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RESEARCH ARTICLE

Focused transcription from the human *CR2/CD21* core promoter is regulated by synergistic activity of TATA and Initiator elements in mature B cells

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Complement receptor 2 (CR2/CD21) is predominantly expressed on the surface of mature B cells where it forms part of a coreceptor complex that functions, in part, to modulate B-cell receptor signal strength. CR2/CD21 expression is tightly regulated throughout B-cell development such that CR2/CD21 cannot be detected on pre-B or terminally differentiated plasma cells. CR2/CD21 expression is upregulated at B-cell maturation and can be induced by IL-4 and CD40 signaling pathways. We have previously characterized elements in the proximal promoter and first intron of *CR2/CD21* that are involved in regulating basal and tissue-specific expression. We now extend these analyses to the *CR2/CD21* core promoter. We show that in mature B cells, *CR2/CD21* transcription proceeds from a focused TSS regulated by a non-consensus TATA box, an initiator element and a downstream promoter element. Furthermore, occupancy of the general transcriptional machinery in pre-B *versus* mature B-cell lines correlate with *CR2/CD21* expression level and indicate that promoter accessibility must switch from inactive to active during the transitional B-cell window.

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INTRODUCTION

Human complement receptor 2 (CR2/CD21) is predominantly expressed on the surface of mature B cells and follicular dendritic cells.[1,2] At the cell surface, CR2/CD21 forms the ligand binding component[3,4] of the B-cell receptor coreceptor complex. Upon interaction with ligands iC3b, C3d, C3dg and the Epstein–Barr virus (EBV)[3,5] the CR2/CD21–CD19 coreceptor complex crosslinks with the B-cell receptor leading to a 10- to 1000-fold decrease in the threshold for B-cell activation.[6–8]

In mice, Cr2/CD21 expression is first evident at low levels on late-immature B cells exiting the bone marrow, a critical checkpoint for B-cell autoreactivity, and Cr2/CD21 expression increases with B-cell maturation.[1,9] Following B-cell activation and differentiation, Cr2/CD21 is downregulated and is not detected on plasma cells.[10,11] In this window, Cr2/CD21 expression varies according to the stage of B-cell development and differentiation, with the highest level of expression observed on marginal zone B cells and B10 cells.[12] The major population of mature B cells, follicular B cells, expresses an intermediate level of Cr2/CD21 that fluctuates according to immunogenic challenge.[13] Research conducted in mouse models has been integral to the current understanding of B-cell development. However, there are important differences in *CR2/CD21* between mouse and human (reviewed in Ref. 14). In mice, two proteins Cr2/CD21 and Cr1/CD35 are transcribed

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by alternative splicing of the *Cr2/CD21* gene.[15] In humans, CR1/CD35 is transcribed from a separate downstream gene and therefore, human CR2/CD21 and CR1/CD35 may have additional functions compared to their mouse counterparts.

Aberrant regulation of CR2/CD21 is observed in systemic lupus erythematosus, an inflammatory autoimmune disorder of the connective tissue involving production of auto-antibodies to DNA and chromatin in more than 90% of patients.[16] B cells derived from systemic lupus erythematosus patients express increased CD19 and decreased CR2/CD21 compared to healthy controls.[17–19] Further, the appropriate restriction and regulation of CR2/CD21 expression is critical to the development of a healthy B-cell repertoire. Transgenic mice expressing human CR2/CD21 at the pre/pro stage of B-cell development in the bone marrow develop B cells with reduced antigen responses, potentially driven by impaired B-cell activation and B-cell receptor-dependent signaling.[20,21] This implies that timing of CR2/CD21 expression is critical to shaping a functional B-cell repertoire, however the mechanisms driving CR2/CD21 expression during B lymphopoiesis are not defined.

Signaling *via* CD40 and IL-4 has been shown to increase surface density of CR2/CD21 by 20%–30% and activate the cAMP pathway in human B lymphocytes.[22,23] The inducible expression of *CR2/CD21* is mediated through elements in the *CR2/CD21* proximal and core promoter. Previously we have identified various elements that regulate the basal and cell-specific expression of *CR2/CD21* in the proximal promoter and first intron respectively.[24,25] Important regulatory regions include an SP1 site located at −120 and two functionally distinct E-boxes located between −47 and −60 relative to the transcriptional start site (TSS).[25] Recent studies have attributed the core promoter with a more complex role in regulation of gene expression.[26–29] The concepts that have emerged are that core promoters are tailored to their biological function and act as the convergence point for long-range and cis-acting regulators of transcription. In the experiments outlined in this report, we assessed the role of the *CR2/CD21* core promoter in driving transcription initiation in B cells. We identified a single major transcription initiation site in two mature B-cell lines and demonstrated that general transcriptional machinery occupancy surrounding the TSS correlates with CR2/CD21 expression level *in vivo*. Moreover, we identified functional regulatory elements in the core promoter that modulate transcriptional activity *in vitro* including a TATA box, initiator element (Inr), downstream promoter element (DPE), SP1 binding site and a functional single nucleotide polymorphism (SNP).

MATERIALS AND METHODS

Cell culture

Suspension cell lines Reh (CRL-8286), Ramos (CRL-1596), Raji (CCL-86), SKW 6.4 (TIB-215) and K562 (CCL-243) were obtained from ATCC (ATCC, Manassas, VA, USA) and were maintained at 37 °C with 5% CO_2 in RPMI-1640 supplemented with 10% FBS 50 U/ml penicillin and 50 μg/ml streptomycin. We selected cell lines blocked at various stages of development to represent pre-B (Reh),[30] mature-B (Ramos, Raji),[31] terminally differentiated-B (SKW 6.4)[32] or erythroid precursor (K562)[33] cells.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described[34] with Protein A/G Agarose/Salmon sperm DNA (Upstate Biotechnology, Lake Placid, NY, USA) and 5 μg of α-SP1 (ab13370; Abcam, Milton, Cambridge, UK), α-TBP (ab63766; Abcam), α-RNA polymerase (RNAP) II CTD YSPTSPS phosphoS2 (ab5095; Abcam), α-RNAP II CTD YSPTSPS phosphoS5 (ab5131; Abcam), α-E12 (Sc-762X; Santa Cruz Biotechnology, Dallas, TX, USA), α-E47 (sc-763X; Santa Cruz) or IgG (ab554121; Abcam) (BD Pharmingen, San Jose, CA, USA). Quantitative PCR utilized 2 μl of ChIP samples and the Illumina Eco Real-Time PCR system V.4 (Illumina, San Diego, CA, USA). Primers spanning the −42/+139 portion of the *CR2/CD21* promoter (forward 5′-CGTGTGCCGGA-CACTATTT-3′ and reverse 5′-GGTGCGACGAGAGCCAAG-AA-3′, annealing temperature 60 °C) were used to detect specific enrichment across the *CR2/CD21* TSS. Primers spanning the −8/+291 portion of the *CR2/CD21* gene (forward 5′-GCT-CACAGCTGCTTGCTGCT-3′ and reverse 5′-GGTCCCTCA-AAGCTAGCGGGAGGCG-3′, annealing temperature 60 °C) were used to detect specific enrichment across the *CR2/CD21* DPE. Serially diluted chromatin input (10%–0.01%) was used to construct a standard curve against which samples were quantified. Specific enrichment generated by immune complexes was normalized to the background enrichment generated by the isotype control. Amplicons from a representative qPCR for each experiment were run on a 1.5% agarose gel stained with ethidium bromide for visualization to ensure specificity and correct amplicon size.

Electrophoretic mobility shift assay (EMSA)

Protein-DNA binding reactions utilized 2–4 μg of cell line nuclear extract (Thermo FISHER Scientific, Scoresby, VIC, Australia) in chilled binding buffer(4% Ficoll, 20 mM HEPES, 1 mM EDTA, 0.5 mM DTT, 1 μg poly dI : dC) and 25 fmol of biotin-labelled oligonucleotide for 30 min on ice. Oligonucleotides encompassed the TATA box (plus 5′-CCGG-ACACTATTTAAGGGCCCGCCTCTCCTGG-3′ and minus 5′-CCAGGAGAGGCGGGCCCTTAAATAGTGTCCGG-3′), the putative SP1 site (plus 5′-TTAAGGGCCCGCCTCTCCT-GGCTCACAGCTGC-3′ and minus 5′-GCAGCTGTGAGCC-AGGAGAGGCGGGCCCTTAA-3′) or the TSS incorporating the major (plus 5′-CCGCCTCTCCTGGCTCACAGCTGCTT-GCTGCT-3′ and minus 5′-AGCAGCAAGCAGCTGTGAGC-CAGGAGAGGCGG-3′) or the minor (plus 5′-CCGCCTCTC-CTGGCTCATAGCTGCTTGCTGCT-3′ and minus 5′-AGCA-GCAAGCAGCTATGAGCCAGGAGAGGCGG-3′) allele of rs182309299. For competition reactions, cold competitor was incubated with nuclear extract for 10 min. For supershift assays, 2 μg of α-SP1 (Abcam) or α-TBP (Abcam) was incubated for 30 min prior to addition of labelled oligonucleotide. Binding reactions were electrophoresed in pre-cast 6% DNA retardation gels (Life Technologies, Mulgrave, VIC, Australia) at 100 V for 60 min, transferred to a nylon membrane at 30 V for 60 min. Protein–DNA complexes were visualized with the Chemiluminescent Nucleic Acid Detection Module (Thermo FISHER Scientific, Scoresby, VIC, Australia).

Identification of putative core promoter elements

The −50 to +50 region of the *CR2/CD21* promoter was manually interrogated for sequences with similarity to known core promoter motifs including TATA box, Inr, upstream or downstream TFIIB recognition element (BRE^U and BRE^D), motif ten element and DPE at consensus locations. Putative transcription factor binding sites were identified using the LASAGNA-search web tool.[35]

Flow cytometry

Cultured B-cell lines were harvested and 1×10^6 cells washed twice with cold staining buffer (PBS containing 5% FBS) at 300*g* for 5 min at 4 °C. Cells were resuspended in 80 µL staining buffer and incubated with 20 µl of PE-conjugated mouse anti-human CD21 antibody (555422; BD Biosciences, San Jose, CA, USA) or PE-conjugated mouse IgG1κ isotype control (BD Biosciences, 551436), for 20 min. Unstained cells were also included. Cells were washed twice, resuspended in 0.5 ml staining buffer and analysed using a BD Accuri C6 flow cytometer (BD Biosciences) and FlowJo software V10.0.5 (Tree Star, Ashland, OR, USA).

Rapid amplification of cDNA ends (RACE)

5′RACE was optimized and performed based on the Scotto-Lavino *et al.*[36] new RACE protocol with modifications. Total mRNA was prepared from 1×10^7 cells using RNAzol RT (Sigma Aldrich, St Louis, MO, USA), dephosphorylated with 20 U CIP (New England Biolabs, Ipswich, MA, USA) and mRNA cap removed using 0.5 U TAP (Epicentre Biotechnologies, Madison, WI, USA). An RNA oligonucleotide (5′-CGACUGAAGCACGAGGAUAUUGACAUGGACUGAA-GGAGUAGAAA-3′) was added using 10 U T4 RNA ligase (New England Biolabs). RNA was incubated with 2.5 fmol gene-specific reverse primer and annealing buffer (0.25 M NaCl, 6.25 mM EDTA, 50 mM Tris-Cl pH 7.5), for 5 min at 65 °C, snap cooled and incubated for 4 h at 40 °C. RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). Primary PCR (FWD 5′-CGACTGAAGCACGAGGATATTGA-3′, REV 5′-GGAGCAA-TGGAGCCAACATT-3′, annealing temperature 55 °C) was followed by two nested reactions (REV 5′-CGGCCCCCACAT-ATTATTT-3′, annealing temperature 55 °C and FWD 5′-GGATATTGACATGGACTGAAGGAGTA-3′, REV 5′-GGGT-GTAGAGCCTCTAATTTT-3′, annealing temperature 54 °C). PCR was performed using the PTC-100 Thermocycler (MJ Research, Waltham, MA, USA) and GoTaq green master mix (Promega, Madison, WI, USA). Briefly, 1 µl template was amplified in 50 µl reactions with 0.2 µM each primer. PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide. Amplicons were cloned using the TOPO-TA cloning kit (Life Technologies) and sequenced.

Transfection and quantitation of promoter activity

The luciferase reporter containing −1250/+75 (1.2 LUC) of the CR2/CD21 promoter was prepared as described previously.[25] Bioinformatics were generated with LASAGNE-Search 2.0[35] and site-directed mutagenesis was performed using the Quik-Change mutagenesis kit (Stratagene, La Jolla, CA, USA). Plasmid DNA was prepared using the EndoFree Plasmid Maxi kit (QIAGEN, Valencia, CA, USA) followed by transient transfection with the Amaxa Nucleofector Device and Cell Line Nucleofector Solution V (Lonza, Basel, Switzerland). Cell lysates were sequentially assayed for *Firefly* and *Renilla* luciferase using the Dual-Luciferase Reporter Assay system (Promega). Luminescence was analysed using Tropix Winglow software (Applied Biosystems, Foster City, CA, USA). *Firefly* luciferase was normalized to *Renilla* luciferase and the activity of each mutant was normalized to the wild-type 1.2 LUC plasmid.

Statistical analysis

Differences in transcriptional activity or ChIP enrichment were assessed using Student's paired *t*-test with a confidence interval of 95% ($P<0.05$). Statistics and graphs were generated using GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA). All graphed values represent the mean±SEM of at least three independent experiments.

RESULTS

Transcription of *CR2/CD21* proceeds from a focused TSS in mature B cells

Four potential TSS for *CR2/CD21* have been identified 89–99 base pairs (bp) 5′ of the translational start codon in EBV-positive human B-cell lines.[37,38] To evaluate this further, we performed 5′ rapid amplification of cDNA ends (RACE) using mRNA from CR2/CD21-positive Ramos (EBV-negative) and Raji (EBV-positive) cells, alongside CR2/CD21-negative Reh and K562. No PCR amplicons were detected in CR2/CD21-non-expressing cell lines (Reh and K562) (Figure 1a, lanes 2 and 5) and no-template controls (NTC) (Figure 1a, lanes 4 and 7). Only one major PCR product was detected in Raji (Figure 1a, lane 6), while a major band and a minor band were observed in Ramos samples (Figure 1a, lane 3). Sequencing of all PCR products indicate that the major TSS in both Raji and Ramos is an adenine residue (Figure 1b, red box) located 92 bp 5′ of the translational start codon, and directly 3′ of the conventional site identified by Rayhel *et al.*[38] in 1991. The minor product observed in Ramos samples mapped to an adenine residue precisely 30 bp downstream of the major TSS (Figure 1b, blue box). To determine if the results generated in our cell lines accurately represent that of primary B cells, we interrogated $polyA^+$ cap analyses of gene expression (CAGE) tags from donor derived $CD20^+$ primary human B cells, which were freely available from the ENCODE server.[39] The vast majority of CAGE tags aligned to a narrow region (25 bp) downstream to the major TSS identified previously (Figure 1c), however, a small number of CAGE tags mapped to a broader 100 bp region either side of the major TSS. Shorter fragments are potentially an artefact of

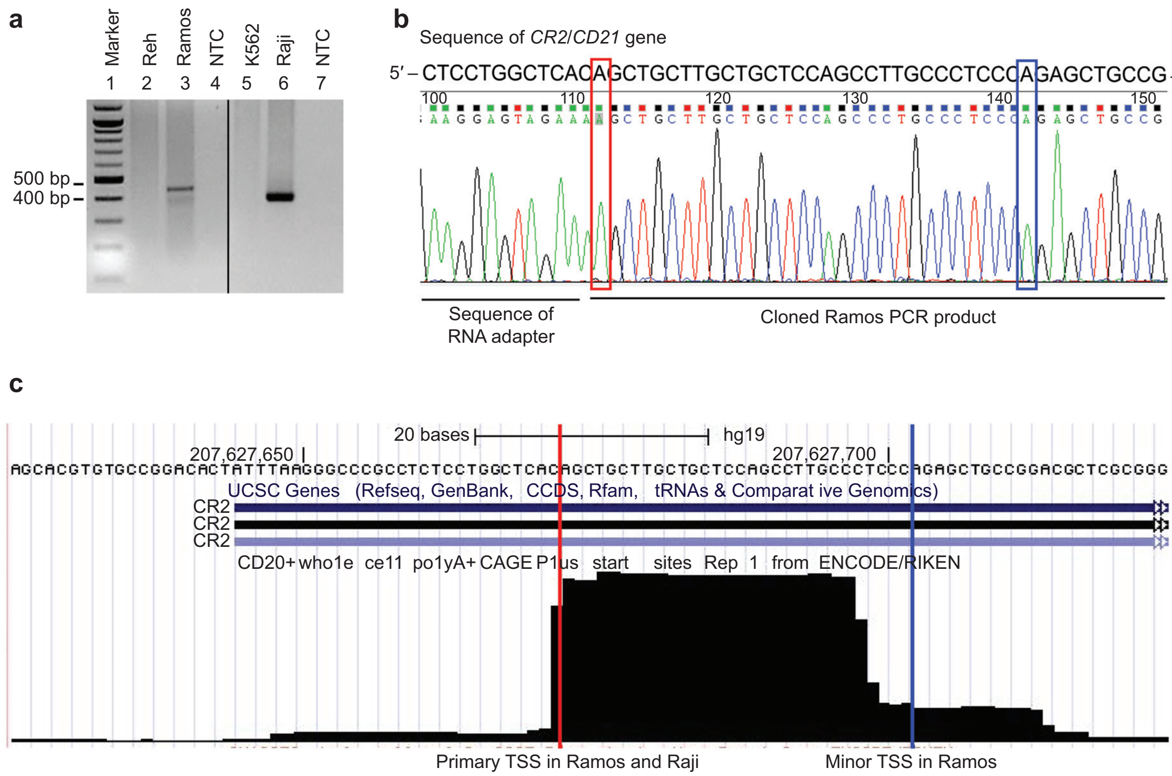

Figure 1 Transcription of *CR2/CD21* proceeds from a single predominant start site in the majority of mature B cells. (**a**) 5′RACE generates a single major PCR product in Ramos (lane 3) and Raji (lane 6), while no PCR amplicons are detected in Reh, K562 or NTCs (lanes 2, 5, 4 and 7, respectively). (**b**) A representative chromatogram generated by sequencing the Ramos 5′RACE PCR product indicates that the first nucleotide in the predominant *CR2/CD21* mRNA is an Adenine (red box) while the minor mRNA start site is located 30 bp downstream (blue box). (**c**) The ENCODE track of CD20$^+$ cell CAGE tags indicates that *CR2/CD21* TSSs are focused around a single 25 bp peak. The major (red line) and minor (blue line) TSS identified in Ramos and Raji cells are indicated. CAGE, cap analyses of gene expression; CR2/CD21, complement receptor 2; NTC, no template control; RACE, rapid amplification of cDNA end; TSS, transcriptional start site.

RNA quality or methodology, but could indicate the presence of minor start sites in B cell subpopulations. Our results and those of others[37,38] indicate that transcription of *CR2/CD21* is focused around a single peak spanning approximately 30 bp.

The *CR2/CD21* core promoter contains putative TATA, Inr, DPE and GC box motifs

We next identified potential core promoter elements and transcription factor binding sites surrounding the TSS (Figure 2a). A non-consensus Inr (*CR2/CD21*; ACAGCTG, consensus; Py-Py-A$_{+1}$-N-T/C-Py-Py) was identified that was spatially aligned with a TATA-like element (*CR2/CD21*; TATTTAAG, consensus; TATAWAWA) located at −29 to −22 relative to the A$_{+1}$ in the putative Inr. A GC box potentially bound by SP1 (*CR2/CD21*; GGGCCC, consensus; GGGCGG) was identified directly downstream and slightly overlapping the TATA-like element. A putative DPE was also identified with a near-consensus sequence (CR2/CD21; AGAGC, consensus; A/G-G-A/T-C/T-A/C/G) located at +31 to +35 and predicted to bind E2A. E2A is associated with gene expression changes during B-cell development[40] and is essential for the development of pro-, pre- and immature B cells in the bone marrow.[41] To determine if E2A is bound to the *CR2/CD21* gene encompassing the DPE in mature B cells, we performed chromatin immunoprecipitation with antibodies specific for the E2A proteins E12 and E47 as well as RNAP as a positive control (Figure 2b). Using Ramos cells, robust enrichment of E12, E47 and RNAP could be detected upstream of the *CR2/CD21* TSS (Figure 2b, n=2).

TATA box, Inr and DPE sequences contribute to transcriptional regulation of the *CR2/CD21* promoter

To test the functionality of putative core promoter elements, luciferase assays were performed using various mutants of the −1250/+75 *CR2/CD21* promoter (Figure 3a). Robust activation of the wild-type (WT) promoter was observed when transfections were performed in Raji cells. Mutation of the TATA box, Inr or DPE sequences resulted in a significant 30%–40% decrease in 1.2 LUC promoter activity ($P \leqslant 0.01$) (Figure 3b). The −22/−20 mutation resulted in reduced luciferase activity comparable to that of the adjacent TATA box mutation, while the overlapping −21/−17 mutant had no effect, suggesting that the guanine (G) residue which forms the overlap between the TATA box and SP1 site, is critical for TATA box function. Mutation of the GC box at either two (SP1 2 bp), or five (SP1 5 bp) nucleotides had no effect. As mutation of a single promoter element was insufficient to abolish transcriptional

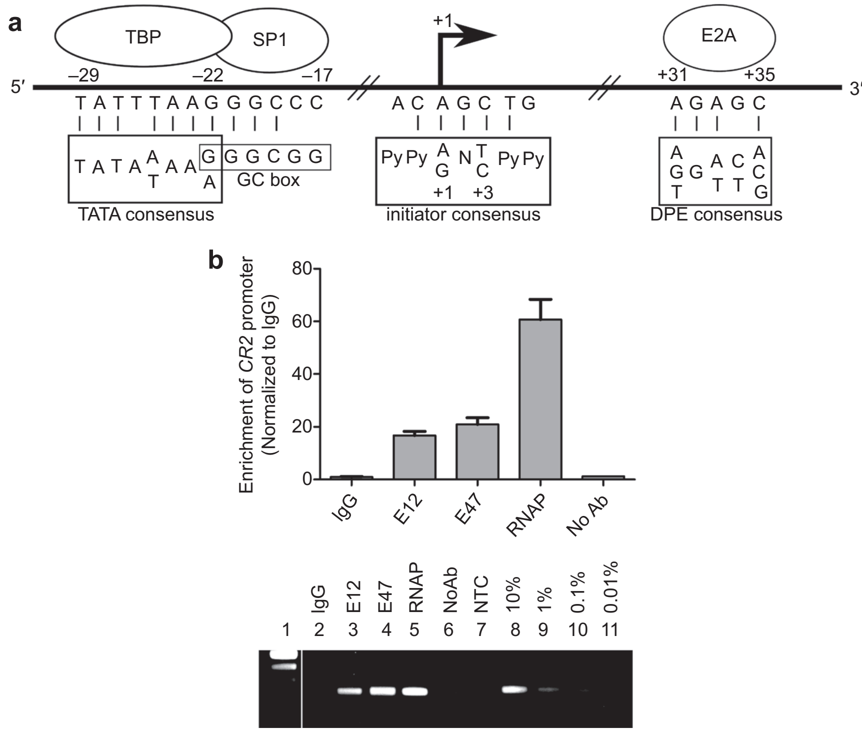

Figure 2 The *CR2/CD21* promoter contains near consensus sequences matching TATA box, GC box, Inr and DPE motifs. (**a**) The *CR2/CD21* promoter sequence spanning −50/+50 relative to the TSS (curved arrow) was manually interrogated for sequences matching known core promoter elements. Identity to TATA box (−22/−29), GC box (−17/−22), Inr (−2/+5) and DPE (+31/+35) motifs is demonstrated with vertical lines between the *CR2/CD21* promoter sequence and the consensus sequence (black box). The LASAGNE-search web tool was used to predict binding of TBP, SP1 and E2A to the TATA box, GC box and DPE, respectively. (**b**) Binding of E2A (E12 and E47) to the *CR2/CD21* gene surrounding the DPE was confirmed in Ramos cells by chromatin immunoprecipitation (n=2). Enrichment generated by antibodies specific for E12, E47 and the positive control (RNAP) was quantified by comparison to serially diluted chromatin input (10%–0.01%) and normalized to the enrichment generated by the IgG control. Representative qPCR amplicons were run on a 1.5% agarose gel stained with ethidium bromide to ensure reaction specificity. No specific enrichment was generated in the no antibody control sample (No Ab) and no PCR amplicons were detected in the NTC. CR2/CD21, complement receptor 2; DPE, downstream promoter element; NTC, no template control; TSS, transcriptional start site.

activity, we hypothesized that transcription is regulated by a synergistic combination of elements. Concomitant mutation of the TATA and GC boxes resulted in a 50% reduction in luciferase activity compared to the WT promoter ($P<0.001$) (Figure 3c), but was not significantly different from mutation of the TATA box alone. Simultaneous mutation of the TATA box and Inr element decreased transcriptional activity by 65% ($P<0.0001$), and was significantly reduced compared to the individual TATA ($P<0.001$) and Inr ($P<0.01$) mutants. As the effects of the individual TATA and Inr mutations were not additive, we conclude that these two elements do not function independently. Additional mutation of the DPE did not further reduce transcriptional activity. Similar results were observed when transfections were performed in the CR2- and EBV-negative cell line K562 (Figure 3d and e).

Sequence-specific protein-DNA complexes containing TBP and SP1 interact with −26 to +6 of the *CR2/CD21* promoter *in vitro*

To delineate the transcription factor binding sites spanning the TATA box (TATA probe, −37 to −6) and putative SP1 site (GC box probe, −26 to +6) partially overlapping oligonucleotides were designed (Figure 4a). When electrophoretic mobility shift assays (EMSA) was performed with CR2-negative (K562, Reh) or CR2-positive (Ramos, Raji) nuclear extract, three protein–DNA complexes were detected in the presence of the TATA oligonucleotide (I, III, IV) and four major complexes bound to the GC box oligonucleotide (I –IV) (Figure 4b). All complexes formed resulted from specific protein-DNA interactions, as they were successfully out-competed by a 30- to 60-fold excess of unlabeled oligonucleotide (Figure 4b). Supershift assays performed with Raji nuclear extract using either α-TBP or α-SP1 with the TATA oligonucleotide did not yield any differences in complex formation (Figure 4c, lanes 2–4). When similar supershift assays were performed with the GC oligonucleotide, addition of α-TBP resulted in a decrease in complex B (Figure 4c, lane 8, white arrow) whereas α-SP1 resulted in a decrease of complex A (Figure 4c, lane 9, black arrow). Addition of an IgG control antibody did not result in removal of any complexes (Figure 4c, lanes 5 and 10). Similar results were obtained using Ramos nuclear extract (data not shown).

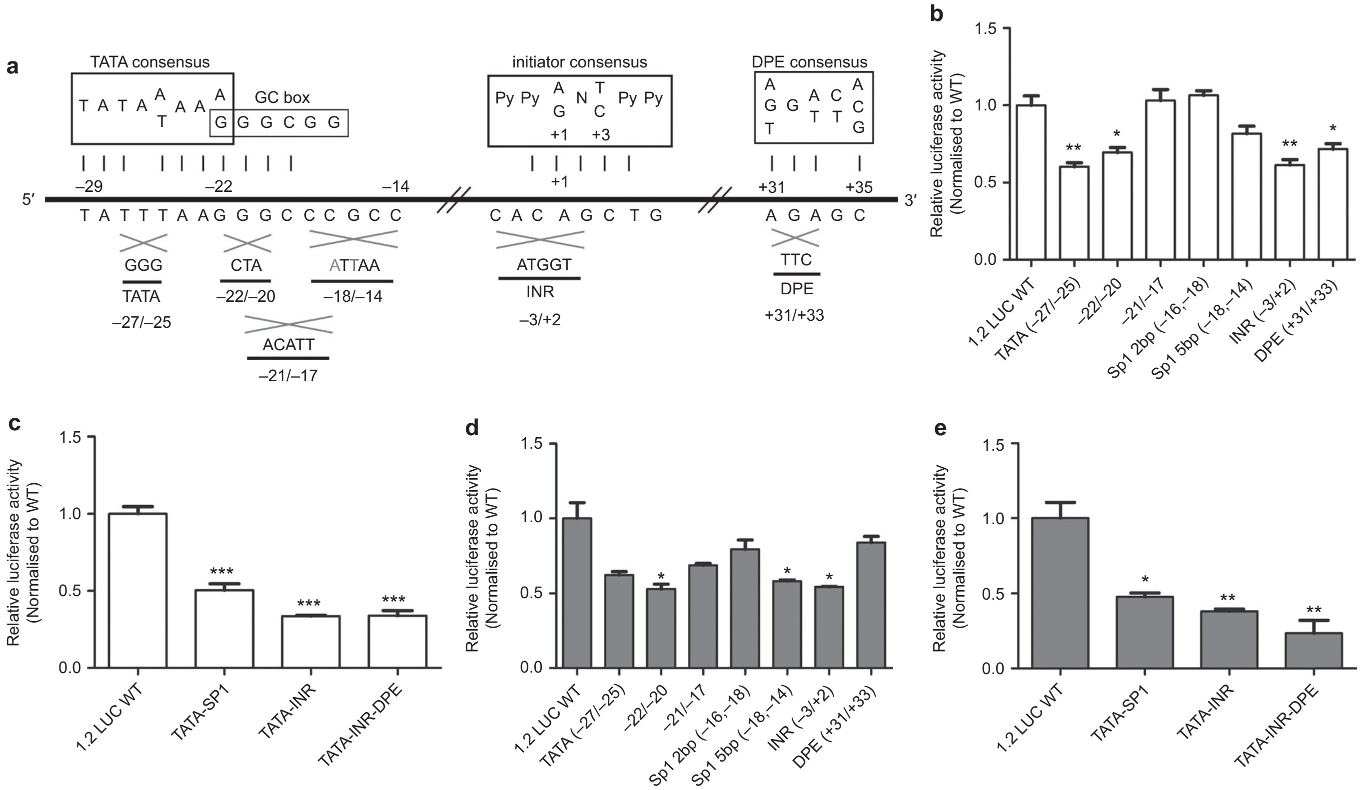

Figure 3 TATA box, Inr and DPE sequences contribute to transcriptional regulation of *CR2/CD21 in vitro*. (**a**) Site directed mutagenesis was performed at multiple sites in the *CR2/CD21* core promoter surrounding the TATA box, GC box, TSS and DPE (consensus sequences identified with black boxes). Sites of individual mutations are indicated with a grey cross above their sequence, name and location. (**b**) In Raji cells, mutation of the TATA box, Inr or DPE results in a significant 30%–40% decrease in luciferase activity compared to the WT *CR2/CD21* 1.2LUC construct. (**c**) In Raji cells, concomitant TATA–SP1 mutation resulted in a 50% reduction in luciferase activity, while TATA–INR mutation resulted in a 65% decrease in luciferase activity but tripartite mutation of TATA–INR–DPE did not reduce luciferase activity below 30% of the WT promoter. (**d**) In CR2/CD21$^-$ and EBV-negative K562 cells, mutation of the GC box and Inr sequence resulted in a 50% reduction in luciferase activity compared to the WT promoter. (**e**) In K562 cells, concomitant TATA–SP1 mutation resulted in a 50% reduction in luciferase activity, while TATA–INR mutation resulted in a 65% decrease in luciferase activity but tripartite mutation of TATA–INR–DPE did not reduce luciferase activity below 25% of the WT promoter. Data are the results of three independent replicates presented as mean±SEM Significance is indicated by *$P<0.05$, **$P<0.005$ and ***$P<0.0005$. CR2/CD21, complement receptor 2; DPE, downstream promoter element; EBV, Epstein–Barr virus; Inr, initiator element; WT, wild-type.

Occupancy of general transcriptional machinery at the CR2/CD21 promoter corresponds to *CR2/CD21* expression level and development stage

As CR2/CD21 is upregulated upon B-cell maturation, we hypothesized that the core promoter is poised for transcriptional activation at the pre-B-cell stage. To investigate this, we performed ChIP using antibodies specific for TBP, SP1 and RNAP phosphorylated at either serine 2 (RNAPpS2) or serine 5 (RNAPpS5). *CR2/CD21* non-expressing pre-B (Reh), or terminally differentiated B (SKW) and *CR2/CD21*-expressing mature B (Ramos and Raji) cell lines were used to assess general transcriptional machinery occupancy at the *CR2/CD21* promoter during B-cell development. The status of CR2/CD21 surface expression for each cell line was confirmed by flow cytometry. In the Reh and SKW cell lines, which do not express CR2/CD21, no significant enrichment was generated with any of the antibodies tested (Figure 5a and d), indicating that *CR2/CD21* is not poised for transcriptional activation prior to induction of *CR2/CD21* expression or after cells have undergone terminal differentiation. In both mature B-cell lines, TBP and RNAPpS5 could be detected at the *CR2/CD21* promoter ($P<0.05$) (Figure 5b and c). Consistently, the levels of enrichment generated by TBP and RNAPpS5 were higher in Raji than Ramos cells, and correlated with *CR2/CD21* expression. Capture of RNAPpS2 at the CR2/CD21 promoter was slight in both Raji and Ramos, whereas the results for anti-SP1 differed between the mature B-cell lines. In Ramos cells, anti-SP1 enrichment was on average sevenfold greater than the isotype control, however this was not observed for Raji cells. Interestingly, although SP1-specific pull-down in Ramos cells did not reach statistical significance, a significant negative correlation was observed between enrichment of SP1 and RNAPs5 within each biological replicate ($P<0.05$, $R^2=0.91$) (Figure 5e). Therefore, higher levels of RNAPpS5 appear to correlate with lower levels of SP1 enrichment and this is consistently observed in the Raji samples.

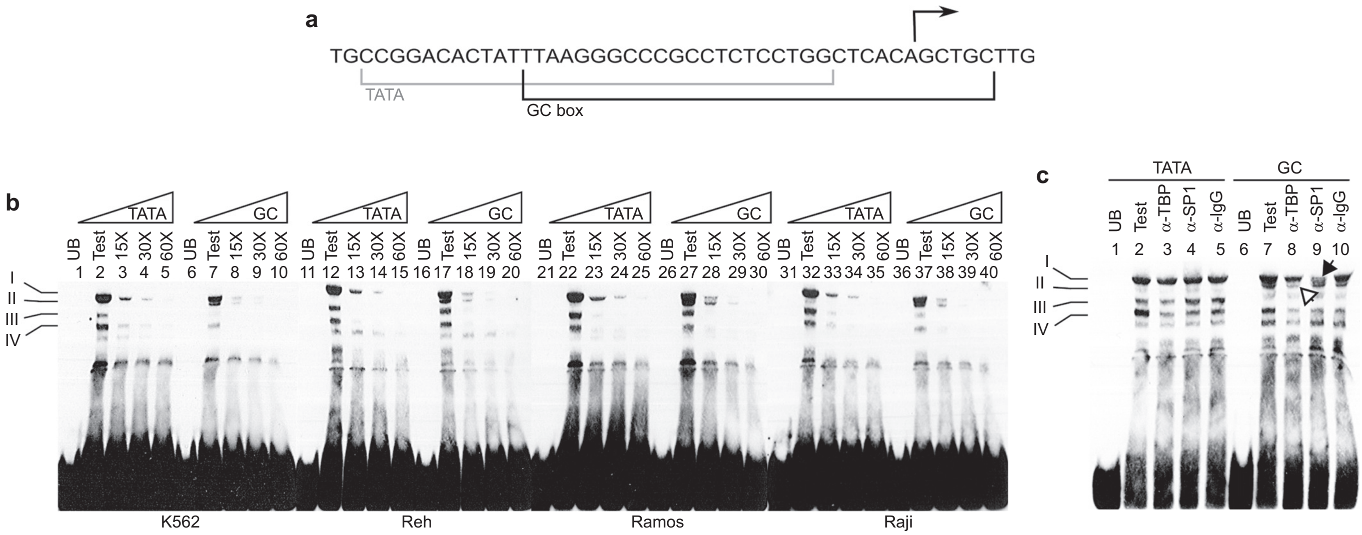

Figure 4 SP1 and TBP are present in sequence-specific complexes bound to −26 to +6 of the *CR2/CD21* promoter *in vitro*. (**a**) To investigate protein–DNA interactions at the *CR2/CD21* core promoter, overlapping oligonucleotides spanning the TATA box (TATA probe, −37 to −6, light grey line) or GC box (GC probe, −26 to +6, dark grey line) were designed. (**b**) When oligonucleotides were incubated with K562 (lanes 2 and 7), Reh (lanes 12 and 17), Ramos (lanes 22 and 27) or Raji nuclear extract (lanes 32 and 37) and either the TATA or GC probe (former and latter lanes respectively), four sequence-specific complexes (I–IV) and one low molecular weight non-specific complex were detected. UB indicates unbound control. Specificity and binding affinity was determined by incubation with increasing amounts (15×–60× molar excess) of unlabelled competitor (K562, lanes 3–5 and 8–10; Reh, lanes 13–15 and 18–20; Ramos lanes 23–25 and 28–30; Raji, lanes 33–35 and 38–40). (**c**) Supershift assays indicate that TBP forms part of complex II bound to the GC probe (lane 8, white arrow) while SP1 forms part of complex I bound to the GC probe (lane 9, black arrow). Specificity was determined based on comparison to the IgG control (lanes 5 and 10). Data are representative of three independent replicates. CR2/CD21, complement receptor 2.

The *CR2/CD21* promoter is unmethylated and enriched for activating histone marks in CD20$^+$ human B cells

We have previously shown that chromatin accessibility surrounding the *CR2/CD21* TSS also correlates with CR2/CD21 expression status in the model cell lines K562, Reh, Ramos, Raji and SKW.[34] In addition, enrichment of histone H3/H4 acetylation and H3K4 dimethylation was also correlated with CR2/CD21 expression status in Reh, Ramos and Raji cells.[34] To determine if these results are representative of primary human cells, we interrogated the ENCODE database for enrichment of histone modifications and CpG dinucleotide methylation in CD20$^+$ or EBV-immortalized peripheral blood B-cells (Figure 6). In general, enrichment of histone modifications associated with active transcription (H3K4m2, H3K27ac, H3K4m3, H3K9ac, H3K79m2)[42] was greater in CD20$^+$ or EBV-immortalized peripheral blood B cells than in the CR2/CD21-negative cell line K562 (Figure 6, red box). Further, CpG dinucleotides surrounding the *CR2/CD21* TSS in peripheral blood B-cells were unmethylated, while CpG dinucleotides were frequently methylated in K562 cells (Figure 6, blue box).

The minor allele of rs182309299 is associated with increased *CR2/CD21* transcript abundance in mature B-cell lines but does not alter protein–DNA interactions

Genetic variants in the core promoter potentially contribute to differential transcriptional regulation and gene expression. We have previously reported the transcriptional effects of the SNP rs3813946 in the core promoter of *CR2/CD21*, which alters chromatin accessibility and transcription factor binding.[43] We identified a second SNP, rs182309299 (C>T), located at the −1 position of the *CR2/CD21* gene that we hypothesized could affect Inr activity, since single nucleotide variations located at −1 to +3 can result in differences in promoter activity of up to ninefold.[44,45] Therefore, we performed luciferase assays in Raji cells using a −1250/+75 *CR2/CD21* promoter construct expressing either the major or minor allele of the −1 C>T SNP. Expression of the minor allele (−1T) resulted in significantly higher luciferase activity when compared to the major allele (−1C) ($P<0.001$) (Figure 7a). Similar results were observed in the CR2/CD21$^-$ and EBV-negative cell line K562 (Figure 7b). To determine if altered transcriptional activity was mediated by altered protein–DNA interactions, we performed EMSA analysis using oligonucleotides spanning the TSS (−18 to +14) and containing either the C or T allele of rs182309299. Using K562, Reh, Ramos and Raji nuclear extracts, we detected two weak sequence-specific protein–DNA complexes (Figure 7c). These complexes correspond to complexes I and IV (Figure 4b and c) previously observed to bind directly upstream of the TSS; however, complex formation did not differ between the alleles.

DISCUSSION

The dynamics of core promoter regulation are made possible by the integrated interaction between nucleotide sequence, core-promoter-element spacing, epigenetic regulation and

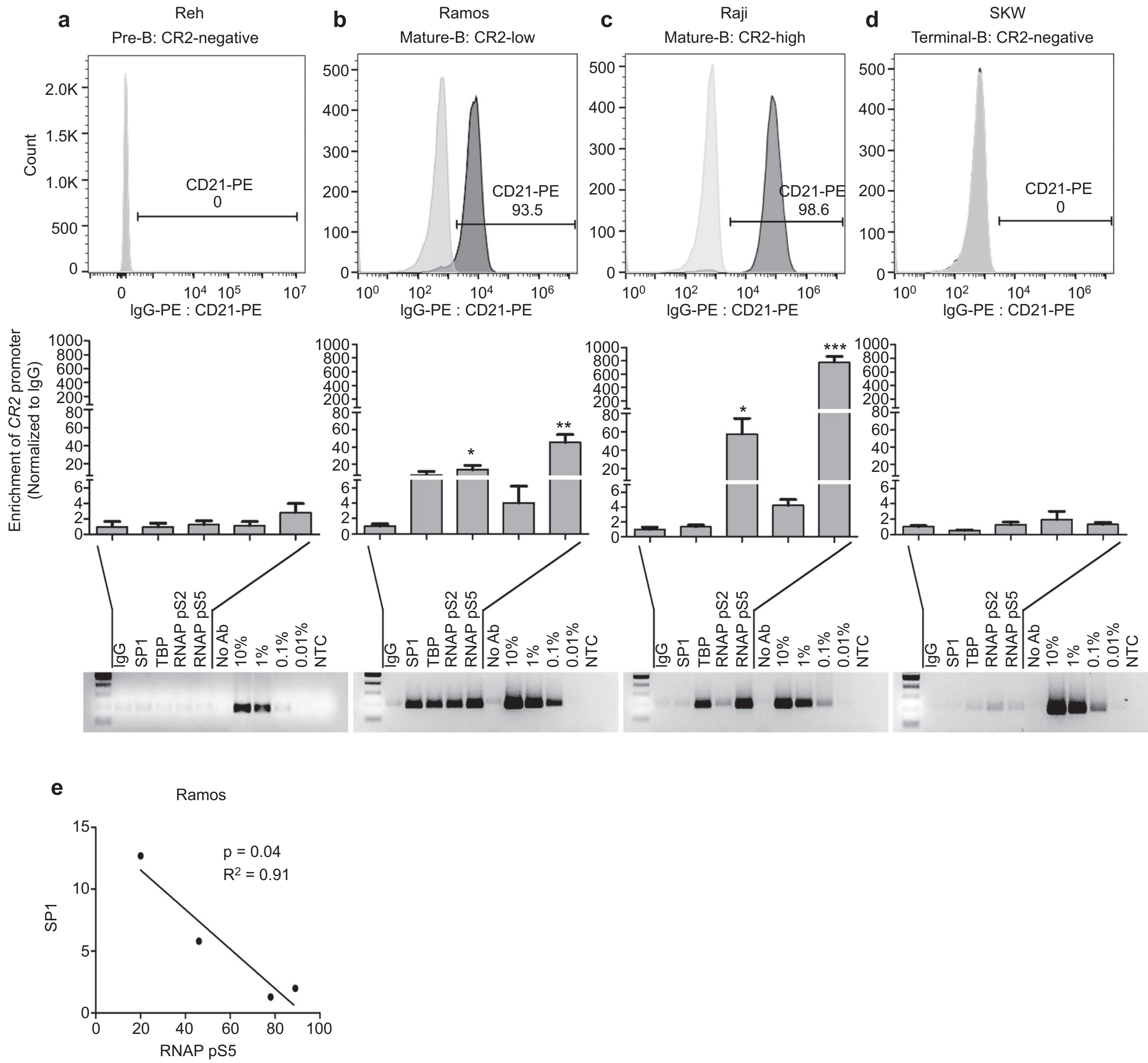

Figure 5 Occupancy of RNAPpS5 at the *CR2/CD21* promoter correlates to CR2/CD21 expression level *in vivo*. CR2/CD21 cell-surface expression was determined by flow cytometry (upper panel). Specific enrichment of the *CR2/CD21* promoter (middle panel) was observed in mature B-cell samples Ramos (**b**) and Raji (**c**) immunoprecipitated with TBP and RNAPpS5. Specific enrichment of SP1 was only generated in Ramos samples (**b**), and was negatively correlated with RNAPpS5 enrichment (**e**). No specific enrichment was generated in CR2/CD21-negative Reh (**a**) or SKW (**d**) with any antibodies tested. Enrichment was quantified by comparison to serially diluted chromatin input (10%–0.01%). A representative sample from each cell line was run on a 1.5% agarose gel (lower panel). Graphs were generated from three independent experiments and represent the mean±SEM Significance is indicated by **P*<0.05, ***P*<0.005 and ****P*<0.0005. CR2/CD21, complement receptor 2.

three dimensional conformation.[28,46–48] Here we discuss the complex core promoter regulation of *CR2/CD21*, the expression of which is cell type-specific and inducible.

We have identified core promoter elements in the *CR2/CD21* core promoter including TATA box, GC box, Inr and DPE sequences. This core promoter architecture is of interest as the combination of TATA, Inr and DPE is rare in human promoters. While human genes containing DPE have been identified,[49,50] very few functional studies on human DPEs have been carried out.[51–54] Interestingly, all of the human DPEs so far characterized are found in genes containing functional Inr elements and in close proximity to an SP1 site.[49,51–54]

We find that no single element is capable of directing *CR2/CD21* transcription initiation, although the TATA box and Inr

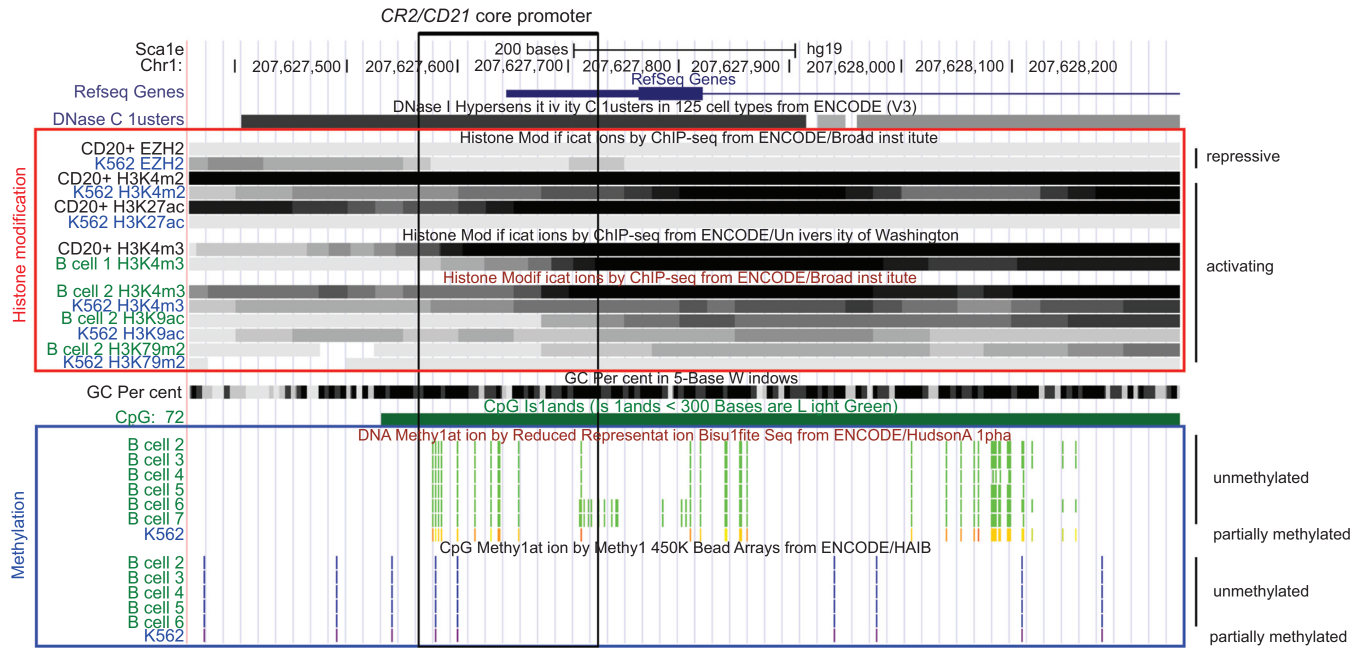

Figure 6 Histone modification and DNA methylation at the *CR2/CD21* core promoter correspond to CR2/CD21 expression in B cells *versus* K562 cells. We interrogated the ENCODE database to determine the status of histone modification (red box) and methylation (blue box) surrounding the *CR2/CD21* core promoter (black box). In general CD20$^+$ (black) or EBV-transformed primary human B cells (green) had a greater enrichment (indicated by darker grey bars) of activating histone marks (H3K4m2, H3K27ac, H3K4m3, H3K9ac, H3K79m2) than K562 (blue) cells. The *CR2/CD21* promoter was unmethylated (green or blue lines) in B cells compared to partially methylated (orange or purple lines) in K562 cells. CR2/CD21, complement receptor 2; EBV, EBV, Epstein–Barr virus.

function synergistically in regulating *CR2/CD21* transcription. We show that the DPE contributes to transcriptional regulation but does not act in conjunction with the TATA and Inr elements *in vitro*. The −57/+75 region of *CR2/CD21* core promoter has previously been shown to direct cAMP inducible expression of *CR2/CD21 in vitro*.[23] The DPE may be involved in regulating inducible expression of *CR2/CD21* as the specific elements mediating this effect have not been defined. Consistent with this, the MHC class I gene promoter contains a TATA box, an Inr, a DPE and an SP1 binding site, none of which are strictly necessary for transcription.[53,54] Rather, each element uniquely regulates tissue-specific or inducible expression levels accordingly.

The role of the GC box is harder to interpret as our EMSA analyses suggest that SP1 competes for binding with unidentified factors bound to the adjacent TATA box, a feasible possibility since the two binding sites overlap by a single nucleotide. Further, our ChIP data show an inverse correlation between SP1 and RNAPpS5, consistent with binding site competition. However, since our luciferase assays indicate that the GC box does not control basal levels of transcript abundance, the role of this element may be mechanical. For example, it has been suggested that constitutive SP1 binding to the core promoter of the Lymphotoxin-α gene in T cells is required to maintain the promoter in an accessible conformation in the absence of TFII-I and RNAP.[55] Further, SP1 is known to interact with both histone acyltransferase[56] and histone deacetylase[57] and may therefore be involved in regulating the chromatin conformation surrounding the TSS. Such interactions would not likely have an effect on reporter gene expression, but may be important to the regulation of inducible gene expression requiring rapid transcript upregulation upon receiving a specific biological signal.

We mapped the major *CR2/CD21* TSS in mature B-cell lines to a single nucleotide located within the Inr. This observation is in line with the current view that tissue-specific and developmentally regulated genes are more likely to be controlled by core promoter elements (TATA, Inr, DPE) and initiate transcription at a single precise location or narrow window.[26,58] This is supported by primary cell data which show that in the major population of peripheral blood B cells, *CR2/CD21* transcription initiates from a focused TSS (ENCODE). However, a small population of CD20$^+$ CAGE tags initiated transcription over a broader 100 bp range. Since CD20$^+$ cells encompass the entire B-cell pool ranging from late pro-B to mature B, it is possible that minor start sites may be utilized in specific B-cell subsets.

We find that general transcription factor occupancy surrounding the major TSS correlates with *CR2/CD21* expression level in pre-B- and mature B-cell lines. It has been suggested that developmentally regulated genes are enriched for poised promoters, while tissue-specific genes are more likely to be strictly active or inactive.[59] However, inducible promoters of the broad peak class are also frequently poised for transcription.[60] We did not find any evidence that *CR2/CD21* is poised prior to B cell maturation. These results are supported by

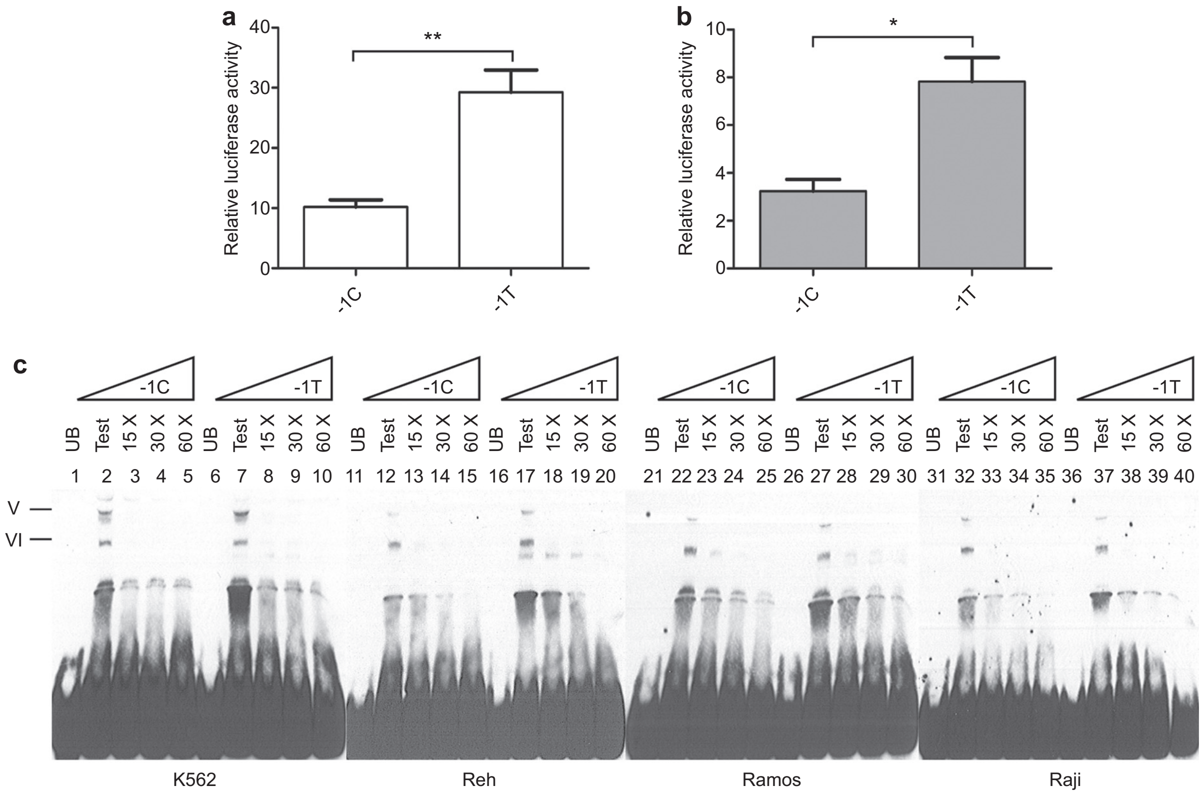

Figure 7 The minor allele of rs182309299 increases transcript abundance *in vitro*, but does not alter protein–DNA complex formation. (**a**) The minor allele of rs1872309299 (−1T) is associated with a 3-fold increase in transcript abundance over the major allele (−1C) in transient transfections performed with Raji B cells. (**b**) The rs1872309299 minor allele (−1T) is associated with a 2.5-fold increase in transcript abundance over the major allele (−1C) in transient transfections performed with K562 cells. (**c**) EMSA was performed with oligonucleotides spanning the *CR2/CD21* TSS and containing either the major (−1C) or minor (−1T) allele of the rs1872309299 SNP. Oligonucleotides were incubated with K562 (lanes 2 and 7), Reh (lanes 12 and 17), Ramos (lanes 22 and 27) or Raji nuclear extract (lanes 32 and 37) and either the −1C or −1T probe (former and latter lanes, respectively), two weak sequence-specific complexes (V and VI) were detected. UB indicates unbound control. Specificity and binding affinity was determined by incubation with increasing amounts (15×–60× molar excess) of unlabeled competitor (K562, lanes 3–5 and 8–10; Reh, lanes 13–15 and 18–20; Ramos lanes 23–25 and 28–30; Raji, lanes 33–35 and 38–40). Results were generated from three independent experiments and graphs are represented as mean±s.e.m. Significance is indicated by $^*P<0.05$ and $^{**}P<0.005$. EMSA, electrophoretic mobility shift assay.

chromatin accessibility assays which show that in *CR2/CD21* expressing cell lines Ramos and Raji, chromatin is significantly more accessible compared to the *CR2/CD21* non-expressing cell line Reh.[34] Chromatin was also more accessible directly surrounding the transcriptional start site in Raji cells compared to Ramos cells.[34] Therefore, *CR2/CD21* expression level correlates with chromatin accessibility and general transcription factor occupancy which suggests that between the pre-B- and mature B-cell stages, the *CR2/CD21* promoter switches from strictly inactive to active and requires significant chromatin rearrangement.

rs182309299, located at −1, modifies the transcriptional activity of the *CR2/CD21* core promoter. Since phenotypic differences in gene expression between populations can be attributed to differences in frequencies of genetic variants,[61] it is possible that rs182309299 contributes to variation in *CR2/CD21* expression levels between populations. Genotyping performed through the 1000 Genomes Project Phase 1 demonstrated the minor allele of this SNP is detected only in 1%–2% of individuals of African or African-American ancestry (Ensembl release 76, August 2014). The retention of this variant in individuals of African descent could confer a functional benefit, potentially relevant to a specific immunological challenge. We did not detect differences in protein binding between the two alleles of rs182309299 *in vitro* despite a threefold increase in transcriptional activity associated with the minor allele. These data suggest that sequence variants in the core promoter may regulate the structural dynamics of transcription. The major allele of rs182309299 is present in a complementary tri-nucleotide pair $\underline{C}A_{+1}$G-CTG which scores among the highest for bendability and flexibility compared to all trinucleotide combinations assessed by DNase I accessibility and nucleosome position matrices.[46] The minor allele disrupts this complementarity ($\underline{T}A_{+1}$G-CTG) and could therefore alter the curvature of the DNA directly surrounding the transcriptional start site. Similarly, point mutations surrounding a Super-core Promoter TSS that did not alter transcription factor binding *in vitro* resulted in a fourfold decrease in reporter gene activity, which was attributed to a decreased seeding of TSS bubble formation.[62] It is likely that DNA structural dynamics act in conjunction with TF binding. The promoters' structural

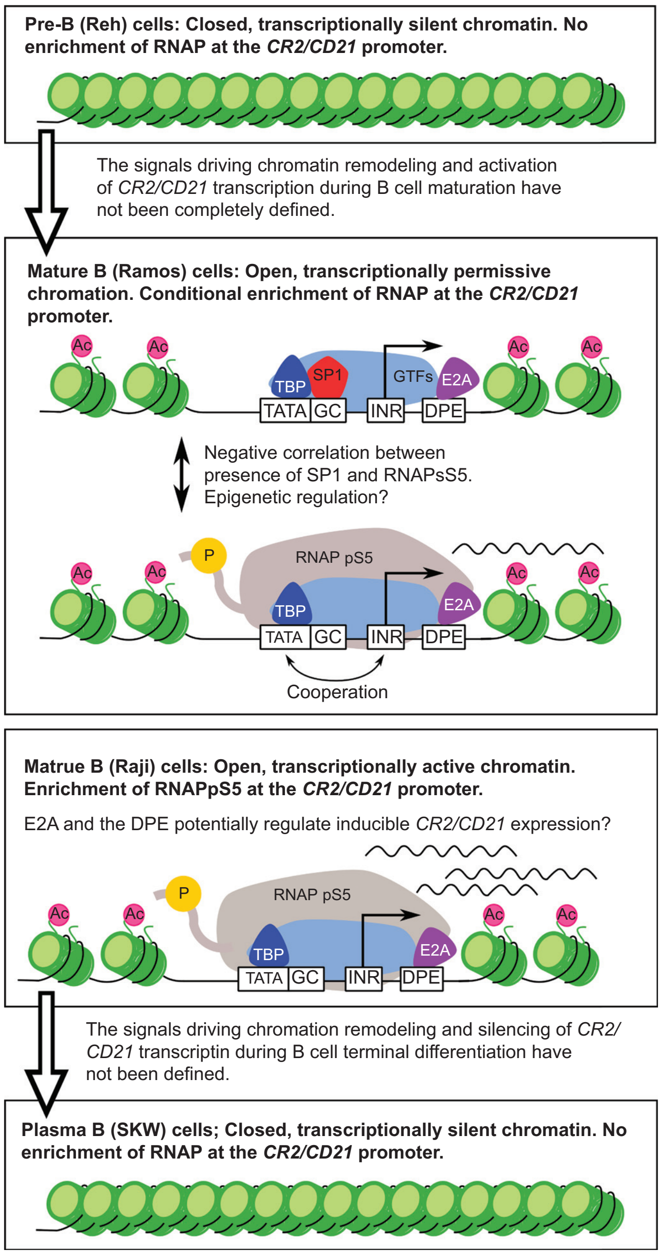

Figure 8 Transcriptional regulation *via* the *CR2/CD21* core promoter in B cells. In Reh pre-B cells, the *CR2/CD21* promoter is in a closed/silent conformation and no binding of RNAP is detected. Between the pre-B- and mature B-cell stages, chromatin is remodeled to allow recruitment of the general transcriptional machinery. At precisely which stage of transitional development this occurs remains to be defined. In Ramos mature B cells, chromatin surrounding the *CR2/CD21* promoter is open, conditionally bound by RNAP and transcriptionally active. Synergistic activity of TATA and Inr efficiently positions RNAP to direct transcription from a focused TSS. In Raji mature B cells, chromatin is open and high levels of *CR2/CD21* transcription take place. Inducible expression of *CR2/CD21* may be modified by E2A binding to the DPE. At B-cell terminal differentiation, chromatin is remodeled by unknown mechanisms to render the *CR2/CD21* promoter closed and transcriptionally silent in SKW plasma cells. CR2/CD21, complement receptor 2; DPE, downstream promoter element; Inr, initiator element; RNAP, RNA polymerase; TSS, TSS, transcriptional start site.

properties seed the 3D conformation required for initiation, which simultaneously enhances recognition of DNA sequence motifs required for TF assembly and directing TSS placement and strength.

CONCLUSION

Using human B-cell lines frozen at specific stages of B-cell development, we show a marked difference in promoter accessibility and RNAP occupancy surrounding the *CR2/CD21* core promoter in pre-B *versus* mature B cells. Based on the results presented here, we propose that consensus motifs in the *CR2/CD21* core promoter become accessible during B cell ontogeny *via* chromatin rearrangement, allowing a developmental switch from inactive to active and recruitment of the general transcriptional machinery (Figure 8). The precise stage of transitional B cell development (T1–T3) at which chromatin remodeling occurs, and the developmental signals driving this transition, remain to be defined. Subsequently, synergistic activity of TATA and Inr efficiently positions RNAP to direct transcription from a focused TSS, while inducible expression of *CR2/CD21* may be modified by TF interactions with GC box and DPE motifs. Lastly, we highlight the potential for single nucleotide variants in the core promoter to contribute to transcriptional regulation and variation in gene expression between populations.

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