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Analysis of tandem E-box motifs within human *Complement receptor 2* (*CR2/CD21*) promoter reveals cell specific roles for RP58, E2A, USF and localized chromatin accessibility

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ABSTRACT

Complement receptor 2 (CR2/CD21) plays an important role in the generation of normal B cell immune responses. As transcription appears to be the prime mechanism *via* which surface CR2/CD21 expression is controlled, understanding transcriptional regulation of this gene will have broader implications to B cell biology. Here we report opposing, cell-context specific control of *CR2/CD21* promoter activity by tandem E-box elements, spaced 22 bp apart and within 70 bp of the transcription initiation site. We have identified E2A and USF transcription factors as binding to the distal and proximal E-box sites respectively in *CR2*-positive B-cells, at a site that is hypersensitive to restriction enzyme digestion compared to non-expressing K562 cells. However, additional unidentified proteins have also been found to bind these functionally important elements. By utilizing a proteomics approach we have identified a repressor protein, RP58, binding the distal E-box motif. Co-transfection experiments using RP58 overexpression constructs demonstrated a specific 10-fold repression of *CR2/CD21* transcriptional activity mediated through the distal E-box repressor element. Taken together, our results indicate that repression of the *CR2/CD21* promoter can occur through one of the E-box motifs *via* recruitment of RP58 and other factors to bring about a silenced chromatin context within CR2/CD21 non-expressing cells.

1. Introduction

The human cell surface receptor Complement receptor 2 (CR2/CD21) shows a restricted tissue distribution and is expressed on sub-populations of B cells (Alvarez et al., 2000), follicular dendritic cells (Reynes et al., 1985), T cells (Levy et al., 1992), epithelial cells (Levine and Davidson, 2005), basophils (Bacon et al., 1993), mast cells (Gommerman et al., 2000) and keratinocytes (Hunyadi et al., 1991). During B-lymphopoiesis, CR2/CD21 is first observed on newly formed immature B cells in the bone marrow (Takahashi et al., 1997; Tedder et al., 1984) and is up-regulated following negative selection and migration to the periphery as transitional intermediates (Melamed et al., 1998; Melamed and Nemazee, 1997; Hartley et al., 1993). CR2/CD21 is undetectable during early stages of development and upon terminal differentiation into antibody secreting plasma cells.

Since the developmental timing and level of *CR2/CD21* transcription is crucial for generating a normal B cell response, we have conducted an analysis of factors regulating the human *CR2/CD21* proximal promoter. We have previously characterized the basal requirements for transcription of the *CR2/CD21* gene and identified tandem E-box elements that are crucial for cell- and B cell stage-specific expression (Ulgiati et al., 2002; Ulgiati and Holers, 2001).

Abbreviations: 2DE, two dimensional electrophoresis; bHLH, basic helix-loop-helix; CAGE, capped analysis of gene-expression; CHART-PCR, chromatin accessibility measured by real-time PCR; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; IEF, isoelectric focusing; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; NE, nuclear extract; TF, transcription factor.

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E-boxes often serve as binding sites for E-proteins, including E2A (E12 and E47 isoforms encoded by the *TCF3* gene), E2-2 (E2-2A and E2-2B isoforms) and HEB proteins.

E-proteins comprise the class I basic helix-loop-helix (bHLH) transcription factors (TFs) which are widely expressed in different cell-types (Massari and Murre, 2000) and are known to be crucial for regulating various aspects of B cell development (Kwon et al., 2008). E-proteins interact with E-boxes (with a consensus core CANNTG) as homodimers or heterodimers with other bHLH family proteins and can activate transcription by recruitment of coactivators p300 and CBP (Guo et al., 2009). While E-proteins are promiscuously expressed (though not ubiquitous), a wide variety of genes controlled by E-box motifs are expressed in a highly restricted developmental pattern. Tissue specific interactions with distinct classes of HLH-containing proteins plays a key role in regulating the target preference of E proteins (Murre, 2005); however, the mechanisms preventing wide-spread spurious binding of homodimers are incompletely understood.

Here we investigate tandem E-box motifs, spaced 22 bp apart and within 70 bp of the transcription initiation site, revealing a critical role in controlling *CR2/CD21* transcription. These two motifs show opposing function and appear only to be active depending on the cellular context. Furthermore, we have identified E2A transcription factors as binding to one of the E-box motifs using EMSA and ChIP analyses; however, as yet unidentified proteins are also binding these functionally important elements. Utilizing a proteomics approach to identify unknown components interacting with E-box site 2 motifs, we successfully isolated and identified RP58 (encoded by *RP58/ZNF238/ZBTB18*); and reconstituted its binding activity *in vitro*. Further, *in vitro* transfection studies confirmed repression specifically through the E-box site 2 repressor element. Conversely, CR2/CD21-expression in B cells was associated with E2A and USF binding *via* E-box sites 1 and 2 respectively and localized chromatin hypersensitivity.

2. Materials and methods

2.1. Cell culture

Cell 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, USA) including Raji (CCL-86) and K562 (CCL-243).

2.2. B cell isolation from peripheral blood

Peripheral blood was obtained from healthy donors and the PBMC fraction was isolated by Ficoll gradient centrifugation using standard protocols. B cells were purified by positive selection using MACS CD19 MicroBeads (Miltenyi Biotec, Australia, Cat#130-050-301) according to the manufacturer's instructions.

2.3. Construction of CR2/CD21 mutant reporter constructs

A *Nhe*I/*Xho*I fragment of the *CR2/CD21* promoter containing the −315/+75 of the *CR2/CD21* 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). Correct orientation and sequence of constructs was verified by restriction enzyme digestion and nucleotide sequence analysis.

2.4. 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 5×10^5 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 performed in triplicate. Promoter activity is expressed as relative *Firefly* luciferase activity normalized against *Renilla* luciferase activity.

2.5. Nuclear extract (NE) preparation and Sephacryl S-300 gel filtration

5–10 l of K562 culture were harvested at a density of 8×10^5 cells/ml in 250 ml centrifuge bottles by centrifugation at 3500 rpm for 10 min at room temperature with a Beckman J2-MC Centrifuge (JA-14 rotor; Beckman Instruments). NE was prepared by scaling a previously described method (Li et al., 1991), snap frozen in liquid nitrogen and stored at −80 °C or proceeded directly to ammonium sulphate precipitation. Crude nuclear proteins were first purified by cutting in 0.33 g of ammonium sulphate per ml of NE as previously described (Marshak, 1996). NE was further purified using HiPrep® Sephacryl S-300 high resolution columns (Amersham Biosciences, USA) in accordance with the manufacturer's instructions. The fractions were tested for protein content (Bio-Rad, USA) and binding activity, using EMSA as described (Woo et al., 2002), snap frozen in liquid nitrogen and stored at −80 °C.

2.6. Electrophoretic mobility shift assay (EMSA)

Oligonucleotides representing the sense and anti-sense strands of the promoter region of interest were synthesized with a non-native 5′ G-overhang added to allow ^{32}P end-labeling. Purified double-stranded oligonucleotides were ^{32}P labeled by filling the 5′ G-overhang with ^{32}P-dCTP (Amersham Pharmacia Biotech, Little Chalfont, England) using the Klenow enzyme (Promega Corp., Madison, WI, USA). 1–5 μg of crude NEs or 0.1–1 μg of Sephacryl S-300 gel filtrated fractions were pre-incubated for 10 min on ice with 1 μg (0.5 μg where indicated) of poly dI-dC (poly [dI-dC]·poly [dI-dC]; Sigma–Aldrich) in a final reaction volume of 20 μl which also consists of 4% Ficoll, 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM DTT and 50 mM KCl. Following the pre-incubation, 200 fmol of ^{32}P labeled probe oligonucleotides were added and the binding reaction was incubated for an additional 30 min before loading onto a pre-run (3 h) 6% non-denaturing polyacrylamide gel containing 0.25× Tris–Taurine/EDTA. Gels were electrophoresed at 150 V for 3 h, vacuum dried and exposed to a phosphoimaging plate (Fuji Photo Film Co.), and processed by the Fuji BAS 1000 Phosphoimaging System (Fuji Photo Film Co.).

2.7. Molecular weight (MW) determination by coupled SDS-PAGE and EMSA

50 μg of Sephacryl S-300 gel filtrated fractions or 250 μg of crude NEs were first concentrated using a Micro-con 10 micro-concentrator (Amicon Inc., USA) in accordance with the manufacturer's recommendations. The concentrated sample was