**Quantitative differences in chromatin accessibility across regulatory regions can
be directly compared in distinct cell types**

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Running Title: Chromatin accessibility across regulatory regions detected by CHART-
PCR

Abstract

Transcriptional activation in eukaryotes is often accompanied by alterations to chromatin structure at specific regulatory sites while other genomic regions may remain unchanged. In this study, we have examined the correlation between expression and chromatin accessibility of the human *CR2* gene in a panel of cell lines (U937, REH, Ramos and Raji) using the CHART-PCR assay with the accessibility agent micrococcal nuclease (MNase). To validate the use of this assay for comparing multiple cell types, we first tested a series of genomic regions to determine if we could observe consistent, site specific levels of MNase chromatin accessibility. Promoter regions of the ubiquitously expressed genes *GAPDH* and *β -actin* were similar and showed high accessibility to MNase digestion in each of the cell lines, while on the other hand, promoter regions of developmentally restricted genes *PAX-7* and *SP-A2* showed consistently reduced chromatin accessibility. Since CHART-PCR detected site specific differences in chromatin accessibility in a manner that could be compared between cell types, we next examined chromatin accessibility over the *CR2* core promoter in the panel of cell lines representing either *CR2* expressing or *CR2* non-expressing cell types. Our data revealed significantly enhanced accessibility over the -289 to -101 and the -115 to -12 regions of the *CR2* promoter in expressing B-cells (Ramos, Raji) compared to non-expressing cells (U937, REH). Thus, CHART-PCR assays detected a correlation between chromatin accessibility and expression of the human *CR2* gene, while the accessibility of other genomic regions was site-specific, but was not significantly altered between cell types.

Keywords

Chromatin accessibility, transcriptional activation, *CR2*, CD21, B-cell differentiation.

Introduction

There are a variety of techniques that examine site specific chromatin structure and involve treating isolated chromatin or cell nuclei with a digestion agent and assessing resulting genomic DNA digestion patterns [1, 2]. More recently, an assay using real-time PCR to measure chromatin accessibility (CHART-PCR) was developed to define specific regions which are subject to chromatin structural variation during induction of gene expression [3]. It is clear that the accessibility of genomic DNA both within chromatin and to nuclear factors is highly regulated to control gene expression.

We are interested in understanding the molecular processes underling B-cell restricted expression of the human *Complement receptor type 2* (*CR2*) gene. On B-cells, CR2 forms the BCR co-receptor complex together with CD19, CD81 and Leu13 [4] and can lower the threshold of antigen necessary for activation [5]. Expression of human CR2 is restricted during B-cell development and is observed on newly formed immature and mature B cells, initiated at approximately the same stage as IgD and CD23. In contrast, CR2 is undetectable during early stages of development (including pro-B cells and pre-B cells in the bone marrow) and upon terminal differentiation into antibody secreting plasma cells [6, 7]. Stringent regulation of CR2 on B-cells provides a model system of differentiation regulated transcription.

Presently, there are no physiologically relevant *in vitro* cell culture models available for initiating expression of the human *CR2* gene. In this study, we used a panel of cell lines representing CR2 non-expressing (U937, REH) and CR2 expressing (Ramos, Raji) cell types to examine the correlation between accessibility of the *CR2* core

promoter and transcriptional activation. To verify the utility of CHART-PCR for analysing multiple cell types we first compared the level of accessibility at a series of regulatory locations to determine if the assay produced comparable site-specific chromatin accessibility among the cell lines examined. To this end, we targeted promoter regions of both broadly expressed (*β-actin* and *GAPDH*) and developmentally restricted (*PAX-7* and *SP-A2*) gene loci. *PAX-7* encodes a transcription factor (TF) predominantly expressed during embryonic development and in adult myogenic precursor cells [8], while *SP-A2* encodes a cell surface receptor expressed at high level in human lungs and on some other epithelial cell types [9, 10]. Our results demonstrate consistent, site specific chromatin digestion by MNase over these regulatory regions, which supports the efficacy of CHART-PCR assays for comparing chromatin accessibility between cell types.

Our laboratory has discovered several functional regulatory elements involved in cell type-specific repression and basal activation of human *CR2* [11-13]. Now, having demonstrated the applicability of CHART-PCR for quantifying variation in accessibility in different cell types, we were able to apply this assay to examine the *CR2* core promoter using the panel of cell lines representing *CR2*-expressing B-cells or non-expressing cell types. Our analyses showed enhanced chromatin accessibility in *CR2*-expressing cell lines (Ramos, Raji), while highly inaccessible chromatin was observed flanking the transcription start site (TSS) in non-expressing cell lines (U937, Reh). Sequence analysis of these regions of the *CR2* core promoter revealed several TF consensus binding sites which could play a role in generating chromatin modifications or alternatively may become accessible to bind TFs as a result of the change in chromatin structure.

Materials and methods

Cell culture

Cell cultures were maintained at 37 °C with 5% CO₂ in RPMI-1640 supplemented with 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 4 mM L-Glutamine. Cell lines were obtained from the ATCC (Manassas, VA). REH (CRL-8286) and U937 (CRL-1593.2) cells were used to represent non-expressing cell types while Ramos (CRL-1596) and Raji (CCL-86) cell lines were used to represent CR2-expressing mature B-cells.

Real-time quantitative PCR (Q-PCR)

Q-PCR reactions were carried out using the QuantiTect™ SYBR® Green PCR kit (QIAGEN, Valencia, CA) according to the manufacturers' instructions with 5 µl template and 0.5 µM each primer (Table 1) in 20 µl reaction volumes on the Rotor-Gene™ 3000 (Corbett Research, Mortlake, Australia) system. Thermal cycling conditions were as follows: 95 °C for 15 min; followed by 40 cycles of 95 °C for 15 s, 50-60 °C (Table 1, annealing temperature) for 20 s and 72 °C for 30 s; followed by a melt-curve cycle which involved a gradual increase in temperature from 60 °C up to 99 °C. Acquisition of sample fluorescence occurred after 72 °C step of cycle and at 1 °C increments during melt-curve cycle. Q-PCR primers were verified by amplification of serially diluted genomic DNA or plasmid DNA to ensure linear detection of target template and by agarose gel electrophoresis of PCR products and routine melt curve analysis to ensure specificity.

mRNA expression analysis

Total RNA from $5\text{-}10 \times 10^6$ cells was isolated using Trizol® reagent (Gibco BRL, Melbourne, Australia) according to the manufacturer's instructions. cDNA was prepared from 1 µg total RNA using SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions in 20 µl reactions with random primers (Promega, Madison, WI). Synthesised cDNA was diluted 5 times with 0.1X TE (1 mM Tris, 0.1 mM EDTA [pH 8.0]) supplemented with 0.008% (w/v) BSA (New England Biolabs, Beverly, MA). Q-PCR was performed using primer pairs targeting CR2 or β-actin cDNA sequences (Table 1, CR2-E2/3 and β-actin) to amplify cDNA or serially diluted plasmid DNA. Relative CR2 levels were normalised to β-actin using the Quantitative Method (Rotor-Gene™ software v6.1).

Flow cytometry

Cell surface expression of CR2 was assessed as described previously [14] using b-anti-hCR2 (mAB171) or an isotype matched control (MOPC-21; BD Biosciences PharMingen, San Diego, CA) followed by secondary staining with phycoerythrin (PE)-conjugated streptavidin (BD Biosciences PharMingen). Stained cells were analyzed using the FACSCanto™ II flow cytometer (BD Biosciences PharMingen). Data was collected with the FACSDiva™ software (BD Biosciences PharMingen) and processed using FlowJo v7.2.1 (TreeStar, Inc., San Carlos, CA).

CHART-PCR

Chromatin accessibility assays were performed as described previously with minor modifications [3]. Approximately 1.25×10^6 nuclei in 100 µl digestion buffer (10 mM Tris-HCl [pH 7.4], 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM

spermidine, 1 mM CaCl₂) $\pm$ 5 U MNase/ml (Worthington Biochemicals, Lakewood, NJ) were incubated at 37 °C for 10 min. Reactions were terminated by adding 20 μ l stop solution (100 mM EDTA, 10 mM EGTA [pH 8.1]) and 10 μ l SDS 10% (w/v). DNA was isolated using the QIAmp DNA blood mini-kit (QIAGEN), eluted in 150 μ l 0.1X TE (1mM Tris, 0.1mM EDTA [pH 8.0]), supplemented with 0.008% BSA (w/v). Genomic DNA samples were used as templates in real time Q-PCR assays to measure the relative abundance of targeted promoter fragments recovered from “Cut” (+MNase) and “Uncut” (no MNase) samples using a series of primer pairs (Table 1). Chromatin accessibility ratios were calculated using the Rotor-Gene™ software v6.1, Comparative Quantitation method to determine the ratio of “Uncut”/“Cut” from pairs of samples from independent accessibility reactions.

Bioinformatic analyses

Mouse and human sequence alignment was performed using CLC Combined Workbench Version 3.02 (CLC bio A/S, Cambridge, MA) with the online Clustal W program [15]. Consensus TF binding sites were identified using the online search tool, MatInspector (Genomatix Software GmbH, Munich, Germany). Predicted TSS motifs were detected using the online Eponine Transcription Start Site finder [16].

Statistical analyses

Two-way ANOVA and Bonferroni post-test analyses were used to determine statistically relevant differences in chromatin accessibility among cell lines for each genomic region examined. All values described in the text represent the mean $\pm$ S.E.M. Statistics and graphs were generated with GraphPad Prism v4 (GraphPad Software, San Diego CA).

Results

CR2 mRNA levels correlate with cell-surface expression

CR2 expression levels were examined in cell lines by measuring relative transcript abundance using quantitative RT-PCR and cell surface density using flow cytometry. We found clear differences in *CR2* transcript levels and cell surface expression when comparing U937, REH, Ramos and Raji cells (Fig. 1A, B). *CR2* was undetectable at both the transcript and surface levels in U937 and REH cells (Fig. 1A, B) suggesting that the *CR2* gene is transcriptionally silenced in these cells. In contrast, analysis of mature B-cell lines, Ramos and Raji revealed distinct *CR2* expression patterns. Ramos cells were bimodal for *CR2* surface expression (Fig. 1B) and *CR2* mRNA could readily be detected by Q-PCR (Fig. 1A), while Raji cells comprise a population of cells expressing high surface levels of *CR2* and expressing high levels (27-fold greater than Ramos) of *CR2* mRNA (Fig. 1) possibly reflecting differences in transcriptional activity of the *CR2* gene.

Uniform chromatin digestion across all cell lines in CHART-PCR assays

To confirm that the previously described CHART-PCR assay [3] was a valid method to examine multiple cell types, MNase digestion patterns were compared in each of the cell lines. Electrophoresis of genomic DNA from MNase treated samples showed similar periodic banding patterns commonly observed in partially MNase digested chromatin (Fig. 2A) due to the preference of MNase to cleave linker DNA separating nucleosomes [17].

To compare the extent of MNase digestion in cell lines, a series of primer pairs were used to target genomic regions representing chromatin associated with developmentally restricted (*SP-A2* and *PAX-7*) or ubiquitously expressed (*β-actin* and *GAPDH*) gene loci. The -228 to -64 region of the *PAX-7* promoter sequence [8] was highly protected from digestion in all cell lines, with accessibility ratios ranging from 1.11 ± 0.08 (Fig. 2B, SKW) to 1.34 ± 0.06 (Fig. 2B, Ramos). By comparison, the -161 to -16 region of the *SP-A2* promoter sequence [18], showed a modestly increased degree of MNase chromatin accessibility ranging from 1.79 ± 0.05 in Raji cells up to 1.95 ± 0.06 in REH cells. There was no significant difference in mean chromatin accessibility values between cell-types at either *PAX-7* or *SP-A2* regions examined ($p > 0.05$ comparing any pair of cell types). Therefore, in the panel of cell lines tested, developmentally restricted promoter regions of either *SP-A2* or *PAX-7* show consistently low levels of chromatin accessibility (Fig. 2B). Sequences near the TSS of the constitutively active *β-actin* (-239 to -62 [19]) and *GAPDH* (-217 to -68 [20]) genes showed enhanced MNase chromatin accessibility compared to the regulatory regions of either *PAX-7* or *SP-A2* (Fig. 2B) in all cell lines examined. Mean chromatin accessibility ratios were observed within the range of 3.56 ± 0.18 (Fig. 2B, U937) and 6.05 ± 0.40 (Fig. 2B, REH) for *β-actin* sequences and within the range of 4.19 ± 0.28 (Fig. 2B, Ramos) and 9.36 ± 0.94 (Fig. 2B, REH) for *GAPDH* sequences. These experiments demonstrate that, when using MNase as the digestion agent, CHART-PCR assays detect site specific variation in chromatin accessibility in a manner that is reproducible in multiple cell lines. Also, this data implies that CHART-PCR assays can detect subtle differences in chromatin accessibility at developmentally restricted gene loci.

Enhanced MNase accessibility within the -289 to -12 region of the human CR2 proximal promoter in CR2 expressing cells

Following verification of the utility of the CHART-PCR assay in assessing chromatin variation in different cell types, we then analysed changes across the *CR2* gene promoter using cell lines representing different stages of B-cell ontogeny. Non-expressing cell types were highly resistant to MNase digestion over the -115 to -12 region of the *CR2* proximal promoter with accessibility ratios approaching one (Fig. 3; U937: 0.977 ± 0.07 and REH: 1.02 ± 0.07). Low levels of accessibility across the -289 to -101 region was also observed in these cell types (Fig. 3; U937: 1.76 ± 0.15 and REH: 1.78 ± 0.30). In contrast, both regions of the *CR2* proximal promoter showed enhanced MNase digestion in the CR2 expressing B-cell lines (Raji and Ramos) compared to each of the other cell lines examined (Fig. 2A). The level of chromatin accessibility across the -289 to -101 region was similar in Raji (3.60 ± 0.13) and Ramos (3.06 ± 0.50) cell lines (Fig. 3, $p > 0.05$). In contrast, the level of chromatin accessibility across the -115 to -12 region was significantly higher in Raji cells (3.08 ± 0.38) compared to Ramos cells (2.21 ± 0.21 ; $p < 0.05$), which were in turn significantly more accessible across this region than both REH ($p < 0.01$) and U937 ($p < 0.01$). These results imply that variation in chromatin structure extends through the *CR2* core promoter and correlates with transcription initiation. Furthermore, since an intermediate level of chromatin accessibility was observed specifically within the -115 to -12 region flanking the *CR2* TSS in Ramos cells, this region may contain sites which vary in accessibility depending on transcriptional status.

Sequence analysis of the CR2 core promoter

Since distinct differences in chromatin accessibility were observed within the *CR2* proximal promoter we wished to analyse these regions to identify candidate sites which may bind TFs that could generate or respond to chromatin structural alterations. Aligning the human *CR2* core promoter with corresponding mouse sequences revealed 61% identity. Consensus binding sites for several TFs were common to both human and mouse sequences including Sp-family members, CBF-1, CREB, C/EBP- β , BLIMP-1 as well as several E-box motifs (Fig. 4), several of which are known to be involved in regulating chromatin structure or function in response to changes in chromatin structure.

Discussion

Previous studies examining chromatin structure during gene induction have shown that activation may be accompanied by increased chromatin accessibility at specific regulatory sites while other genomic regions remain unchanged. This has been observed at 5' flanking sequences of the TSS of *E-selectin* during endothelial cell activation [21] and also of *GM-CSF* [22], *IL-2* [23] and *IL-10* [24] genes during T-cell activation. Since dynamic *in vitro* models for developmental initiation of human *CR2* expression have not been described, the studies presented here utilised cell lines representing expressing and non-expressing cell types. To determine if CHART-PCR assays could reasonably be used to compare several cell lines we first examined chromatin accessibility at promoter regions chosen to represent constitutively active or developmentally restricted genomic sites. These analyses revealed a range of accessibility states at different genomic regions from highly inaccessible sequences at the *PAX-7* promoter to highly accessible sequences at the *GAPDH* and β -*actin* promoters. The *SP-A2* promoter region showed consistent intermediate chromatin accessibility ratios suggesting that this region was a suitable target to monitor chromatin digestion in the haematopoietic cell types examined. Therefore, when using MNase as an accessibility agent, CHART-PCR reveals site specific differences in chromatin accessibility that can be detected across several cell lines.

Previously, we have demonstrated that *CR2* transcription is regulated by a cell type-specific repressor element within the proximal promoter designated E-box site 2 [11]. Here we show that this repressor element is located within a region which is highly inaccessible to MNase in non-expressing cell lines, between -115 to -12 relative to the

TSS. Interestingly, across this region we also observed 1.4-fold greater accessibility in Raji cells compared to Ramos cells. This difference in accessibility may therefore correlate with *CR2* expression as there is approximately a 2.1-fold greater proportion of positive cells in Raji compared to Ramos and *CR2* transcript abundance is 27-fold greater in Raji compared to Ramos. This result may reflect that enhanced chromatin accessibility across the -115 to -12 region of the *CR2* proximal promoter is a function of the probability of productive transcription rather than the rate of transcription. However, we can not rule out the possibility that chromatin accessibility within this region is graded to control transcription levels.

In addition to the highly protected segment flanking the *CR2* TSS, non-expressing cell types showed significantly lower levels of MNase accessibility over the -289 to -101 region of the *CR2* proximal promoter compared to *CR2* expressing cell types. Since the sequence between -315 to +75 relative to the TSS comprises the minimal sequence necessary to drive basal activity of reporter constructs [13], the results presented above suggest that extensive regions of the core promoter of the human *CR2* gene are subject to chromatin structural alterations to regulate gene expression.

Transgenic mouse studies suggest that the precise timing of *CR2* transcription activation during B-cell development is important for generating a robust, healthy B-cell repertoire [25, 26]. Presently, the signalling cues and transcription factors driving B-cell differentiation at the stage(s) when *CR2* is activated are poorly defined. The results presented in this paper show that CHART-PCR assays can reliably detect chromatin structural heterogeneity in different cell types and demonstrate a correlation between the accessibility of sequences within the *CR2* core promoter and

gene expression in different B-cell lineages. The identification of consensus TF binding sites within these regions which are also found within the mouse *Cr2* promoter may reflect the involvement of several common TFs regulating chromatin structure and *CR2* expression including E-box binding bHLH family proteins, Sp-family proteins, CBF-1, CREB, C/EBP- β and BLIMP-1. It is possible that these common factors function in combination or in response to different stimuli to regulate transcription of *CR2* at the same stages during B-cell differentiation in both species.

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References

- [1] M.A. Keene, S.C. Elgin, Micrococcal nuclease as a probe of DNA sequence organization and chromatin structure, *Cell* 27 (1981) 57-64.
- [2] K. Zinn, T. Maniatis, Detection of factors that interact with the human beta-interferon regulatory region in vivo by DNAase I footprinting, *Cell* 45 (1986) 611-618.
- [3] S. Rao, E. Procko, M.F. Shannon, Chromatin remodeling, measured by a novel real-time polymerase chain reaction assay, across the proximal promoter region of the IL-2 gene, *J Immunol* 167 (2001) 4494-4503.
- [4] R.C. Rickert, Regulation of B lymphocyte activation by complement C3 and the B cell coreceptor complex, *Curr Opin Immunol* 17 (2005) 237-243.
- [5] P.W. Dempsey, M.E. Allison, S. Akkaraju, C.C. Goodnow, D.T. Fearon, C3d of complement as a molecular adjuvant: bridging innate and acquired immunity, *Science* 271 (1996) 348-350.
- [6] K. Takahashi, Y. Kozono, T.J. Waldschmidt, D. Berthiaume, R.J. Quigg, A. Baron, V.M. Holers, Mouse complement receptors type 1 (CR1;CD35) and type 2 (CR2;CD21): expression on normal B cell subpopulations and decreased levels during the development of autoimmunity in MRL/lpr mice, *J Immunol* 159 (1997) 1557-1569.
- [7] T.F. Tedder, L.T. Clement, M.D. Cooper, Expression of C3d receptors during human B cell differentiation: immunofluorescence analysis with the HB-5 monoclonal antibody, *J Immunol* 133 (1984) 678-683.
- [8] Y.V. Syagailo, O. Okladnova, E. Reimer, M. Grassle, R. Mossner, S. Gattenlohner, A. Marx, J. Meyer, K.P. Lesch, Structural and functional

characterization of the human PAX7 5'-flanking regulatory region, *Gene* 294 (2002) 259-268.

[9] J. Madsen, I. Tornøe, O. Nielsen, C. Koch, W. Steinhilber, U. Holmskov, Expression and localization of lung surfactant protein A in human tissues, *Am J Respir Cell Mol Biol* 29 (2003) 591-597.

[10] J.K. Kim, S.S. Kim, K.W. Rha, C.H. Kim, J.H. Cho, C.H. Lee, J.G. Lee, J.H. Yoon, Expression and localization of surfactant proteins in human nasal epithelium, *Am J Physiol Lung Cell Mol Physiol* 292 (2007) L879-884.

[11] D. Ulgiati, V.M. Holers, CR2/CD21 proximal promoter activity is critically dependent on a cell type-specific repressor, *J Immunol* 167 (2001) 6912-6919.

[12] K.W. Makar, D. Ulgiati, J. Hagman, V.M. Holers, A site in the complement receptor 2 (CR2/CD21) silencer is necessary for lineage specific transcriptional regulation, *Int Immunol* 13 (2001) 657-664.

[13] D. Ulgiati, C. Pham, V.M. Holers, Functional analysis of the human complement receptor 2 (CR2/CD21) promoter: characterization of basal transcriptional mechanisms, *J Immunol* 168 (2002) 6279-6285.

[14] J.M. Guthridge, K. Young, M.G. Gipson, M.R. Sarrias, G. Szakonyi, X.S. Chen, A. Malaspina, E. Donoghue, J.A. James, J.D. Lambris, S.A. Moir, S.J. Perkins, V.M. Holers, Epitope mapping using the X-ray crystallographic structure of complement receptor type 2 (CR2)/CD21: identification of a highly inhibitory monoclonal antibody that directly recognizes the CR2-C3d interface, *J Immunol* 167 (2001) 5758-5766.

[15] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting,

- position-specific gap penalties and weight matrix choice, *Nucleic Acids Res* 22 (1994) 4673-4680.
- [16] T.A. Down, T.J. Hubbard, Computational detection and location of transcription start sites in mammalian genomic DNA, *Genome Res* 12 (2002) 458-461.
- [17] A.D. Mirzabekov, A.V. Belyavsky, S.G. Bavykin, V.V. Shick, Primary organization of nucleosomes and its functional implications, *Biosystems* 12 (1980) 265-271.
- [18] J. Li, E. Gao, S.R. Seidner, C.R. Mendelson, Differential regulation of baboon SP-A1 and SP-A2 genes: structural and functional analysis of 5'-flanking DNA, *Am J Physiol* 275 (1998) L1078-1088.
- [19] S.L. Danilition, R.M. Frederickson, C.Y. Taylor, N.G. Miyamoto, Transcription factor binding and spacing constraints in the human beta-actin proximal promoter, *Nucleic Acids Res* 19 (1991) 6913-6922.
- [20] N. Nasrin, L. Ercolani, M. Denaro, X.F. Kong, I. Kang, M. Alexander, An insulin response element in the glyceraldehyde-3-phosphate dehydrogenase gene binds a nuclear protein induced by insulin in cultured cells and by nutritional manipulations in vivo, *Proc Natl Acad Sci U S A* 87 (1990) 5273-5277.
- [21] L.C. Edelstein, A. Pan, T. Collins, Chromatin modification and the endothelial-specific activation of the E-selectin gene, *J Biol Chem* 280 (2005) 11192-11202.
- [22] A.F. Holloway, S. Rao, X. Chen, M.F. Shannon, Changes in chromatin accessibility across the GM-CSF promoter upon T cell activation are dependent on nuclear factor kappaB proteins, *J Exp Med* 197 (2003) 413-423.
- [23] X. Chen, J. Wang, D. Woltring, S. Gerondakis, M.F. Shannon, Histone dynamics on the interleukin-2 gene in response to T-cell activation, *Mol Cell Biol* 25 (2005) 3209-3219.

- [24] J. Shoemaker, M. Saraiva, A. O'Garra, GATA-3 directly remodels the IL-10 locus independently of IL-4 in CD4⁺ T cells, *J Immunol* 176 (2006) 3470-3479.
- [25] J.M. Ahearn, M.B. Fischer, D. Croix, S. Goerg, M. Ma, J. Xia, X. Zhou, R.G. Howard, T.L. Rothstein, M.C. Carroll, Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen, *Immunity* 4 (1996) 251-262.
- [26] K.J. Marchbank, L. Kulik, M.G. Gipson, B.P. Morgan, V.M. Holers, Expression of human complement receptor type 2 (CD21) in mice during early B cell development results in a reduction in mature B cells and hypogammaglobulinemia, *J Immunol* 169 (2002) 3526-3535.
- [27] E.J. Rayhel, M.H. Dehoff, V.M. Holers, Characterization of the human complement receptor 2 (CR2, CD21) promoter reveals sequences shared with regulatory regions of other developmentally restricted B cell proteins, *J Immunol* 146 (1991) 2021-2026.
- [28] S.M. Christensen, B.K. Martin, S.S. Tan, J.H. Weis, Identification of sites for distinct DNA binding proteins including Oct-1 and Oct-2 in the Cr2 gene, *J Immunol* 148 (1992) 3610-3617.

Figure legends

Figure 1: Amount of CR2 surface expression correlates with mRNA levels.

(A) Q-PCR was performed on reverse transcribed cDNA from U937, REH, Ramos and Raji cells to determine normalized CR2 levels as described. CR2 mRNA abundance was plotted on the y-axis relative to β -actin and results show the mean $\pm$ S.E.M. (n=2).

(B) Flow cytometric profiles (PE-fluorescence, x-axis versus cell counts, y-axis) for U937, REH, Ramos and Raji cells incubated with anti-CR2 (solid histograms) or an isotype matched control antibody (MOPC-21; open histograms) followed by secondary PE staining. The percentage of cells within the positive gate is shown for CR2-specific stained cells (above the gate) and for the isotype matched control (below the gate).

Figure 2: CHART-PCR assays detect consistent, site specific accessibility across different cell lines.

(A) Genomic DNA recovered from CHART-PCR assays were electrophoresed on a 1.5% agarose gel stained with ethidium bromide. Representative samples from each of the cell lines incubated with (+) or without (-) MNase are shown in lanes 2-9 and the pGEM DNA marker was run in lanes 1 and 10.

(B) Pairs of “Uncut” and “Cut” samples from CHART-PCR assays were used as template in Q-PCR reactions with primer pairs targeting promoter regions of *PAX-7* (-228 to -64), *SP-A2* (-161 to -16), *β -actin* (-239 to -62) and *GAPDH* (-217 to -68) genes. MNase chromatin accessibility ratios were calculated as described and plotted

as the mean $\pm$ S.E.M. (Raji, U937 [n=6], REH [n=5] and Ramos [n=3]) from independent pairs of “Uncut” and “Cut” samples.

Figure 3: CHART-PCR assays show enhanced accessibility over the *CR2* core promoter in expressing cell types.

MNase chromatin accessibility of *CR2* promoter sequences -289 to -101 and -115 to -12 regions relative to the TSS. *CR2* promoter specific primers were used in Q-PCR reactions to calculate chromatin accessibility ratios (“Uncut”/“Cut”) plotted as the mean $\pm$ S.E.M (Raji, U937 [n=6], REH [n=5] and Ramos [n=3]) from independent pairs of “Uncut” and “Cut” samples.

Figure 4: Common TF binding motifs can be identified in the human and murine *CR2* core promoter.

The human minimal core promoter sequence (Hs) is numbered relative to the major TSS (dotted arrow denotes +1) as previously described [13, 27] and aligned to the murine promoter sequence (Mm) [28]. Potential TSS motifs detected using the online Eponine Transcription Start Site finder are denoted (bold). Core consensus TF binding sites identified using MatInspector (Genomatix Software GmbH) which were common to both human and mouse sequence in addition to canonical E-box motifs (CANNTG) are shown in bold or underlined. Solid arrows indicate the location of primers used in Q-PCR assays targeting the *CR2* promoter.

Table 1: Sequences of the primers used for quantitative RT-PCR and CHART-PCR.

Primer	Sequence forward primer (5' → 3')	Sequence reverse primer (5' → 3')	Product size	Anneal Temp
SP-A2p	CTAAGTATTCTCCAGCCTGAGTGTTTC	GGTGGACAACAGCATTTATAGCATG	152 bp	60
PAX-7p	CCGAACCTATCAGATCGCGCTCAC	GTCACCCCTGTCTCCTCCGTCCAG	165 bp	60
β-Actinp	CAGCACCCCAAGGCGGCCAACGC	GCAACTTTCGGAACGGCGCACGC	178 bp	60
GAPDHp	CACGCTCGGTGCGTGCCCAAGTTG	CCGCCTCCCGCCAGGCTCAGCC	150 bp	60
CR2pB	TCCCGCTTGTCACACCTCA	AGGCGGGCCCTTAAATAGTGTCC	104 bp	50
CR2pC	GATTACTAAGGGTGAGTACTAAGTTG	GTGGGACAAGCGGGAG	189 bp	60
CR2-E2/3	TGCCTGTAAACCAACTTCTC	AGCAAGTAACCAGATTACAG	219 bp	55
β-actin	GATGACCCAGATCATGTTTGAG	GACTCCATGCCAGGAAGGAA	459 bp	55