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Human complement receptor (CR) type 2 (CR2/CD21) is a 145-kDa membrane protein encoded within the regulators of complement activation gene cluster localized on human chromosome 1q32. Understanding the mechanisms that regulate CR2 expression is important because CR2 is expressed during specific stages of B cell development, and several lines of evidence suggest a role for altered CR2 function or expression in a number of autoimmune diseases. Additionally, even modest changes in CR2 expression are likely to affect relative B cell responses. In this study we have delineated the transcriptional requirements of the human CR2 gene. We have studied the human CR2 proximal promoter and identified sites important for controlling the level of transcription in CR2-expressing cells. We have determined that four functionally relevant sites lie within very close proximity to the transcriptional initiation site. These sites bind the transcription factors USF1, an AP-2-like transcription factor, and Sp1. The Journal of Immunology, 2002, 168: 6279 – 6285.

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uman complement receptor (CR)3 type 2 (CR2/CD21) is a 145-kDa protein encoded within the regulators of complement activation gene cluster localized on human chromosome 1q32 (1). CR2 is the receptor for complement activation fragments of C3, specifically iC3b, C3dg, and C3d (2, 3). CR2 plays a central role in the generation of normal B cell responses to T-dependent Ags. Cr2−/− mice demonstrate a defect in the Ag-specific response to T-dependent Ags, manifested primarily by the lack of a robust switched IgG response (4–6).

The significance of mechanisms that regulate CR2 expression is apparent by studies of human B cell expression in a number of autoimmune and rheumatic diseases. It has been shown that patients with systemic lupus erythematosus (SLE) have abnormalities in the expression of CR2 on B cells (∼50% of normal levels) that may correlate with disease activity (7–9). Studies of lupus-prone mice have also found an early decrease in CR2 expression. This decrease was progressive and initially detectable before any major clinical manifestations (10). A recent study using congenic mice containing the major murine SLE susceptibility locus demonstrated a defect within the Cr2 gene (11). This Cr2 gene contained a single-nucleotide polymorphism that introduced a novel glycosylation site. Moreover, this polymorphism was located within the C3dg binding domain and was shown to reduce ligand binding and receptor-mediated signaling, suggesting a role for this Cr2 allele as a lupus susceptibility gene.

Further analysis of the biological effects of CR2 has shown the importance of CR2 expression in the maintenance of B cell tolerance and anergy (12). These studies demonstrated that combining mice that are genetically deficient in CR2 and CR1 with B6/lpr mice resulted in exacerbation of lupus disease activity. Additionally, B cells from soluble hen egg lysozyme anti-egg lysozyme-Ig double transgenic mice that are deficient in CR2 failed to be appropriately anergized in response to self Ag (12). Furthermore, a recent study has also reported a marked decrease in CR2 expression on B cells that was associated with a breakdown of tolerance in anergic mice and with induction of an SLE-like syndrome in graft-vs-host-induced mice (13).

Therefore, several lines of evidence exist suggesting that marked down-regulation of CR2 may play roles both in driving a breakdown in tolerance and in the pathogenesis of autoimmunity. As modest changes in levels of CR2 are likely to affect relative B cell responses (4), understanding CR2 regulation is imperative.

Our laboratory has discovered several critical elements involved in cell type-specific silencing or repression of human CR2. We have shown that the cell- and stage-specific expression of human CR2 is controlled by an intronic transcriptional silencer, designated the CR2 silencer. Use of a stable transfection system and transgenic mice has shown that the CR2 silencer element in conjunction with the CR2 proximal promoter is able to repress transcription in CR2-negative cell lines and tissues (14, 15). Recently, we have also demonstrated the existence of a cell type-specific repressor element within the CR2 proximal promoter that is critical for inhibiting expression in CR2 nonexpressing cell lines (16). In this study, we extend the understanding of the transcriptional regulation of the human CR2 gene by characterizing the requirements for basal transcription in a human CR2-expressing cell line.

Materials and Methods

Cell lines and culture conditions

The human Burkitt’s lymphoma cell line Raji (CCL-86) was obtained from the American Type Culture Collection (Manassas, VA). The cell line was maintained at 37°C with 5% CO2 in RPMI 1640 with t-glutamine supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 IU/ml penicillin.
Construction of CR2 promoter deletion and mutant luciferase fusion constructs

A NheI/XhoI fragment of the CR2 promoter containing either −315/+75 or −1250/+75 of the CR2 promoter was cloned into the luciferase reporter pGL3-basic vector (Clontech Laboratories, Palo Alto, CA). Site-directed mutagenesis was performed using the Quickchange mutagenesis kit (Stratagene, La Jolla, CA), which enabled the incorporation of MluI restriction sites extending 3′ from the positions −220 (site 4), −140 (Sp1), −120 (NF-κB), −90 (AP-2), −81 (AP-1), −65 (E box 2) and −47 (E box 1). Deletion constructs were then prepared using the newly incorporated MluI sites together with the MluI site situated in the pGL3-basic vector polylinker. Restriction enzyme digestion of the mutant plasmids with MluI resulted in varying lengths of upstream CR2 promoter sequence being deleted from the full-length −315/+75 construct. All constructs were confirmed by both restriction enzyme digestion and nucleotide sequence analysis.

Transfection and quantitation of promoter activity

Before each transfection, Raji cells were split and grown in log phase to 5 × 10⁵ cells/ml. Cells were then transfected using the Qiagen Superfect transfection reagent with plasmid DNA prepared using Qiagen Maxiprep-500 columns (Qiagen, Valencia, CA). Briefly, 10 μg of plasmid DNA and 300 ng of pRL-TK control vector was complexed together with Superfect reagent for 10 min at room temperature. The transfection complexes were then added dropwise to the cells, which had been plated in 5 ml of medium (17). Protein-DNA binding was performed for 20–30 min at room temperature in a binding buffer containing 10 mM Tris (pH 7.5), 40 mM NaCl, 1 mM EDTA, 1 mM 2-ME, and 4% glycerol. Following electrophoresis, wet gels were exposed to film at 4°C and autoradiographs were developed. Bands of interest were cut out and then electroeluted and phenol/chloroform extracted, precipitated, and dried. The pellet was resuspended in 100 μl of a 1/10 dilution of piperidine and heated at 90°C for 40 min. Following lyophilization, samples were then analyzed by electrophoresis on 6 or 8% acrylamide plus urea gels in Tris-borate buffer.

DNase I footprint analysis

DNase I footprint analysis was performed according to Dynan and Tijan (18). Probes (20,000 cpm) were labeled on the sense or antisense strand, incubated with increasing amounts of nuclear extracts (25–75 μg), and incubated on ice for 30 min in a buffer containing 2 mM HEPES (pH 7.8), 12.5 mM MgCl₂, 1 mM DTT, 10 μM ZnSO₄, 20% glycerol, and 0.1% Nonidet P-40, with 2 or 4 μg of poly(dI-C). Samples were then digested with 2 μl of increasing concentrations of DNase I at room temperature for 1 min. Reactions were stopped by addition of 90 μl of a solution containing 20 mM EDTA, 1% SDS, 0.2 M NaCl, and carrier RNA. Samples were then phenol/chloroform extracted, ethanol precipitated, dried, and resuspended in 90% formamide loading buffer. The samples were then analyzed on 6 or 8% acrylamide-urea gels.

Results

High basal activity is achieved by the −315/+75 proximal promoter

To determine which regions of the proximal promoter contributed to basal CR2 expression. −1250/+75 and −315/+75 bp of proximal promoter sequence was cloned upstream of a luciferase reporter gene. The −1250/+75 luciferase construct was shown to be highly active in Raji cells (Fig. 1, construct 1). Deletion of the proximal promoter sequence from −1250 to −315 resulted in no significant difference in promoter activity, indicating that all elements required for basal transcriptional activity are localized to the

FIGURE 1. Deletion analysis of the CR2 proximal promoter reveals that a high level of basal transcriptional activity is maintained by the −315/+75 sequence in Raji cells and that several additional regions are critical for basal promoter activity. Normalized transcriptional activity of the CR2 5′ promoter deletions is shown. Results are shown as mean normalized transcriptional activity vs 315 wild-type construct ± SEM (n = 5–10).
-315/+75 sequence (Fig. 1, construct 2). Further truncation of the CR2 promoter had very little effect on promoter activity (Fig. 1, constructs 3–5). Deletion of the CR2 promoter sequence up to −93 resulted in a 50% decrease in promoter activity (Fig. 1, construct 6). Further truncation to −83 and −75 resulted in a small decrease in promoter activity and, interestingly, deletion from −75 to −60 (Fig. 1, construct 9) resulted in a 1.3- to 1.7-fold induction in promoter activity, and, interestingly, deletion from −75 to −60 (Fig. 1, construct 9) resulted in a 1.3- to 1.7-fold induction in promoter activity. However, truncation of the promoter sequence from −60 to −47 resulted in a marked decrease in transcriptional activity (−60%). This truncation results in a construct containing only a small amount of upstream sequence plus the TATA box at position −29. These results show that these latter sequences are responsible for start site selection and mediate low-level basal activity, but that high-level basal transcription is mediated by elements within the entire −315/+75 sequence.

**DNase I footprinting and methylation interference analysis across the functionally relevant sites reveal several protected regions and methylated nucleotides**

Transcriptional analysis revealed important roles for sequences spanning nucleotides −120 to −93 and −75 to −47 in the regulation of CR2 expression (Fig. 1). To examine these regions further and identify candidate regulatory sites, DNase I footprinting and methylation interference analyses were performed. First, a 165-bp probe encompassing sequence from −90 to +75 (Fig. 2) was designated site 1. A second sequence, located at −67/−60, was also shown to be protected, albeit weakly (Fig. 2, site 2).

Methylation interference assays on both the sense (Fig. 2Bi) and the antisense (Fig. 2Bii) strand demonstrate methylation of several G residues. Methylation of residues −46, −42, −40, and −38 on the sense strand and methylation of G residues −45 and −43 on the antisense strand was shown to interfere with protein binding. Results are summarized in graphical form (Fig. 2A). Sequence analysis of these two sites shows homology to two classes of E box motif shown to bind the helix-loop-helix (HLH) family of transcription factors (19). These elements share a motif consisting of a core hexanucleotide sequence, CANNTG, and bind multiple classes of HLH transcription factors, including E12, E47, c-Myc, USF, and Mad/Max.

Deletion analysis also revealed a strong activator domain localized between nucleotides −120 and −93 within Raji cells (Fig. 1). To examine NF binding to this region, a second probe was designed and used in DNase I footprinting. The probe was an 80-bp fragment spanning nucleotides −149 to −69 (Fig. 3). Surprisingly, a large protected region was seen over 40 bp of the probe from −131 to −92 (Fig. 3B). Sequence analysis of the footprinted region demonstrates a cytosine-rich sequence that includes many Sp1 sites and several CACC boxes. Additionally, there are AP-1-like and AP-2-like elements within this region between −90 and −69; however, as was also seen with the probe in Fig. 2 (−90/+75), we could not demonstrate a clear footprint of these two elements (data not shown).

**FIGURE 2.** A, DNase I footprinting reveals two protected sequences from −70 to −30, designated sites 1 and 2. Protected sequences are boxed. Methylated G residues as shown by interference assays are shown by stars. The arrow indicates a hypersensitive site. B, Footprint and methylation interference assays from −90 to +75. i, DNase I cleavage was performed with no extract or with 25, 50, or 75 μg of Raji extract. A Maxam-Gilbert G+A sequence reaction is shown in the left lane. The arrow indicates a DNase I hypersensitive site. Positions of the TATA box, AP-1, and AP-2 sites are shown. ii and iii, Methylation interference assay is shown for the sense and antisense strands. Methylated G residues interfering with protein binding are shown as stars. F and B represent the free and bound probes, respectively.

**FIGURE 3.** A, Summary of DNase I footprint analysis of the −149 to −69 region. The protected sequence designated as site 3 is shown as the boxed sequence. B, DNase I footprint assay. A Maxam-Gilbert G+A sequence reaction is shown in the left lane. DNase I cleavage was performed with no extract or increasing amounts of Raji extract. The protected area is designated site 3.
Deletion analysis as well as DNase I footprinting and methylation interference analysis revealed several potentially important sites within the CR2 promoter involved in basal transcriptional control in the CR2-expressing Raji cell line. Sequence analysis of these regions using TFSEARCH (version 1.3) database search revealed several consensus transcription factor binding sites within the functional and DNase I footprinted regions (Fig. 4). These sites include two adjacent E box motifs within the footprinted sequences at positions −47 to −38 and −67 to −60 and are designated E box sites 1 and 2, respectively (Fig. 4). The large protected sequence located at position −132 to −92 contained many Sp1-like sites and CACC boxes, and is designated site 3.

Mutation analysis of the CR2 promoter reveals important roles for the −81 AP-2 sequence, the −60 E box site 2 motif, and the −47 E box site 1 motif in regulating basal expression of CR2.

To further assess whether the consensus sequences shown by footprinting and methylation interference (Fig. 4) were functional, a series of site-specific mutations were made and then used in transient transfections of CR2-expressing Raji cells. Introduction of a 6-bp mutation into the putative Sp1 site localized within the site 3 footprinted region had a modest but reproducible effect on transcriptional activity (Fig. 5, construct 3). Site-specific mutation of the consensus AP-1 site at −90 had no effect on promoter activity as compared with the −315/+75 wild-type construct. Interestingly, introduction of the 6-bp mutation into both the consensus AP-2 sequence at −81 (Fig. 5, construct 5) and the E box site 2 motif at −60 (Fig. 5, construct 6) resulted in a 1.3- to 1.7-fold induction of transcriptional activity. Deletion of the AP-2 site and upstream sequence (Fig. 1, construct 7) did not show a similar induction in transcriptional activity. This is most likely due to the interaction of this AP-2 element with sequences upstream. Mutation of the E box site 1 motif located at −47 resulted in a marked 80% decrease in transcriptional activity, indicating a particularly important role for this site in maintenance of CR2 basal transcription.
Characterization of the factors binding the functionally relevant sites within the CR2 proximal promoter

Sequence analysis of the DNase I footprinted regions revealed consensus binding sequences for known transcription factors (Fig. 4). Additionally, transcriptional assays revealed important roles for the two adjacent E box motifs at -47 and -60 as well as the AP-2 site located at -81, together with the entire site 3 motif spanning nucleotides -130 to -90. To determine whether these functionally important sequences do indeed bind the known transcription factors within their consensus binding sites, EMSA was performed using nuclear extracts prepared from Raji cells.

EMSA spanning the entire site 3 DNase I footprinted region from -130 to -90 resulted in the presence of two major protein-DNA complexes (Fig. 6A, complexes A and D) and two minor protein-DNA complexes (Fig. 6A, complexes B and C). Addition of increasing amounts of cold self oligonucleotide resulted in competition of all complexes, indicating the existence of highly specific complexes. Sequence analysis of the site 3 region revealed a match for a Sp1 sequence. Competition using a Sp1 consensus oligonucleotide resulted in abolishment of the major protein-DNA complex A as well as the minor complex C (Fig. 6A, Sp1). To further elucidate the presence of Sp1 binding to the site 3 motif, supershift analysis was performed (Fig. 6B). Addition of the Sp1 Ab to the binding reaction resulted in a supershift of protein-DNA complex A (Fig. 6B, A*). These results indicate that the transcription factor contained within complex A is a member of the Sp1 family. Protein-DNA complexes B and D contain as-yet-unidentified proteins.

EMSA spanning the AP-2 consensus binding site from -90 to -69 comprised one specific protein-DNA complex (Fig. 7A, complex A). Cold competition using self oligonucleotide indicated competition of protein-DNA complex A at a 50-fold molar excess, indicating the presence of highly specific binding. Competition assays using a consensus oligonucleotide for AP-2 also revealed competition of complex A, indicating the potential presence of AP-2 in this complex. The AP-2 consensus oligonucleotide used in these experiments is a binding site for the transcription factor AP-2α. However, to further investigate the role of AP-2α in binding this site, supershift analysis (Fig. 7B) using an Ab recognizing the AP-2α transcription factor was performed. Addition of this Ab to the binding reaction did not result in competition of complex A, indicating that the protein within complex A shares DNA binding specificity with AP-2 but is serologically distinct from AP-2α.

Functional assays revealed a major role for maintenance of transcriptional activity for the E box site 1 motif. This motif is a member of the E box family of transcription factors and has been shown to bind several proteins of the basic HLH-leucine zipper class, including USF1, TFE3, Max, and c-myc. EMSA spanning the E box site 1 motif from -63 to -40 resulted in the presence of three protein-DNA complexes (Fig. 8A, complexes A–C). Addition of increasing amounts of cold self competitor oligonucleotide revealed the competition of complexes A–C, indicating that these complexes were of high affinity. Supershift analysis (Fig. 8B) using an Ab directed against USF1 resulted in the abolishment of protein-DNA complex A and decreases of complexes B and C. As a negative control, an Ab directed to a different class of HLH protein (E2A) was used (Fig. 8B). As expected, addition of this Ab had no effect on the binding of the specific proteins to the E box site 1 motif.

Discussion

This study has investigated further the requirements for basal transcription of the human CR2 gene. Early studies (20, 21) on the CR2 promoter demonstrated that the transcriptional initiation site was localized to 92–94 bp upstream of the ATG codon. Additionally, in transfection experiments the 5’ promoter sequence was
found to be active, but the specific requirements for basal transcription have not been further characterized until now. We cloned various lengths of the upstream promoter sequence in front of a luciferase reporter and found several regions involved in basal transcription. Promoter constructs containing either −1250/+75 or −315/+75 of upstream sequence had the same high level of basal transcriptional activity when transfected into the CR2-expressing Raji cell line. These results indicated that all of the necessary machinery for driving basal CR2 expression was localized in this −315/+75 sequence. Interestingly, a recent report (22) has shown that further upstream regions are responsible for PMA- and cAMP-inducible expression of the CR2 promoter.

Deletion analysis of the −315/+75 sequence revealed several regions within the promoter important to function. The first element was localized upon deletion of the promoter sequence from −120 to −93. This deletion resulted in a 50% decrease in transcriptional activity, indicating the presence of an activator motif within this sequence. DNase I footprinting across this region revealed a large protected sequence from −69 to −90 of the upstream promoter. This sequence contains a number of GC and GT boxes. It has been demonstrated previously (23) that G-rich elements and GT/CACC boxes such as the ones seen in this protected footprint are important elements in housekeeping as well as many tissue-specific genes. It was previously thought that the ubiquitous transcription factor Sp1 acts through these GC/GT boxes; however, recently it has become clear Sp1 is only one of many transcription factors belonging to a family characterized by a highly conserved DNA-binding domain consisting of three zinc fingers that bind to these sites. Site-specific mutagenesis of one of many consensus Sp1 motifs within the footprinted region had only a modest effect on transcriptional activity of the CR2 promoter. This is not surprising, as the large footprinted region could indicate a complex pattern of NF binding, which was uninterrupted by the mutation introduced. EMSA using an oligonucleotide spanning the footprinted region from −130 to −90 resulted in the presence of two major protein-DNA complexes. Competition using both a Sp1 consensus oligonucleotide and a Sp1 supershift Ab indicated the presence of this protein in binding the large footprinted region. However, other as-yet-unidentified proteins, perhaps one of the newly characterized Sp1-like zinc finger transcription factors, are also likely involved in binding the site 3 motif.

Mutational analysis also revealed an important role for the AP-2 consensus sequence localized at position −81. Mutation of several base pairs spanning this motif resulted in a 1.5- to 1.8-fold induction in promoter activity, suggesting the presence of a repressor element at this site. EMSA and supershift analysis using an oligonucleotide spanning −90 to −69 encompassing the AP-2 consensus sequence within the CR2 promoter showed that this site appeared to bind a protein that shared DNA specificity with AP-2 but was serologically distinct from AP-2α. The AP transcription factor family was first isolated from HeLa cells and was initially named for its transcriptional activation function (24, 25). It has been shown to be an activator in regulating many genes, including HIV type 1 (26), type IV collagenase (27), and the dopamine β-hydroxylase gene (28). However, the AP-2 site within the human CR2 gene acts as a transcriptional repressor and recently more data have been assembled suggesting that this well-known activating transcription factor may act as a transcriptional repressor. For example, an AP-2 site functions as a repressor and contributes to the liver-specific expression of the serum amyloid A1 gene (25). Additionally, an AP-2 site within the T cell-restricted CD2 gene has been shown to be acting as a repressor; however, the factor binding to this site was shown to be serologically distinct from AP-2 (29), similar to that seen in the CR2 gene.

Finally, a third region within the CR2 proximal promoter was determined to be functionally significant using both mutant and deletion analysis. An E box motif located at −47 was found to be particularly important in maintenance of basal transcriptional control of the CR2 promoter. Interestingly, a recent paper by Vereshchagina et al. (22) has also shown a role for this site in induction of CR2 promoter activity by cAMP and PMA. Deletion and mutation analysis of E box site 1 at −47 demonstrated the existence of a strong activator motif within this sequence. DNase I footprinting demonstrated clearly protected sequences across this site. Additionally, methylation interference assays revealed several nucleotides that were methylated across E box site 1. EMSA and supershift analysis demonstrated the binding of USF1 to this site.

Table I. Summary of the functionally relevant sites within the CR2 proximal promoter*

<table>
<thead>
<tr>
<th>Nucleotide Position (bp)</th>
<th>Footprint</th>
<th>Methylation State</th>
<th>Functional Role</th>
<th>Proteins Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>−47</td>
<td>+</td>
<td>+</td>
<td>Activator</td>
<td>USF1 and unknown</td>
</tr>
<tr>
<td>−63</td>
<td>+</td>
<td>−</td>
<td>Cell type-specific repressor</td>
<td>E2A proteins (16)</td>
</tr>
<tr>
<td>−81</td>
<td>−</td>
<td>ND</td>
<td>Repressor</td>
<td>AP-2-like</td>
</tr>
<tr>
<td>−120/−93</td>
<td>+</td>
<td>−</td>
<td>Activator</td>
<td>Sp1 and unknown</td>
</tr>
</tbody>
</table>

* Shown are the nucleotide positions of the localized elements and the functional role for each motif. Also shown are the proteins that were shown to bind these sites and the footprint and methylation status of each functional motif.
USF1 is a member of the basic HLH/leucine zipper family of transcription factors (30, 31). USF transcription factors are involved in the regulation of many E box-containing genes, includingmurine metallothionenI (32), murine p53 (33), human CD2 (34), and human β-globin gene (35). Interestingly, there is also evidence to suggest that USF functionally interacts with basal transcriptional machinery such as TFIID (36). USF has also been shown to bind cooperatively with TFI-I and sequences close to the initiation of transcription (37, 38). It is interesting to speculate that, due to the proximity of the E box site 1 motif to the transcriptional initiation site (−47), USF1 binding to this motif may interact with the basal transcriptional machinery to activate transcription. This may explain the importance of this site in maintenance of basal CR2 transcription as demonstrated by a dramatic loss in promoter activity when this site was mutated within the CR2 proximal promoter.

In conclusion, several sites within the proximal promoter were shown to be important in the transcriptional control of human CR2 in Raji cells, a CR2-expressing cell line (Table I). These sites bind the transcription factors USF1, an AP-2-like factor, and Sp1 as well as other as-yet-unknown proteins. Recently, we have also discovered the presence of a cell type-specific repressor localized to the E box 2 motif at position −63 of the proximal promoter (16). The primary control of basal transcription in CR2-expressing cells appears to lie in very close proximity to the TATA box; however, we cannot rule out the possibility of other as-yet-undetected elements further upstream, or even within other areas of the CR2 gene itself. The four main motifs involved in basal transcription control lie within 120 bp of upstream sequence (Table I). Moreover, three of the four functionally relevant sites are localized to within 80 bp of the transcriptional initiation site and are located very close together, within −30 bp. How these elements interplay with one another to control CR2 transcription and how they may interact with the intronic silencer that controls cell- and stage-specific expression is yet to be determined but is currently under investigation.

Acknowledgments

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References