic Systems Inc., Westwood, MA) equipped with an argon laser operated at 488 nm and 250 mW and a krypton laser operated at 568.2 nm and 200 mW, and interfaced with a model 2150 Data Handler (Ortho Diagnostic Systems Inc.).

**Analysis of CR1 by PAGE.** CR1 in detergent lysates of erythrocytes or neutrophils that had been surface-labeled with  $^{125}\text{I}$  (16) was immunoprecipitated as described (7) using YZ-1 IgG that had been coupled to CNBr-activated-Sepharose (Sigma Chemical Co., St. Louis, MO). The immunoprecipitated CR1 was eluted by incubation for 3 min at  $100^\circ\text{C}$  in 1% SDS and was analyzed after reduction and alkylation by SDS-PAGE (22) on slab gels containing a gradient of 3–10% acrylamide, followed by autoradiography.

**Southern Blot Analysis of Genomic DNA.** Genomic DNA was isolated from peripheral blood leukocytes (23) and analyzed by Southern blot hybridization (24) using the 0.75-kb CR1 cDNA probe, CR1-1, which represents a portion of a 1.2-kb sequence that occurs in at least two highly homologous copies in the coding sequence of the CR1 gene (25, 26).

<sup>1</sup> *Abbreviations used in this paper:* B1-biotin, biotin-conjugated monoclonal anti B-1; FITC-GaM, FITC-conjugated goat IgG anti-mouse IgG; RFLP, restriction fragment length polymorphism; XRITC, rhodamine 600.

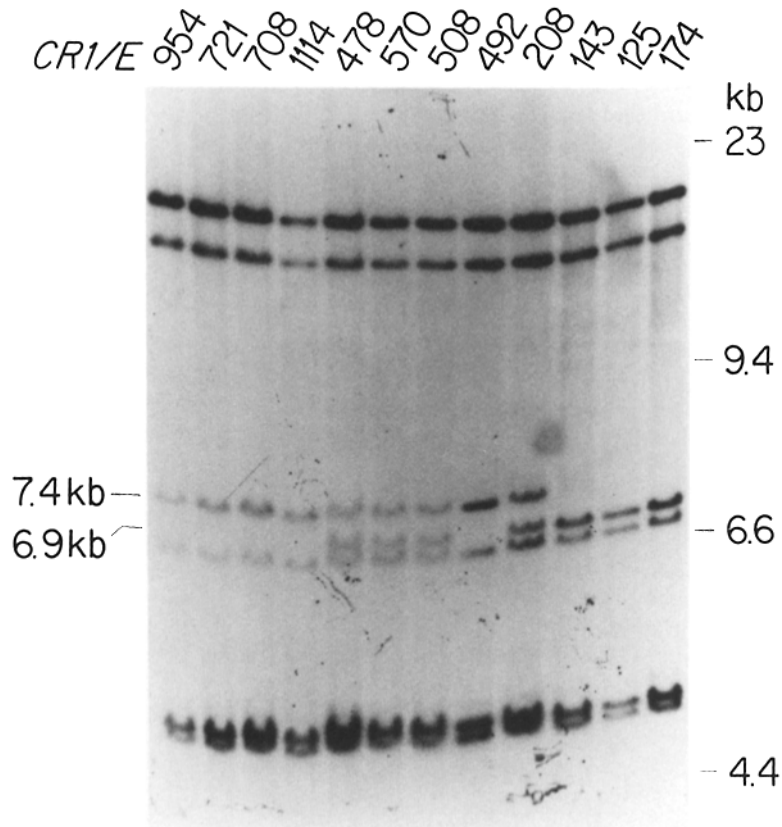


FIGURE 1. Comparison of Hind III RFLP with number of CR1 expressed on E. Genomic DNA from normal individuals was digested with Hind III, Southern blots of the DNA were probed with  $^{32}\text{P}$ -CR1-1, and hybridizing fragments were detected by autoradiography. Numbers of CR1/E are shown above the lanes and kb sizes of DNA fragments are indicated to the right and left of the autoradiogram.

### Results

Leukocyte DNA was prepared from healthy individuals having numbers of CR1 on their E in the high (708–1,114 CR1 sites per cell), intermediate (478–570 sites per cell), and low (174–208 sites per cell) ranges, respectively. The DNA was digested with a panel of restriction enzymes and Southern blots were hybridized with the 0.75-kb CR1 cDNA probe, CR1-1. This probe hybridizes under stringent conditions not only to its complementary sequence but also to other highly homologous sequences that occur within the coding sequence of the CR1 gene (25, 26). The existence of these repeating sequences accounts, at least in part, for the multiple genomic restriction fragments to which the probe hybridizes. Using Hind III, we observed a restriction fragment length polymorphism (RFLP) involving fragments of 7.4 kb and 6.9 kb that appeared to correlate with the variations in the quantitative expression of CR1 (Fig. 1). All four individuals having high CR1 sites/E had only the 7.4-kb fragment, three of the four persons having low CR1 had only the 6.9-kb fragment, and three of the four individuals having intermediate numbers of CR1 had both fragments. Therefore, a larger group of normal individuals was analyzed for CR1 number

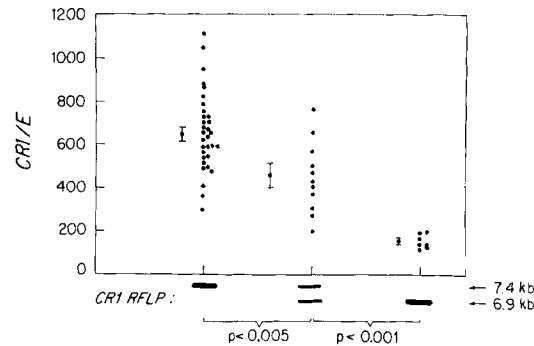


FIGURE 2. CR1/E among normal individuals grouped according to Hind III RFLP. Each point represents data for a single individual. CR1/E are shown in the vertical axis, and the pattern for the Hind III RFLP is shown diagrammatically below each group. Points with error bars to the left of each group represent mean  $\pm$  SEM, and the  $p$  values obtained when groups were compared in a two-sample  $t$  test are shown.

on E and for the occurrence of these Hind III restriction fragments. All 50 individuals had either the 6.9-kb fragment, the 7.4-kb fragment, or both (Fig. 2). The number of CR1 sites on E from persons having only the 7.4-kb restriction fragment was  $611 \pm 33$  (mean  $\pm$  SEM). Although there was considerable overlap, the 11 donors having both restriction fragments had significantly fewer sites per E,  $455 \pm 52$  ( $p < 0.005$ ). Individuals having only the 6.9-kb fragment showed less variation in CR1 expression than did the other two groups, not overlapping with any person homozygous for the 7.4-kb fragment, and had a mean of only  $156 \pm 13$  sites per E (significance of the difference of means for either higher group,  $p < 0.001$ ).

The probe with which the Hind III RFLP was detected, CR1-1, shows in situ hybridization to a single chromosomal locus, 1q32 (26). The linkage of this RFLP to the structural gene for CR1 was confirmed by examining in an informative family whether the Hind III restriction fragments cosegregated with the S, F, and F' structural allotypes of CR1 (Fig. 3A). In individuals II-1, II-2, III-1, and III-2, gels of  $^{125}\text{I}$ -labeled CR1 from both E (Fig. 3A) and neutrophils (Fig. 3B) were obtained to aid in the definitive assignment of CR1 structural allotypes. The individual designated I-2 was heterozygous for the F and F' structural allotypes and homozygous for the 7.4-kb Hind III fragment. Every descendant of I-2 who inherited the F' allotype (II-1, II-2, III-1, and III-2) also had the 7.4-kb Hind III fragment. The F allotype of individual II-1 was necessarily inherited from his father, individual I-1, and this allotype cosegregated with the 6.9-kb Hind III fragment in all cases, including individuals II-1, II-2, and III-3. Finally, the S and F allotypes of individual II-3 cosegregated with the 7.4- and 6.9-kb Hind III fragments, respectively, in individuals III-1, III-2, and III-3. Thus, the element identified by the Hind III RFLP that regulates the quantitative expression of CR1 is linked to the CR1 gene.

Previous studies have indicated that the relative proportions of the CR1 structural allotypes expressed on E of different heterozygous individuals were relatively constant over time (6, 7), and have suggested that these ratios might be genetically controlled (8, 9, Rodriguez de Cordoba, personal communication, New York Blood Center, New York). Having shown that the Hind III RFLP

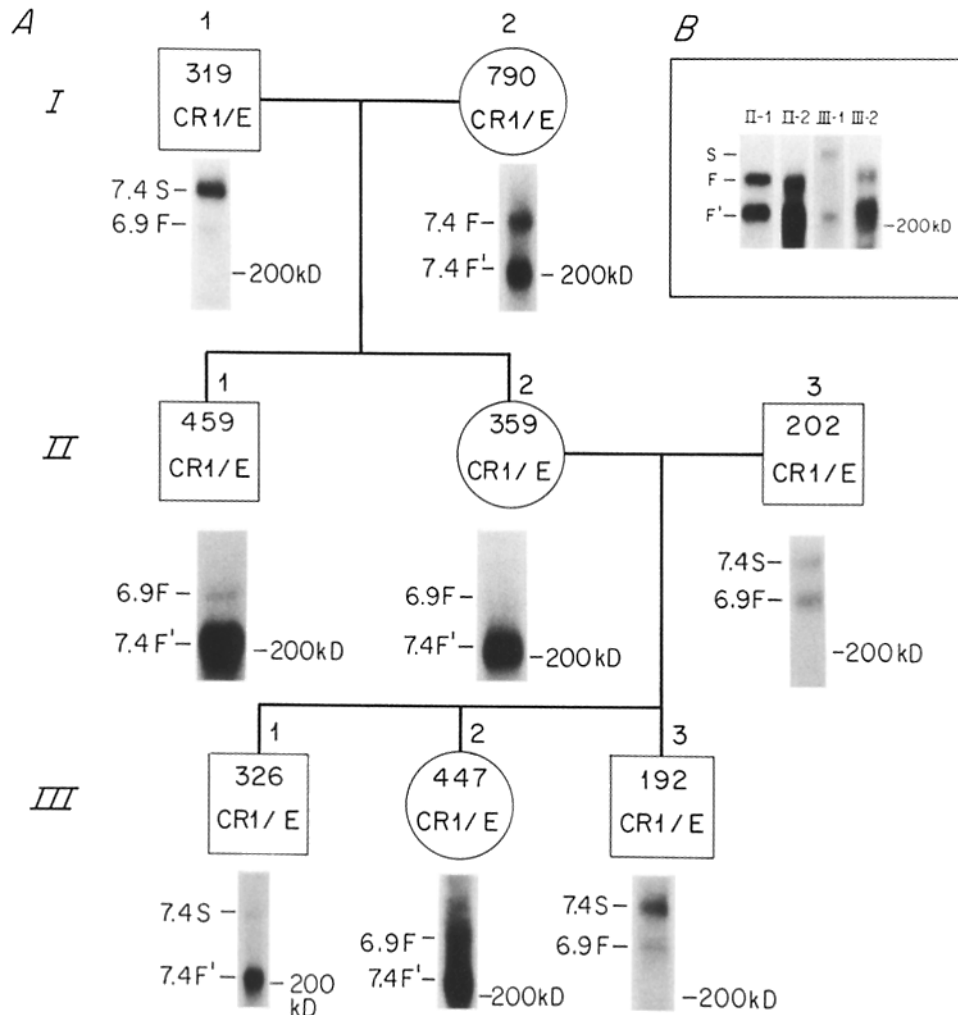


FIGURE 3. Segregation in a normal family of Hind III RFLP, structural allotype of CR1 and CR1/E. Number of CR1/E is shown inside each symbol. An autoradiogram of the SDS-PAGE of CR1 protein immunoprecipitated from each individual's surface-<sup>125</sup>I-labeled E is shown beneath each figure, and the positions of the S, F, and F' allotypes of CR1 and the 200-kD molecular mass marker are indicated. The 7.4-kb and 6.9-kb Hind III fragments that were seen in Southern blots of each individual's DNA are shown adjacent to the allotypic form of CR1 with which they cosegregated. An autoradiogram of the SDS-PAGE of CR1 immunoprecipitated from surface-<sup>125</sup>I-labeled neutrophils of individuals II-1, II-2, III-1, and III-2 is shown in the inset.

and the locus defining the CR1 structural allotypes are linked, it was possible to assess whether the regulatory element associated with this RFLP was *cis*- or *trans*-acting by comparing the relative amounts of the S, F, and F' allotypes expressed on E of the individuals depicted in Fig. 3. The F' allotype that is associated with the 7.4-kb Hind III fragment in individuals I-2, II-1, II-2, III-1, and III-2 is expressed in large quantities on E of each of these individuals. In contrast, the 6.9-kb fragment-associated F allotype of individuals I-1, II-1, II-2, and III-3 is expressed in relatively small quantities. Thus, the regulatory element that is

linked to these 7.4-kb and 6.9-kb Hind III restriction fragments *cis*-regulates CR1 expression by E. The expression of the S allotype of individual II-3 that was associated with the 7.4-kb fragment did not exceed that of his 6.9-kb fragment-associated F allotype. Moreover, the expression of this S allotype on E of individuals III-1 and III-3 was relatively low, and the three individuals having this allele had only 202, 326, and 192 CR1 sites per E, respectively. Therefore, in these individuals the factors causing low CR1 number in association with the 7.4-kb allele also were inherited and were expressed in a *cis*-acting manner.

Visual comparison of the proportions of the structural allotypes of CR1 expressed on E (Fig. 3A) and neutrophils (Fig. 3B) of individuals II-1, II-2, III-1, and III-2 of Fig. 3 suggested that factors regulating the amounts of CR1 on these two cell types may differ, in accord with earlier findings (7, 9, 27). To investigate further whether such regulation might be cell-type-specific, eight normal individuals were selected, four homozygous for the 7.4-kb Hind III fragment and four homozygous for the 6.9-kb fragment, and CR1 was quantified on their E by the binding of  $^{125}\text{I}$ -YZ-1, on their B cells by two-color immunofluorescent flow cytometry, and in their neutrophils by a sandwich RIA of total cellular CR1 (Fig. 4). For B cells and neutrophils, determinations for all eight individuals were performed simultaneously to avoid interassay variation. The mean number of CR1/E for the individuals homozygous for the 7.4-kb Hind III fragment was  $886 \pm 94$ , significantly higher than that for the individuals homozygous for the 6.9-kb fragment,  $159 \pm 18$  ( $p < 0.001$ ). When the amount of CR1 present on B cells, identified by their red fluorescent staining by B1-biotin and XRITC-avidin, was expressed as the relative intensity of green fluorescent staining by YZ-1 and FITC-GaM, B cells of the individuals who were homozygous for the 7.4-kb fragment had a mean relative fluorescence for CR1 of  $26.7 \pm 2$  (SEM), not significantly different from that of B cells from individuals who were homozygous for the 6.9-kb fragment,  $29.1 \pm 6$  ( $p > 0.2$ ). The individuals homozygous for the 7.4-kb fragment and those homozygous for the 6.9-kb fragment also were similar in the total amount of CR1 in their neutrophils,  $43,870 \pm 4,010$  and  $42,985 \pm 2,184$  CR1/cell, respectively ( $p > 0.8$ ). Thus, regulation of total CR1 expression by the element associated with the Hind III RFLP is apparent only in erythrocytes.

### Discussion

In Southern blots of genomic DNA that had been digested with Hind III, the 0.75-kb probe, CR1-1, hybridized to multiple fragments having an aggregate length of  $\sim 80$  kb (Fig. 1). This circumstance permitted analysis of a large portion of the CR1 gene with a probe that was derived from  $<10\%$  of the CR1 transcript (25). Among 50 healthy donors (Fig. 2), a polymorphism involving two restriction fragments of 6.9 kb and 7.4 kb was found to correlate with the level of CR1 expression on E. The occurrence in all individuals of either or both restriction fragments is consistent with their being allelic, and family studies supported this possibility (Fig. 3). The additional finding that a corresponding polymorphism was not observed with the enzymes Eco RI, Bam HI, and Pvu II (data not shown) suggests either that the two alleles differ at only a few nucleotides or that there are cleavage sites for these three enzymes between the polymorphic region and the hybridization site for CR1-1.

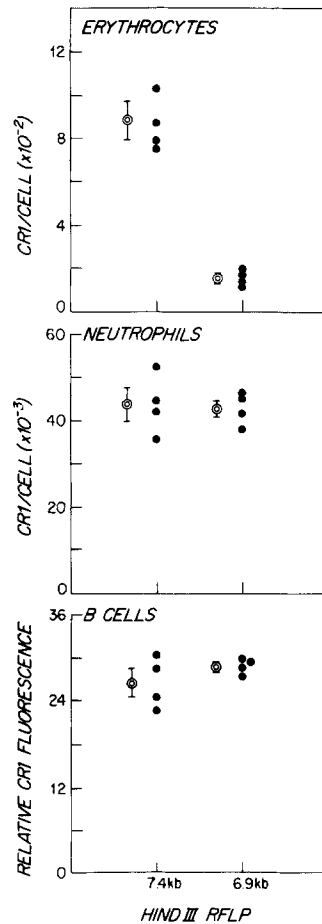


FIGURE 4. Quantitation of CR1 of E, neutrophils, and B lymphocytes of individuals homozygous for either the 7.4-kb or the 6.9-kb *Hind III* RFLP. Each point represents data for a single individual. Open circles with error bars to the left of each group represent mean  $\pm$  SEM.

Presumed homozygosity for the form of the regulatory element identified by the 6.9-kb and by the 7.4-kb restriction fragment was associated with low and high numbers of CR1 on E, respectively, whereas heterozygosity was associated with CR1 number on E in the intermediate range (Fig. 2). This regulatory element was shown to be linked to the CR1 structural gene and *cis*-acting by showing that it regulated the relative amounts of the S, F, and F' structural allotypes of CR1 expressed on E in three generations of a family (Fig. 3). Studies of CR1 expression by leukocytes suggested that the effects of this regulatory element were tissue-specific (Figs. 3 and 4). However, unequal expression of the CR1 structural allotypes that paralleled that on E was also observed on neutrophils of individual III-2 of Fig. 3.

Inherited differences in the expression of CR1 could result from structural alterations affecting its membrane insertion or stability. For example, inherited changes in the transmembrane and cytoplasmic domains of low density lipoprotein receptors resulted in the secretion of most of these receptors from fibroblasts

(28). Although CR1 of E from individuals homozygous for either of the two forms of the Hind III RFLP did not differ on SDS-PAGE and there was no corresponding alteration in CR1 expression by their leukocytes, possible differences in protein structure of their CR1 can be excluded only by comparing the coding sequences of the CR1 genes associated with the 6.9-kb and 7.4-kb restriction fragments. Altered stability of CR1 resulting from differences in primary structure of the receptor might be more apparent on erythrocytes than leukocytes because of the longer life span of the former cell type and its lack of biosynthetic capacity.

Observed differences in CR1 expression also could be caused by factors affecting protein synthesis, including differential transcription initiation, RNA splicing, polyadenylation, rates of mRNA processing and transport, stability of the message in the cytoplasm, or rates of translation or intracellular degradation. In eukaryotic genes the steady state levels of message appear to be regulated primarily by modulation of transcription initiation (29), as has been found for the genes encoding Ig light and heavy chains (30–33), insulin (34), and chymotrypsin (34). Thus, the Hind III RFLP might identify allelic forms of either promoter or enhancer elements, both of which are *cis*-acting, and the tissue-specific nature of the CR1 regulatory element may favor the possibility that it is an enhancer. The threefold span of CR1 number on E of individuals who are homozygous for the 7.4-kb Hind III fragment indicates either that a recombination event has occurred between the regulatory element and the Hind III restriction sites, or that additional genetic factors influencing CR1 expression are superimposed on the regulatory element identified in this study. However, the regulatory element associated with the 6.9-kb Hind III fragment, when present in the homozygous state, overrides the effects of such additional genetic factors (Fig. 2).

### Summary

A genetic basis for the regulation of the number of CR1 on E of different normal individuals was investigated by probing Southern blots of their genomic DNA with a 0.75-kb fragment of CR1 cDNA. Using Hind III, we observed a RFLP involving fragments of 7.4 kb and 6.9 kb that correlated with the number of CR1 on E. 32 individuals having only the 7.4-kb restriction fragment had a mean of  $661 \pm 33$  (SEM) CR1/E, 11 donors having both restriction fragments had a mean of  $455 \pm 52$  CR1/E, and 7 individuals having only the 6.9-kb fragment had a mean of  $156 \pm 13$  CR1/E, all means being significantly different ( $p < 0.005$ ). Cosegregation in a normal family of the Hind III restriction fragments with the S, F, and F' structural allotypes of CR1 confirmed that the regulatory element identified by these fragments is linked to the CR1 gene. Moreover, an analysis of the relative expression on E of these structural allotypes in association with either the 7.4-kb Hind III fragment or the 6.9-kb fragment showed that this regulatory element is *cis*-acting. In contrast, quantitation of CR1 of B lymphocytes and neutrophils revealed no differences in total CR1 expression between individuals homozygous for the 7.4-kb and 6.9-kb Hind III fragments. Thus, we have identified a genomic polymorphism that is linked to



the CR1 gene and is associated with a *cis*-acting regulatory element for the expression of CR1 on E.

*Received for publication 6 March 1986.*

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