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Promoter Structure and Transcriptional Activity of Human Complement Receptor Type I (CR1) Gene

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Until recently, interest in human complement receptor type I (CR1) has focused on immune complex processing, which contributed to our understanding of regulatory mechanism of complement activation. However, the promoter structure and transcriptional regulation of human CR1 gene has not been clear. To study the unique regulation of human CR1 gene expression, we assessed promoter activity of the 5'-flanking region of human CR1 gene using transient transfection and gel mobility shift assays. In this study we demonstrated that NF-Y binds to the inverted CCAAT element and that the functional interaction with protein(s) which bind to the GC-rich motif may be necessary for optimal transcription of human CR1 gene. We also show that sequence elements which located at -95/-58 and +45/ +50 are important for optimal transcription of CR1 gene.

The human complement receptor type 1 (CR1, CD35) plays an essential role in immune complex processing and regulation of complement system (Ross et al., 1985). CR1 is a membrane glycoprotein that is present on erythrocytes, monocytes/macrophages, granulocytes, B cells, some T cells, splenic follicular dendritic cells, and glomerular podocytes (Gelfand et al., 1976; Fearon et al., 1980; Kazatchkine et al., 1982; Wilson et al., 1983; Reynes et al., 1985). CR1 binds C3b and C4b that have covalently attached to immune complexes and other complement activators. The consequences of these interactions depend upon the cell type bearing the receptor (Fearon et al., 1983).

The gene encoding CR1 protein is tightly linked with C4b-binding protein (C4BP), complement receptor type 2 (CR2), decay-accelerating factor (DAF), membrane cofactor protein (MCP), and factor H genes defined as regulators of complement activation (RCA) locus, which maps to band q32 of chromosome 1 (Rodriguez de Cordoba et al. 1985; Weis et al., 1987; Lubin et al., 1987). Since the RCA gene cluster encodes the proteins involved in control of C3 convertases, it represents the regulatory counterpart of the class III gene cluster of major histocompatability complex (MHC) that encodes the structural components of C3 convertases C2, B, and B4. Thus, the RCA gene family consists of structurally homologous, functionally related, and genetically linked proteins. The RCA gene promoters have not been fully characterized, but a large portion of them appears to be TATA-less. However, the TATA-less RCA genes do not appear to have a common structural feature, and in general, individual RCA gene promoters have unique structures.

In our laboratory we have previously isolated and partially characterized the human CR1 gene promoter. This promoter has many features classically observed in housekeeping genes including a typical CpG island and TATA-less promoter. Several potential transcription factor binding sites, such as CArG (Funkhouser et al., 2000), Sp1, ets, inverted CCAAT box, and AML1 binding site (Kim et al., 1999a; Kim et al., 1999b; Rho et al., 2002) were also identified. In this context, here, we set out to further elucidate the promoter elements involved in regulation of human CR1 gene expression.

Materials and Methods

Cell Culture

HEL (human erythroleukemia) cells were maintained in RPMI1640 containing 10% fetal bovine serum. The cells were grown in an atmosphere of 5% CO_2 at 37°C.

Transient transfection and luciferase assays

The HEL cells were transfected with a reporter construct by electroporation and relative luciferase activities were assayed as described previously (Kim et al., 1999a). Compositions of the plasmids used are described in the figure legend. Transfection efficiencies were normalized to the level of β-galactosidase activities expressed from

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co-transfected pCMV-βgal plasmid containing cytomegalovirus promoter directing bacterial β-galactosidase gene expression.

Nuclear extracts and EMSA

Nuclear extracts from HEL cells were prepared by the rapid method of Schreiber et al., (1989) with minor modifications. Electrophoretic mobility shift assays (EMSA) were performed by a modification of the procedures by Pahl et al., (1993). For the binding reaction, 0.2-0.5 ng of 5'-end labeled synthetic double-stranded probe (10,000 cpm) was mixed with 15 μg of nuclear extracts in a final volume of 30 μl containing 10 mM Tris-HCl, pH7.5, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$, 4% glycerol, 3 ng BSA, and 1-5 μg of poly(dI).poly(dC). For competition experiments, an 80-fold molar excess of unlabeled double-stranded probe or mutant oligonucleotide was added to the binding reaction. For super-shift experiments, nuclear extracts were incubated on ice for 1 h with 2 μg of polyclonal antibody against Sp1, NF-YA or NF-YB prior to the addition of labeled probe. Samples were resolved on 4 or 6% non-denaturing polyacrylamide gel. Gels were dried and exposed to film for autoradiography.

Results and Discussion

We had previously shown that the basal human CR1 promoter is located within 376 nucleotides upstream of the transcription initiation site (Kim et al., 1999a) and

(-376) TTTCTTCACATTCCCTGTACACCGTACACATCGCCTGCATTGCCCTTCA

(-327) AACACGGAGAGCAGGCATTTCATTAGCTGACCTTCCCACACACATTCTT

(-278) GCAAAGAGGAAAAGTTAAGCAGGGTGTTTGGAGGCGAGCTGCCATCATC

(-229) CACCGCCTTTGTCTGGAAGCGCAGGGCCTCACACGCGGATCCATCGGA

(-180) AGCCCGAGCATTGTCAAGCTCTGCTGCTGCACCTGGGTCAGCAAGGTGG

(-131) GCTCTGCCAGCGAAACTCGTTAGAAACAATGCAAATGGGGAGTAAACAT

(-82) GACCTCGCCCATGAAGGGGAAGCTGTGGTCAAAAGCATTTTGTCCCGGA

CArG SRE Ets AML

(-33) ACCCCGCAGCCCTCCCCACACTCTGGGCGCGGAGCACAATGATTGGTCA

GC-Rich +1 Inverted CCAAT

(+17) CTCCTATTTTCGTCTGAGCTTTTCCTCTTATTTCAGTTTTCTTCGAGAT

+45/+50

(+66) CAAATCTGGTTTGTAGATGTGCTTGGGGAGAATGGGGGC

Met

Fig. 1. Nucleotide sequences of human CR1 promoter. The location of the transcription initiation site is shown and designated +1. Several potential transcription factor-binding sites were identified by computational analysis and indicated with underline.

identified several potential transcription factor-binding sites (Fig. 1). To further locate element(s) responsible for basal expression in this promoter, we generated a series of deletion mutants and point mutants of the pGL2-CR1 reporter construct between -376 to +77 and fused them with promoter-less luciferase reporter vector (pGL2-Basic). These constructs were transiently transfected into HEL cells by electroporation. Relative luciferase activity of the different reporter constructs were expressed relatively

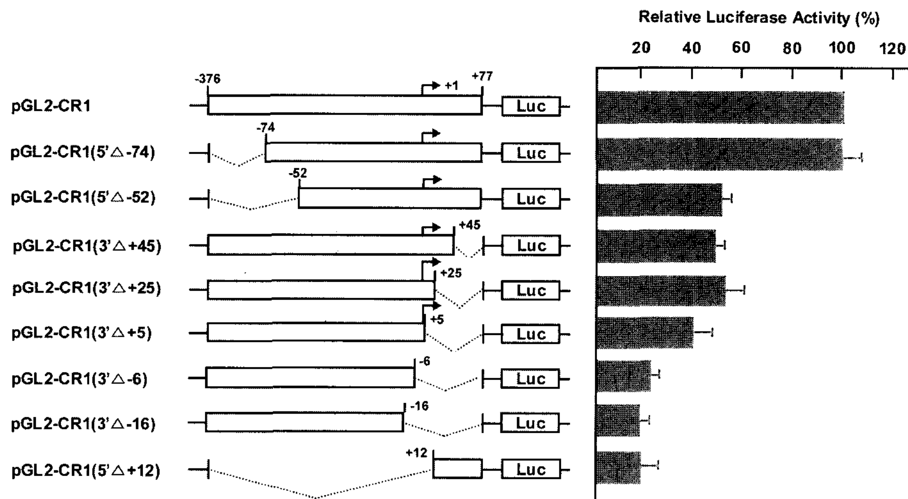

Fig. 2. Deletion analysis of the human CR1 promoter. A series of deletions were generated in the 5'- and 3'-flanking region of human CR1 gene by restriction digestion and PCR. Each construct was transiently transfected into HEL cells, and respective firefly luciferase activities were measured 48 hr after transfection. In each assay, the pCMV-βgal was cotransfected, and then the luciferase assays were normalized according to the β-galactosidase activity. The luciferase activity values obtained from the different plasmids were shown relative to the value from pGL2-CR1 plasmid, which was taken as 100%. Each value is an average of at least three separate experiments performed each in triplicate. The standard deviations are indicated with bars. The pGL2-Control plasmid was used as a positive control, and the promoter-less plasmid, pGL2-Basic, was used to measure the background level.

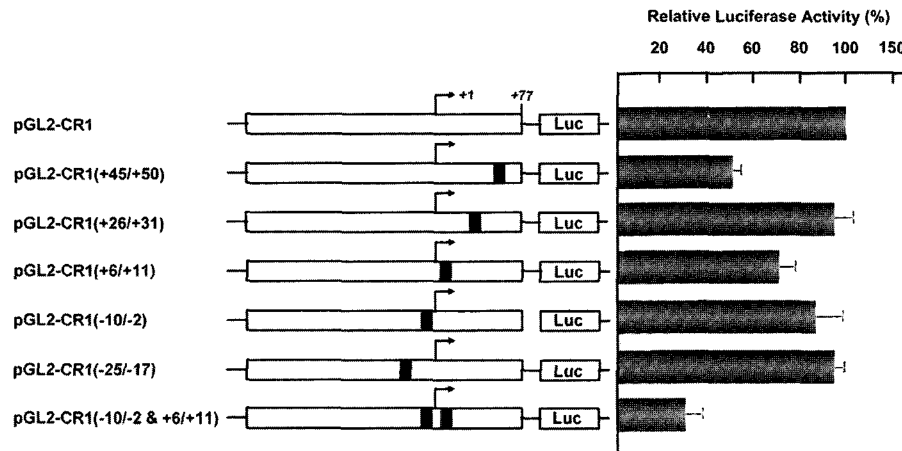

Fig. 3. Direct site mutagenesis of the human CR1 promoter. Point mutations on the some potential transcription binding sites were performed by site-directed mutagenesis. The activities of these mutants driving a firefly luciferase reporter gene were determined by transient transfection assay in HEL cells cotransfected with pCMV-βgal plasmid. The data (right panel) represent the mean firefly luciferase activity of three separate experiments.

to the pGL2-CR1 plasmid, which was taken as the 100% value.

As shown in Fig. 2, the reporter construct deleted to -52 showed 50% transcriptional activity compared with the wild type promoter (pGL2-CR1), whereas deletions of DNA segments located upstream of position -74 had no effect on transcriptional activity of human CR1 gene. These results are consistent with our previous findings and findings by others that the sequence element between -95 to -58 is necessary for the transcriptional activity of CR1 gene (Funkhouser et al., 2000) and the integrity of an AML1 binding site, 5'-TGTGGT-3', is critical for the human CR1 promoter activity (Kim et al., 1999b).

When the CR1 promoter was deleted to +45 from the 3' end, the transcription activity was significantly reduced (Fig. 2). In addition, the construct point mutated in the +45/+50 region also showed 50% transcription activity compared with the wild type CR1 promoter (Fig. 3). We also used EMSA to identify HEL cell nuclear protein(s) binding to the +45/+50 region of the human CR1 promoter. A single, slowly migrating band was observed which was effectively competed by unlabeled wild type oligonucleotide but not by its mutated counterpart (Fig. 4). We could not identify the nuclear protein. However, these results suggest that a yet undefined transcription factor present in HEL cell extracts specifically binds to the +45/+50 region in the human CR1 promoter and this region contains an important regulatory sequence for CR1 expression.

On the other hand, point mutations in the nucleotides between +6 to +11 also decreased the transcription to 70% and the constructs point mutated in the region -10/-2 showed 85% level of expression compared with the wild type promoter. However, the double mutated construct in the -10/-2 and +6/+11 showed a more dramatic decrease in the promoter activity to 30% compared to each single site mutant (Fig. 3). Furthermore, we identified a GC-

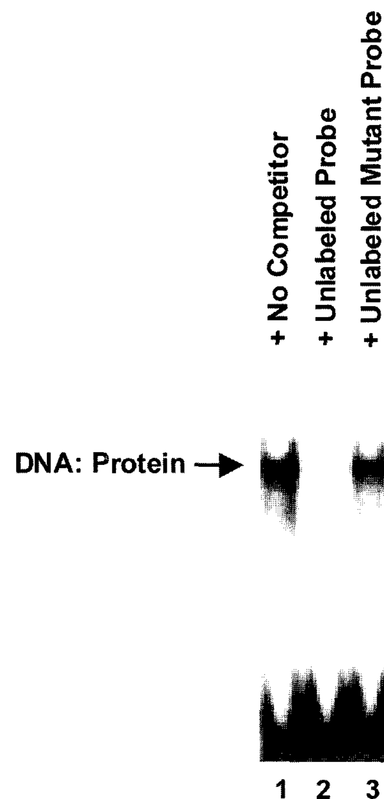

Fig. 4. EMSA analysis of the interaction of HEL nuclear extract with the sequence element located at +45/+50 in the human CR1 promoter. The nuclear extracts prepared from HEL cells were incubated in the presence of 26-mer radiolabeled double-stranded oligonucleotide probe, 5'-CTTTTC-CTCTTATTTCAGTTTTCTTC-3', corresponding to the human CR1 promoter region extending from position +35 to +60. Where indicated, an unlabeled probe and nucleotide substituted unlabeled (5'-CTT-TTCCTCTGTCGACAGTTTTCTTC-3', the underlined sequences represent mutated bases) were added to the reaction mixture as a competitor. The position of the DNA-Protein complex is indicated by the arrow.

rich motif (-10 to -2) and an inverted CCAAT element (+9 to +13) by computational analysis in the proximal 5'-flanking sequence.

The identification of the GC-rich motif (-10 to -2) and the inverted CCAAT element (+9 to +13) within the 5'-UTR prompted us to determine which nuclear factors could bind to these sites, because the former region contained a matched SP1 binding sequence and the latter region a NF-Y binding sequence. To investigate the nuclear factor binding to the CCAAT element, we tested DNA-protein complex formation with HEL cell nuclear extracts using EMSA as described in Materials and Methods. The nuclear extracts from HEL cells were incubated in the presence of a radiolabeled double-stranded 20-mer oligonucleotide probe, 5'-GCACAATGATTGGTCACTCC-3', corresponding to the human CR1 promoter region extending from positions +1 to +20 and containing the inverted CCAAT element (underlined sequence). As shown in Fig. 5A, a DNA-protein complex was detected and its formation was specifically inhibited by excess amounts of unlabelled probe. To determine that the binding involved the inverted CCAAT element, we introduced nucleotide substitutions at the ATTGG motif (GAGTT) in the double-stranded oligonucleotide probe and demonstrated lack of competition in its presence (Fig. 5, lane3).

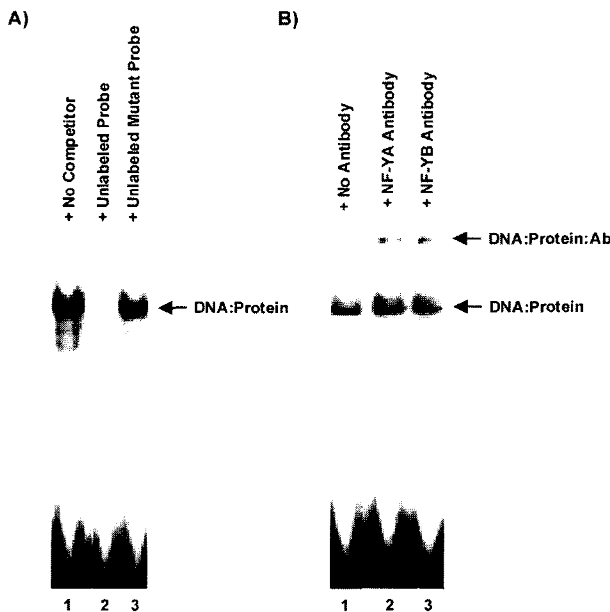

Fig. 5. NF-Y recognizes and binds to the inverted CCAAT element in the human CR1 promoter. (A) EMSA shows protein(s) present in HEL nuclear extracts bind to the CCAAT element in the human CR1 promoter. A gel mobility shift assay was performed using double-stranded synthetic oligonucleotide corresponding +1 to +20 sequence of the human CR1 promoter which contains inverted CCAAT element and fifteen μg of HEL nuclear extracts. To determine the specificity of binding activity, molar excess amounts (80X) of cold probe as a competitor was incubated in the reaction. (B) Identification of CCAAT binding complexes by EMSA. Fifteen μg of HEL nuclear extract was preincubated on ice for 1h with 2 μg of antibody against NF-YA or NF-YB. The specific DNA-Protein complex and the super-shifted complexes generated in the presence of antibodies were shown on the right.

We next tested whether NF-Y binds to the inverted CCAAT element of the human CR1 promoter using super-shift assays with polyclonal antibodies against NF-YA or NY-YB (Serotec, UK). As shown Fig. 5B, the DNA-protein complex was super-shifted by antibodies against NF-YA and NF-YB. The NF-Y protein has been identified as the major protein binding to the inverted CCAAT element (Hooft van Huijsduijnen et al., 1987; Dorn et al., 1987; Chodosh et al., 1988). The NF-Y complex is a ubiquitous heterotrimeric transcription factor, also referred to as CP1 or CBF, that consists of NF-YA, NF-YB, and NF-YC subunits with molecular masses of 42, 36, and 40 kDa, respectively (Vuorio et al., 1990; Maity et al., 1990; Sinha et al., 1995; Kim et al., 1996). All three NF-Y subunits are required for binding to the CCAAT element (Sinha et al., 1995; Kim et al., 1996). Therefore, these results suggest that the transcription factor NF-Y complex specifically binds to the inverted CCAAT element and controls the basal expression of human CR1 gene.

We also examined the protein binding ability in the GC-rich motif (-10 to -2) of CR1 gene by EMSA. A slowly migrating band which was effectively competed by unlabeled probe was observed. However, we failed to detect any antibody reactive DNA-protein complex when we performed super-shift assay with SP1 antibody (Fig. 6). An intriguing feature was that the construct double mutated in the GC-rich motif (-10/-2) and inverted CCAAT element (+6/+11) showed significantly reduced transcriptional activity whereas each construct point mutated either in the GC-rich motif (-10/-2) or the inverted CCAAT element (+6/+11) showed relatively mild reduction in the level of expression compared with the wild type promoter as we observed in transient transfection assay (Fig. 3). Therefore, we do not exclude the possibility that functional interaction of NF-Y and other undefined nuclear factor(s) cooperate in the transcription of the human CR1 and its synergistic regulation, although the mechanism has not been investigated. Cooperative interactions among transcription factors have been shown to be important for regulation of a number of gene promoters. The molecular mechanism responsible for this cooperative activity has recently been partially elucidated by demonstration of cooperative DNA binding of NF-YA and Sp1 and the presence of specific protein-protein interaction domains in NF-YA and Sp1 (Roder et al., 1997; Roder et al., 1999).

In summary, we used transient-transfection assay and EMSA to identify promoter regions necessary for optimal promoter activity of human CR1 gene. The studies presented here demonstrated that NF-Y binding to the inverted CCAAT element and nuclear factor(s) binding to the GC-rich motif (-10/-2) seem to operate in conjunction to regulate human CR1 gene transcription. The results also demonstrated that the sequence element which located at -74/-58 and +45/+50 are important for optimal transcription for CR1 gene. Future work should focus on

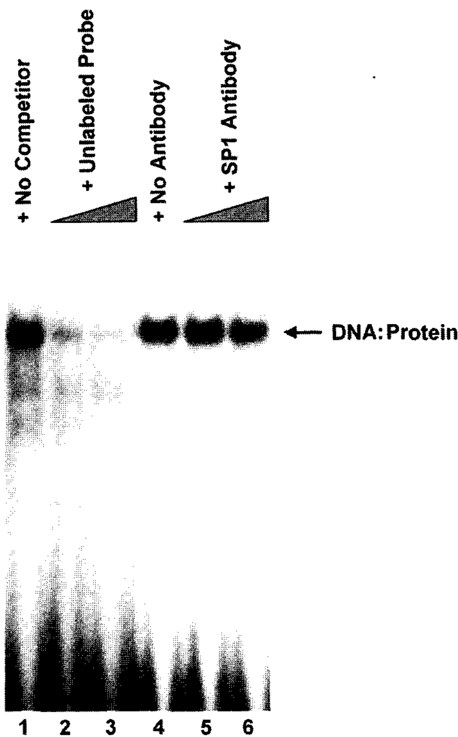

Fig. 6. EMSA analysis of the interaction of HEL nuclear extracts with GC-rich motif in the human CR1 promoter. HEL cell nuclear extracts (15 μg) were incubated with ^{32}P-labeled doubled-stranded synthetic oligonucleotide probe corresponding -17 to +4 sequence at the human CR1 promoter which contains GC-rich motif (TGGGCGCGG). The presence in the reaction mixture of competitor or antibody that specifically reacts with human SP1 is indicated above each lane.

characterization of protein binding activity of the consensus sequences identified in this promoter region. The results of these studies may provide important insight into human CR1 and RCA gene transcription.

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