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# Transcriptional effects of a lupus-associated polymorphism in the 5′ untranslated region (UTR) of human complement receptor 2 (CR2/CD21)

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ABSTRACT

Systemic lupus erythematosus (SLE) is a complex autoimmune disease with a strong genetic component that determines risk. A common three single-nucleotide polymorphism (SNP) haplotype of the complement receptor 2 (*CR2*) gene has been associated with increased risk of SLE (Wu et al., 2007; Douglas et al., 2009), and a less common haplotype consisting of the major allele at SNP1 and minor alleles at SNP2 and 3 confers protection (Douglas et al., 2009). SNP1 (rs3813946), which is located in the 5′ untranslated region (UTR) of the *CR2* gene, altered transcriptional activity of a *CR2* promoter–luciferase reporter gene construct transiently transfected into a B cell line (Wu et al., 2007) and had an independent effect in the protective haplotype (Douglas et al., 2009). In this study, we show that this SNP alters transcriptional activity in a transiently transfected non B-cell line as well as in stably transfected cell lines, supporting its relevance *in vivo*. Furthermore, the allele at this SNP affects chromatin accessibility of the surrounding sequence and transcription factor binding. These data confirm the effects of rs3813946 on *CR2* transcription, identifying the 5′ UTR to be a novel regulatory element for the *CR2* gene in which variation may alter gene function and modify the development of lupus.



## 1. Introduction

Complement receptor type 2 (CR2/CD21) is a 145 kDa glycoprotein that binds C3 degradation products generated during complement activation, specifically iC3b, C3dg, and C3d. It has a number of important functions in normal immunity, including cooperating with the B cell receptor to activate B cells, targeting antigen to follicular dendritic cells in secondary lymphoid organs, processing and presenting complement-coated antigens to T cells, and shaping the natural antibody repertoire (reviewed in Holers, 2005). Its critical role in host defense is apparent from studies showing blunted IgG responses in *Cr2*-deficient mice after immunization with T-dependent or -independent antigens (Ahearn et al., 1996; Croix et al., 1996; Haas et al., 2002; Molina et al., 1996).

CR2 is also believed to participate in the development of autoimmune disease. CR2 levels are decreased by ∼50% in patients with systemic lupus erythematosus (SLE) (Marquart et al., 1995; Wilson et al., 1986), and SLE-prone MRL/*lpr* mice demonstrate a similar phenotype prior to the onset of clinical evidence of disease (Takahashi et al., 1997), suggesting a role in disease pathogenesis. Mice rendered deficient in *Cr2* develop either enhanced or diminished symptoms of autoimmunity, depending on the disease model (Del Nagro et al., 2005; Kaya et al., 2001; Prodeus et al., 1998; Wu et al., 2002). Furthermore, *Cr2* is a strong candidate gene for disease susceptibility in the NZM2410 murine model of SLE due to gene polymorphisms that alter the structure and function of the CR2 protein product (Boackle et al., 2001). The mechanism by which CR2 participates in the onset and evolution of autoimmune disease is not known.

CR2 expression is restricted primarily to B cells and follicular dendritic cells, and its expression on B cells is tightly regulated, first detected at the late immature/transitional stage, increasing as the cells mature into follicular and marginal zone B cells respectively

*Abbreviations:* b, biotinylated; B6, C57BL/6; CR2, complement receptor type 2; NE, nuclear extract; Q-PCR, quantitative PCR; SLE, systemic lupus erythematosus; SNP, single-nucleotide polymorphism; UTR, untranslated region.

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(Takahashi et al., 1997; Tedder et al., 1984; Thomas et al., 2006), and disappearing as cells differentiate into plasma cells (Tedder et al., 1984). The transition of human transitional B cells from $CR2^{low}$ to $CR2^{high}$ is believed to be a checkpoint for the deletion of self-reactivity (Suryani et al., 2010). Surface expression of CR2 is closely linked to the presence of mature mRNA, which is controlled primarily at the level of transcription (Makar et al., 1998). Critical elements that regulate basal transcription of the human *CR2* gene have been targeted to the region spanning −315 to +75 of the genomic sequence. Although this region of the *CR2* promoter is sufficient to drive basal transcription (Ulgiati et al., 2002), the strict developmental regulation and inducible expression of CR2 require additional elements (Makar et al., 1998, 2001; Tolnay et al., 2002; Vereshchagina et al., 2001). A number of regulatory elements have been identified in the human *CR2* gene, including sequences homologous to TATA, SP1, AP-2, AP-1 and Ig enhancer E motif DNA–protein binding sites in the proximal promoter (Rayhel et al., 1991; Ulgiati et al., 2002) as well as distal regions that control inducible expression including a NF-κB site (Tolnay et al., 2002). Furthermore, appropriate tissue-specific expression of *CR2* requires an intronic silencer element (Makar et al., 1998). However, the 5′ UTR of the *CR2* proximal promoter was not known to be relevant in the regulation of *CR2* transcription until our previous report, in which we showed that a lupus-associated polymorphism (rs3813946) located in this region altered transcriptional activity of a *CR2* promoter–luciferase reporter gene construct that was transiently transfected into a B cell line (Wu et al., 2007).

In our initial study conducted in Caucasian and Chinese lupus simplex families, we demonstrated that the major allele of rs3813946 was transmitted preferentially from heterozygous parents to their affected offspring with a $p$ value of 0.007 and an estimated odds ratio of 1.53 (1.11–2.10) (Wu et al., 2007). In our subsequent case–control study conducted in unrelated Caucasian subjects, we identified a protective *CR2* haplotype that contains the major allele of rs3813946 and the minor alleles of 3 other exonic SNPs (haplotype $p$ value 0.003, odds ratio 0.79 [0.68–0.92]), on which the major allele of rs3813946 exhibited an independent genetic effect although it was not associated with increased risk of lupus in single SNP analysis (Douglas et al., 2009). These data suggest that the ability of the major allele of rs3813946 to alter transcription could modify the effects of the causal SNP(s) in the protective *CR2* haplotype. In order to verify that rs3813946 alleles alter gene function and to further characterize the role of the 5′ UTR of *CR2* in transcriptional regulation, we performed the additional studies outlined in this report. Herein, we show allelic differences of rs3813946 in a second non-CR2 expressing cell line as well as in a stably integrated reporter construct. Furthermore, its variants alter chromatin accessibility and binding of several proteins, which may explain its transcriptional effects. These data confirm a functional effect for this polymorphism and demonstrate the contribution of the 5′ UTR in which it is located to the regulation of CR2 transcription, supporting further study of the effects of this region on the complex developmental and tissue-specific expression of CR2.

## 2. Materials and methods

### 2.1. Cell lines and culture conditions

The human Burkitt's lymphoma cell line Raji and the human erythroleukemic cell line K562 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cell lines were maintained at 37 °C with 5% $CO_2$ in RPMI 1640 with L-glutamine (Gibco BRL, Invitrogen Life Technologies, Melbourne, Australia) supplemented with 10% FBS (Gibco BRL, Invitrogen Life Technologies), 100 μg/ml streptomycin, and 100 IU/ml penicillin (Gibco BRL, Invitrogen Life Technologies).

### 2.2. Construction of CR2 mutant reporter constructs

A *Nhe*I/*Xho*I fragment of the *CR2* promoter containing the −315/+75 of the *CR2* promoter was cloned into the luciferase reporter pGL3-basic vector (Clontech Laboratories, Palo Alto, CA, USA) as described previously (Ulgiati et al., 2002). Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA), which enabled the incorporation of the minor +21C allele at rs3813946 in the *CR2* promoter–luciferase reporter fusion construct. Correct orientation and sequence of constructs was verified by restriction enzyme digestion and nucleotide sequence analysis.

### 2.3. Transfection and quantitation of promoter activity

Transfections were performed by harvesting cells (Raji or K562) grown to log phase using the Superfect reagent (QIAgen, Valencia, CA, USA) according to the manufacturer's specifications with plasmid DNA prepared using Endofree Maxiprep-500 columns (QIAgen). Briefly, 4 μg of plasmid DNA and 120 ng of pRL-TK (*Renilla*) control vector were complexed in combination with Superfect reagent for 10 min at room temperature. The transfection complexes were added to suspensions of cells in 24-well plates to a final density of $5 \times 10^5$ cells/ml. Cell lysates from the transfected cells were prepared and assayed for both *Firefly* and *Renilla* luciferase using the Dual-Luciferase Reporter Assay system according to the manufacturer's instructions (Promega, Madison, WI, USA). All transfection data shown are the mean of 3–5 independent transfections, with $n$ values shown in each experiment. Promoter activity is expressed as relative *Firefly* luciferase activity normalized against *Renilla* luciferase activity.

### 2.4. Chromatin accessibility assays

Chromatin accessibility measured by real-time PCR (CHART-PCR) assays were performed as described previously (Cruickshank et al., 2008; Rao et al., 2001) using $1.25 \times 10^6$ nuclei with 5 U MNase/ml (Worthington Biochemicals, Lakewood, NJ, USA). Following digestion of nuclei, DNA was recovered using the QIAamp DNA blood mini-kit (QIAgen) and used in quantitative (Q)-PCR assays with primers targeting the *β-actin* promoter (forward: 5′-CAGCACCCCAAGGCGGCCAACG-3′; reverse: 5′-GCAACTTTCGGAACGGCGCACGC-3′), *PAX7* promoter (forward: 5′-CCGAACCTATCAGATCGCGCTCAC-3′; reverse: 5′-GTCACCCCCTGTCTCCTCCGTCCAG-3′), *CR2* upstream region (forward: 5′-GATGTGGATTCGCCTATCCC-3′; reverse: 5′-CCCTGAAGGTAGTGGTGTAAAGC-3′) and *CR2*-reporter transgene (forward: 5′-GATGTGGATTCGCCTATCCC-3′; reverse 5′-TCTTCCAGCGGATAGAATG-3′). Q-PCR was performed on the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the QuantiTect™ SYBR® Green PCR kit (QIAgen) according to the manufacturers' instructions with 0.5 μM each primer in 20 μl reaction. Thermal-cycling conditions were as follows: 95 °C for 15 min; followed by 40 cycles of 95 °C for 15 s, 57 °C for 15 s and 72 °C for 15 s; followed by a melt–curve cycle (gradual increase in temperature from 60 °C up to 99 °C). Acquisition of sample fluorescence occurred after 72 °C cycles and at 1 °C increments during melt–curve analysis. The efficacy of Q-PCR primers was verified by amplification of serially diluted genomic DNA or plasmid DNA to ensure linear detection of template and by agarose gel electrophoresis of PCR products and routine melt–curve analysis to ensure specificity.

### 2.5. Preparation of nuclear and cytosolic proteins

For preparation of nuclear extract (NE), $4 \times 10^7$ cells from Raji and K562 were harvested and prepared essentially as described