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

Mark Cruickshank [a,b], Emily Fenwick [a,b], Lawrence J. Abraham [a], Daniela Ulgiati [a,b,*]

[a] *Biochemistry and Molecular Biology, School of Biomedical, Biomolecular and Chemical Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, 6009 WA, Australia*

[b] *Laboratory for Cancer Medicine, UWA Centre for Medical Research, The Western Australian Institute for Medical Research, Perth, 6000 WA, Australia*

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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 not altered between cell-types.

Keywords: Chromatin accessibility; Transcriptional activation; Complement Receptor 2; CD21; B-cell differentiation

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 (Q-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) and

* Corresponding author. Address: Biochemistry and Molecular Biology, School of Biomedical, Biomolecular and Chemical Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, 6009 WA, Australia. Fax: +61 864881148.
E-mail address: daniela.ulgiati@uwa.edu.au (D. Ulgiati).

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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 analyzing 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*, *GAPDH*) and developmentally restricted (*PAX-7*, *SP-A2*) gene loci. *PAX-7* encodes a transcription factor (TF) expressed during embryogenesis and in adult myogenic precursors [8], while *SP-A2* encodes a surface receptor expressed on lung cells and 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 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 *CR2* regulatory regions using the panel of cell lines. Our analyses showed enhanced chromatin accessibility over the *CR2* core promoter in expressing cells (Ramos, Raji), while highly inaccessible chromatin was observed flanking the transcription start site (TSS) in non-expressing cells (U937, Reh). Sequence analysis of these regions revealed several consensus TF 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. Cultures were maintained at 37 °C with 5% CO_2 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) cells were used to represent CR2 expressing B-cells.

Q-PCR. Q-PCRs were carried out using the QuantiTect™ SYBR® Green PCR kit (QIAGEN, Valencia, CA) according to the manufacturer's 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). 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 (gradual increase in temperature from 60 °C up to 99 °C). Acquisition of sample fluorescence occurred after 72 °C steps and at 1 °C increments during melt-curve. Primers were 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.

mRNA expression. Total RNA from 5 to 10×10^6 cells was isolated using Trizol® (Gibco-BRL, Melbourne) 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). cDNA was diluted five times with $0.1 \times$ 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, β-actin) to amplify cDNA or serially diluted plasmid DNA. Relative CR2 levels were normalized to β-actin using the Quantitative Method (Rotor-Gene™ 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) and secondary staining with phycoerythrin (PE)-conjugated streptavidin (BD Biosciences PharMingen). Cells were analyzed using the FACSCanto™ II cytometer (BD Biosciences PharMingen). Data were collected with FACSDiva™ (BD Biosciences PharMingen) and processed using FlowJo v7.2.1 (TreeStar, Inc., San Carlos, CA).

CHART-PCR. Chromatin accessibility assays were performed as described [3] with minor modifications. 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 $CaCl_2$) ± 5 U MNase/ml (Worthington Biochemicals, Lakewood, NJ) were incubated at 37 °C for 10 min. Reactions were terminated with 20 μl stop solution (100 mM EDTA, 10 mM EGTA [pH 8.1]) and 10 μl SDS 10% (w/v). DNA was isolated using the QIAmp DNA blood mini-kit (QIAGEN), eluted into 150 μl 0.1× TE supplemented with 0.008% BSA (w/v). DNA recovered from "Cut" (+MNase) and "Uncut" samples were used in Q-PCR assays to measure the relative abundance of targeted regions using a series of primer pairs (Table 1). Chromatin accessibility ratios were calculated using the Rotor-Gene™ v6.1, Comparative Quantitation method to determine the ratio of "Uncut"/"Cut" for independent pairs of samples.

Bioinformatic analyses. Mouse and human sequence alignment was performed using CLC Combined Workbench v3.02 (CLC bio A/S, Cambridge, MA) with Clustal W [15]. Consensus TF motifs were identified using MatInspector (Genomatix GmbH, Munich, Germany). Predicted TSS motifs were detected using Eponine Transcription Start Site finder [16].

Statistical analyses. Two-way ANOVA and Bonferroni post-test analyses were used to detect differences in accessibility among cell lines for each genomic region examined. All values described represent means ± SEM. Statistics and graphs were generated with GraphPad Prism v4 (GraphPad, San Diego, CA).

Results

CR2 mRNA levels correlate with cell surface expression

CR2 expression levels were examined by measuring relative transcript abundance using Q-PCR and cell surface density using flow cytometry. We found clear differences in CR2 transcript levels and cell surface expression in the panel of cell lines (Fig. 1A and B). CR2 was undetectable at both the transcript and surface levels in U937 and REH cells (Fig. 1A and 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