Interaction between the immunoglobulin heavy chain 3' regulatory region and the IgH transcription unit during B cell differentiation

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Abstract

The immunoglobulin heavy (*Igh*) chain locus is subject to precisely regulated processes, such as variable region gene formation through recombination of variable (V_H), diversity (D_H), and joining (J_H) segments, class switching and somatic hypermutation. The 3' regulatory region (3' RR) is a key regulator of the *Igh* locus, and, as revealed by deletions in mouse plasma cell lines and mice, is required for IgH expression as well as class switching. One of the mechanisms by which the 3' RR regulates its targets is through long-range physical interactions. Such interactions between elements of the 3' RR and a target site in the IgH transcription unit have been detected in plasma cells, and in resting and switching B cells, where they have been associated with IgH expression and class switching, respectively. Here, we report that lentiviral shRNA knockdown of transcription factors, CTCF, Oct-2, or OBF-1/OCA-B, had no discernible defects in loop formation or H chain expression in plasma cells. J_H-3' RR interactions in pre-B cell lines were specifically associated with IgH expression. J_H-3' RR interactions were not detected in either Pax5-deficient or RAG-deficient pro-B cells, but were apparent in an Abelson-derived pro-B cell line. These observations imply that the 3' RR has different loop interactions with target *Igh* sequences at different stages of B cell development and *Igh* regulation.

Keywords

Chromosome conformation capture (3C); immunoglobulin gene rearrangements; immunoglobulin heavy chain gene expression; long-range enhancer interactions; lentivirus-mediated shRNA; B cell development

1. Introduction

During B cell maturation and activation, immunoglobulin heavy chain (IgH) genes are subject to DNA rearrangements required for VDJ joining and class switch recombination (CSR), and to V_H gene somatic hypermutation (SHM) (reviewed in (Dudley et al., 2005). These processes are tightly regulated by cis elements of the *Igh* locus, including V region promoters, I/switch region promoters, the intronic enhancer (Eμ) and the hs sites of the 3' regulatory region (3' RR), several of which are associated with the four known enhancers

(hs3a, hs1.2, hs3b and hs4) (Cogne and Birshtein, 2004). Additional 3' RR hs sites contain binding sites for Pax5 and CTCF and display their accompanying insulator activity (hs5-7 and "38") (Fig. 1A) (Chatterjee et al., 2011; Garrett et al., 2005). Functionally, the 3' RR is important for immunoglobulin heavy chain gene expression in plasma cells and for class switching in activated B cells, as revealed by mouse knockout models (Pinaud et al., 2001; Vincent-Fabert et al., 2010). A contribution of the 3' RR to SHM was revealed by a reduction in SHM resulting from a deletion of the entire 3' RR enhancer region from a BAC transgenic mouse (Dunnick et al., 2009).

Chromosome conformation capture (3C) studies have identified loops that juxtapose 3' RR enhancers with target *Igh* sequences, such as J_H sequences associated with the IgH transcription unit. In switching cells, additional interactions of the 3' RR with I/switch region promoters have been identified (Wuerffel et al., 2007; Yan et al., 2011). That J_H-3' RR interactions occur in splenic B cells but not in splenic T cells indicates that these interactions are B cell-specific. Loss of H chain expression in a plasma cell line (Ju et al., 2007) or defects in CSR (Wuerffel et al., 2007), each resulting from deletions of 3' RR enhancers, showed concomitant defects in loop formation. Hence, loop interactions involving the 3' RR are implicated in these roles for expression and recombination. Additional 3' RR loop interactions have been identified, one through DICE elements at the V_H promoter, which is also associated with Igh expression in a plasma cell line (Ren et al., 2011), and a second with CTCF sites upstream of DFL16.1 in pro-B cells that is associated with *Igh* locus contraction (Degner et al., 2011) and predicted to impact on VDJ joining.

The transcription factor CTCF has been shown to contribute to loop formation in various loci. Initially identified as a regulator of the chicken c-myc promoter (Lobanenkov et al., 1990), CTCF was subsequently found to be involved in insulation of enhancers from promoters, potentially regulating boundaries between active and silent chromatin (rev. in (Phillips and Corces, 2009)). CTCF sites identified in the imprinted control region (ICR) regulate H19/Igf2 imprinting through loop-formation as measured by chromosome conformation capture (3C) experiments (Kurukuti et al., 2006), and similarly, contribute to MHC locus regulation (Majumder and Boss, 2010). CTCF also plays a role in looping in the β-globin locus, but apparently without a critical role in gene expression (Splinter et al., 2006). Recently, CTCF has been shown to affect *Igh* locus contraction apparently through an impact on loop interactions (Degner et al., 2011).

Octamer binding motifs are found in many *Igh* regulatory elements, including V gene promoters, Eμ and the 3' RR. Together with the co-factor OBF-1, Oct-1 or Oct-2 binds to octamer motifs in B cells and exerts its function. Oct-1 is ubiquitously expressed while Oct-2 is expressed only in B cells and neuronal cells. Although B cell development in fetal liver is not affected in Oct-2 knockout mice, these mice die soon after birth for unclear reasons (Corcoran et al., 1993). OBF-1 (also known as OCA-B and Bob-1) expression is B cell-specific, and OBF-1 knockout mice show reduced levels of IgG isotypes and no germinal center formation (Schubart et al., 1996). Oct-2 and OBF-1 have been shown to regulate 3' RR activity in B cells (Tang and Sharp, 1999). In human t(14;18) lymphoma cells, the bcl2 gene is involved in a translocation with the *Igh* locus, and there is an interaction between the translocated *bcl2* gene and the 3' RR. The contribution of Oct-2 to the *bcl2*-3' RR interaction was revealed by siRNA knockdown of Oct-2 (Duan et al., 2007). Recent studies have shown a role for OBF-1/OCA-B in interactions between hs4 and DICE sequences downstream of the V_H promoter (Ren et al., 2011)

In this report, we find that loop interactions involving the 3' RR and J_H target sequences in a plasma cell line, although critical for *Igh* expression, do not rely on CTCF, Oct-2 or OBF-1. J_H-3' RR loop interactions detected on the single VDJ rearranged and expressed allele in a

pre-B cell line imply their association with *Igh* gene expression. The analysis of J_H-3'RR interactions in pro-B cell sources gives insight into the ontogeny of 3' RR interactions with the IgH transcription unit during early stages of B cell development. Collectively, these studies monitor the engagement of the 3' RR with the IgH transcriptional unit during B cell development and suggest that the 3' RR participates in a variety of functional loops.

2. Materials and Methods

2.1. B cell lines

Murine EL-4 T cells (ATCC #TIB-39) were used as a control for cell type specificity. The mouse pro-B cell line 63-12 (Shinkai et al., 1992), pre-B cell lines 3-1 and 70Z/3 (Nelson et al., 1983; Paige et al., 1978) were maintained in RPMI-1640 medium with 10% fetal calf serum; plasma cell line MPC11 (Laskov and Scharff, 1970) was cultured in DMEM medium with 10-15% fetal calf serum. All cells were grown at 37°C in an atmosphere of 5% CO_2. 63-12 cells reconstituted with RAG-2 protein were a generous gift from Dr. Kathryn Calame (Columbia University) (Angelin-Duclos and Calame, 1998). Pro-B cells derived from a Pax5$^{-/-}$ mouse and transduced with a Pax5 expression construct coupled with estrogen receptor (ER) (Pax5-ER (B1.1)) (Nutt et al., 1998) were a generous gift from Dr. Meinrad Busslinger (Research Institute of Molecular Pathology, Vienna, Austria), and were received from Dr. Paolo Norio (Montefiore Medical Center, Bronx, NY). Pax5-ER cells were maintained on a layer of ST2 stromal cells (irradiated at 1100 rad) in the presence of IL-7 (1 ng/ml). Upon induction with 4-hydroxytamoxifen (H7904, Sigma), Pax5 protein was expressed. After 48 hr of culture, ~40% CD19+ cells (CD19 is a Pax5 target gene) were detected by FACS (FACScan, BD Biosciences).

2.2. Primary cells

CD19$^+$ pro-B cells were isolated from the bone marrow of RAG1$^{-/-}$ mice (The Jackson Laboratory) by positive selection with anti-CD19 magnetic beads (Miltenyi Biotec). The purity of the pro-B cells was ~90%, as detected by FACS. C57BL/6 splenic B cells and DBA splenic B cells were purified by CD43 depletion (Miltenyi Biotec). The purity of both splenic B cell populations (B220$^+$) was ~95%, as detected by FACS.

2.3. Gene knockdown by lentiviral shRNA

10^6 293T (human kidney epithelial) cells were transfected with packaging plasmids (pMDLg/pRRE, pRSV/Rev and pMD2.VSV.G) and vector plasmid pLKO.1 (Sigma Aldrich), which contains a specific shRNA (Mission). For each desired targeting, multiple shRNA's were tested, and the most efficacious were used. These were: CTCF: TRCN0000039019, termed 19 in text, NM_007994.1- 10301c1, CCGGGCAGAGAAAGTAGTTGGTAATCTCGAGATTACCAACTACTTTCTCTGCTTT TTG; Oct-2: RCN0000081518, termed 1518 in text, NM_011138.1-1503s1c1, CCGGCTTAGTAACCTCGCCTCTCTTCTCGAGAAGAGAGGCGAGGTTACTAAGTT TTTG, and OBF-1/OCA-B: TRCN0000086513, termed 6513 in text, NM_011136.1-1089s1c1, CCGGGCGGCAAATGATAGTGATATTCTCGAGAATATCACTATCATTTGCCGCTTT TTG. Calcium phosphate-precipitated plasmid DNA was applied to 293T cells that have been treated by chloroquine. Supernatants containing lentiviral particles were collected 24 hr and 48 hr after transfection. A GFP lentiviral shRNA was generated as a control following the same procedure. Virus was concentrated by ultra-centrifugation (SW28) at 30,000 rpm for 2.5 hrs. Concentrated virus was stored at -80°C, and a small aliquot of GFP virus was used to calculate viral titer. Low speed spinoculation was used for lentiviral infection at an MOI (multiplicity of infection) of 20:1. After 48 hr of infection, puromycin was added and cells were maintained for as long as two weeks to obtain a stable cell line. The minimum

concentrations of puromycin to kill all the uninfected cells were determined for each cell source in advance of lentivirus infection.

2.4. RT-PCR

2 μg total RNA was applied in each reverse transcription reaction using the Superscript III First-Strand Synthesis System (Invitrogen). After reverse transcription, 50 ng cDNA was used for PCR amplification for 25 to 27 cycles. Primer sequences are listed in Table I.

2.5. Western blot

15 μg nuclear extracts was loaded onto a 10% pre-cast mini acrylamide gel (Bio-Rad), and subjected to electrophoresis for 2 hrs at 100 v prior to transfer to nitrocellulose membrane (Amersham Bioscience). After blocking with 10% fat-free milk in tris-buffered saline tween-20 (TBS-T), the membrane was incubated with primary antibodies: anti-CTCF, Upstate 07-729; anti-GAPDH, sc25778, Santa Cruz Biotechnology; anti-OBF-1, rabbit specific antiserum, a generous gift from Dr. Robert Roeder, Rockefeller University, through Dr. Hilda Ye, Albert Einstein College of Medicine; or anti-TBP (TFIID), sc-273, Santa Cruz Biotechnology. The membrane was washed twice with TBS-T, incubated with goat anti-rabbit IgG-HRP-conjugated secondary antibody, sc2004, Santa Cruz Biotechnology, and washed four times with TBS-T. The membrane was developed using the Amersham ECL Western Blot Analyzing System (GE healthcare).

2.6. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed and quantitated as described previously (Garrett et al., 2005). Briefly, cells were fixed with formaldehyde at RT after which cells were lysed and nuclei were sonicated to yield ~200-100bp fragments. Pre-cleared chromatin from ~4×10^6 cells was taken for each pull down. ChIPs were done with 4 mg of antibodies to CTCF (Upstate 07-729) with 4 mg of rabbit IgG (sc-2027) used as a negative control. 30 ml of salmon sperm protein A+G agarose beads (Upstate) were used to immunoprecipitate the antibody-chromatin complex. The complex was washed several times prior to elution of bound chromatin by elution with SDS and reversal of protein-DNA crosslinks. Chromatin was then digested with proteinase-K and DNA was extracted by Qiagen PCR Purification kit (Cat # 28104). DNA was diluted to a concentration of 4.95 ng (3000 copies)/ml, and a standard curve of C_T vs the log of copy number from successive 5-fold dilutions was obtained for each primer using Real Time PCR analysis. Primers for the 3' RR and controls were previously described (Garrett et al., 2005). For assay of ChIP, a 2% dilution of chromatin was used as input and each pull down (PD) and corresponding input were analyzed in triplicate and calculated for copy number. The results were plotted as the ratio of PD/input (Y axis). ChIP experiments were normalized by comparison to a positive control whenever possible.

2.7. 3C assay

The procedure was performed as previously described (Ju et al., 2007). Briefly, 10^7 cells were cross-linked with formaldehyde for 10 min at room temperature. Nuclei were prepared, and non-cross-linked protein was removed by SDS. Triton X-100 was added to sequester the SDS, after which samples were digested with 1,500 units of HindIII overnight at 37°C with gentle shaking. The digestion was terminated in the presence of SDS at 65°C. Digested nuclei (10^6 nuclei) were subjected to intramolecular ligation with T4 DNA ligase. Samples were then treated with Proteinase K and RNase A, and DNA was purified by phenol:chloroform extraction and isopropanol precipitation. One hundred nanograms of ligated DNA samples were analyzed by 32 to 34 cycles of PCR (94°C/30 s, 55°C/30 s, and 72°C/30 s) in a 25 μl reaction system. These conditions give amplifications in linear range

(Ju et al., 2007). 3C primer sequences are listed in Table I. A control template was generated by mixing DNA from BACs that contain sequences of interest with fragments of the calreticulin (CalR) gene in equal molar ratios. This control template was subjected to HindIII digestion and to DNA ligation and was used to normalize PCR efficiency among different primer pairs. PCR products were subjected to electrophoresis on a 2% gel, and gel signals (S) were analyzed by SYNGENE GENE genius BioImaging System. Crosslinking efficiency between two HindIII fragments was calculated as previously, namely (SIgH/SCalR in selected cell type)/(SIgH/SCalR in control template).

3. Results and Discussion

3. 1 Neither CTCF nor Oct-2 or OBF-1 is essential for 3' RR-J$_H$ long-range interactions of IgH expression in MPC11 cells

3.1.1 CTCF—To address whether CTCF is essential for 3' RR-JH interactions and for IgH expression in the MPC11 plasmacytoma cell line, we utilized lentivirally-cloned shRNA to silence CTCF expression. Of the two shRNAs tested, the 19 shRNA (Materials and Methods) was the more efficient at silencing, achieving an ~70% knockdown as shown by RT-PCR and Western blot (Fig. 1A). ChIP analysis of wild-type and CTCF-KD cells showed considerable although incomplete reduction of CTCF binding to the 3' RR (Fig. 1B). However, no significant change in either heavy chain or light chain expression was detected in CTCF-KD cells (Fig. 1A), and 3C data showed that there was no alteration in loop-formation (Fig. 1C). It should be noted that knockdowns of CTCF at these levels with comparable residual binding to the 3' RR as measured by ChIP resulted in measurable differences in locus contraction and 3C interactions between the 3' RR/CTCF sites and DFL16.1/CTCF sites (Degner et al., 2011). We concluded that 3' RR-J$_H$ loops in MPC11 cells are not critically dependent on these levels of CTCF, implying that these interactions require less CTCF than those between this region and DFL16.1/CTCF, or that proteins other than or in addition to CTCF are essential. Of interest are studies that show that it is not CTCF (Splinter et al., 2006) but rather cohesin (Chien et al., 2011), which binds at both CTCF and non-CTCF sites in the β-globin LCR and globin target genes, that is essential for β-globin expression.

3.1.2 Oct-2 and OBF-1/OCA-B—We addressed the contribution of Oct-2 to loops involving the 3' RR and the IgH transcription unit in the mouse *Igh* locus in MPC11 by using a lentiviral shRNA technique to KD Oct-2 expression. RT-PCR showed that Oct-2 mRNA expression was considerably reduced by 1518 shRNA (see Materials and Methods) (Fig. 2A). However, neither IgH nor Igκ expression was affected. In addition, there were no detectable changes in Oct-1 expression. Considering that Oct-1 might functionally compensate for the loss of Oct-2, we treated MPC11 cells with shRNA to target OBF-1/OCA-B (see Materials and Methods), a coactivator of both Oct-1 and Oct-2 and essential for V$_H$ promoter-driven expression (Luo and Roeder, 1995). RT-PCR and Western blot analysis showed that OBF-1 mRNA expression was substantially reduced by 6513 shRNA (see Materials and Methods) (Fig. 2B); and as was shown in Oct-2 KD cells, neither IgH nor Igκ expression was affected. 3C experiments also showed no significant differences in loop formation in either Oct-2- or OBF-1- silenced MPC11 cells (Fig. 2C). The level of KD achieved in our studies was estimated to be ~85% (Fig. 2B), enabling us to infer that normal IgH expression and loop formation are achieved by as low as 15% of normal levels of OBF-1/OCA-B and Oct-2.

3.1.3. Other studies involving OBF-1 in IgH expression in a plasma cell line—Recent studies have reported that KD of OBF-1/OCA-B in MPC11 cells resulted in a reduction of Igh expression and 3C interactions (Ren et al., 2011), in clear contrast to our

observations. The most likely explanation is differences in the efficiency of the KDs. Our studies used single shRNA's, respectively, to KD OBF-1/OCA-B and Oct-2, and involved considerable cell death during the process of obtaining puromycin-resistant cell lines. Studies by Ren et al used sequential shRNAs (X. Ren and R. Roeder, personal communication). Hygromycin-resistant cell lines with the lowest OBF-1/OCA-B expression were initially selected and then reinfected by lentivirus carrying a second shRNA with puromycin as the selectable marker. Doubly resistant cell lines with the lowest OBF-1/OCA-B expression level were chosen for analyses. Although levels of KD reported by Ren appear to have been similar to ours (Ren et al., 2011), a double KD might be predicted to result in additional reduction in OBF-1/OCA-B expression, thereby crossing a threshold for an effect. It is interesting to consider the possibility that the double KD affected not only the well-characterized OBF-1/OCA-B, but also a predicted and partially redundant OCA-B2 (R. Roeder, personal communication). Notably, even with the double KD approach, there was substantial remaining IgH expression and loop formation, requiring real-time RT-PCR to detect changes in IgH gene expression (X. Ren and R. Roeder, personal communication).

Reporter assays in human B cells have shown that hs4 is regulated by different contributions of NFκB and octamer-binding proteins in different cell lines (Michaelson et al., 1996). In accord with a model proposed by Ren et al (Ren et al., 2011), residual IgH expression in double KD MPC11 cells could result from compensation through the NFκB-mediated pathway: partially in the double KD and essentially fully in the single KD. In this respect, the MPC11 cell line has frequently been shown to generate drug-induced or spontaneous variants that can affect H chain expression (Birshtein et al., 1974; Koskimies and Birshtein, 1976) or loop formation (Ju et al., 2007). In both instances, i.e. as reported here, and by Ren et al (Ren et al., 2011), there was considerable selection pressure, potentially resulting in variant formation.

Collectively, these observations suggest that loop formation and high levels of IgH transcription in MPC11 cells are dependent on synergy or cooperation among multiple factors, such as CTCF, Oct-2 or OBF-1/OCA-B (or Oct-1), or require other, as yet, unidentified, factors.

3.2 Long-range J_H-3' RR interactions occur on the expressed but not unexpressed allele in a pre-B cell line

As noted in the introduction, 3' RR loop interactions important for IgH expression in plasma cells have been reported (Ju et al., 2007) as have those for class switch recombination in B cells (Wuerffel et al., 2007). To determine whether long range J_H-3' RR interactions associated with IgH expression occurred before the B cell stage of maturation, we analyzed the 3-1 pre-B cell line. Our experiments showed that J_H-3' RR interactions in 3-1 cells were equivalent in magnitude to those detected in mature B cells (Fig. 3A). Analysis of the 70Z/3 pre-B cell line permitted us to determine whethr loop interactions were associated with IgH expression. The 70Z/3 pre-B cell line contains an expressed, VDJ rearranged allele, and a non-expressed, DJ recombined allele. 70Z/3 was generated from C57BL/6 X DBA mice (Fig. 3). The expressed IgH allele (C57BL/6) has a VDJ_H1 rearrangement, together with a spontaneous deletion of the region containing 3' RR enhancers hs3a and hs1,2. The IgH non-producing allele, derived from DBA mice, has a D_HJ_H2 rearrangement, and possesses an intact 3' RR (Saleque et al., 1999). Based on these findings, we were able to identify primer pairs to uniquely track the individual alleles. Because J_H1 is retained only on the expressed IgH allele, J_H1-hs3b or -hs4 interactions are specific for the expressed VDJ allele; and because hs3a and hs1.2 are present only on the unexpressed allele, J_H3-hs3a or J_H3-hs1.2 interactions are specific for the non-expressed DJ rearranged allele. 3C data showed that J_H3-hs3a or J_H3-hs1,2 interactions in 70Z/3 cells (both deriving from the non-expressed allele) were at a low level similar to those detected in splenic T cells and substantially less

than those detected in splenic B cells from DBA mice (Fig. 3B). In contrast, there are significant J_H1-hs4 interactions (deriving from the expressed allele), which are at even higher levels than those detected in normal B cells. The relative reduction in signal in normal B cells likely reflects J_H usage and the location of HindIII sites in the J_H genomic regions, HindIII being the enzyme that is used to separate genomic fragments as part of the 3C method. J_H1 and J_H2 are separated by one HindIII site, and JH3 and J_H4 by a second HindIII site. Thus, a J_H3 primer amplifies VDJ rearrangement to both J_H2 and J_H3 during PCR, while the J_H1 primer amplifies only a VDJ_H1 rearrangement.

These data indicating that long-range interactions occur on the expressed allele, but not on the non-expressed allele in 70Z/3 pre-B cells, suggest that J_H-3' RR interactions are associated with transcription rather than recombination.

3.3 Long range J_H-3' RR interactions are detected in a pro-B cell line, but not in primary pro-B cells

We then analyzed long-range J_H-3' RR interactions in an Abelson virus-induced pro-B cell line, 63-12, derived from a RAG-2 deficient mouse (Shinkai et al., 1992). J_H-3' RR interactions were clearly evident in this cell line (Fig. 4A), which because it lacks RAG proteins, cannot undergo DJ or VDJ joining. To determine whether RAG protein expression might increase loop formation, we compared 63-12 with 63-12 cells that were reconstituted with RAG-2 (Angelin-Duclos and Calame, 1998) (Fig. 4A). The levels of interactions were unchanged.

However, J_H-3' RR interactions were not detected in other sources of pro-B cells. CD19^{+} pro-B cells isolated from bone marrow of RAG1^{-/-} mice showed only baseline levels of interaction similar to those detected in EL-4 thymocytes (Fig. 4B). Pax5 deficient mice provide another source of pro-B cells. In Pax5 knockout mice (Urbanek et al., 1994), B cells do not develop at all in fetal liver, and B cell development in the bone marrow is arrested at the pro-B cell stage at the proximal V_H to DJ joining step prior to locus contraction and accessibility of distal V_H genes (Fuxa et al., 2004). Similar to RAG1^{-/-} pro-B cells, an estrogen-sensitive Pax5 inducible pro-B cell line, Pax5-ER (see Materials and Methods), showed only baseline levels of J_H3-3' RR interactions in the absence of Pax5 expression (Fig. 4B); and these levels were unchanged even after induction of nuclear localization and expression of Pax5-ER with tamoxifen (Nutt et al., 1998) when ~40% of the assayed cells were CD19 positive.

4. Overview

Other long-range interactions involving 3' RR sequences have been observed in pro-B cells. In addition to the especially high density of CTCF sites in hs5-7 of the 3' RR (Garrett et al., 2005), CTCF sites are located throughout the V region and are associated with the DFL16.1 segment (Degner et al., 2011; Degner et al., 2009; Featherstone et al., 2010). CTCF-dependent interactions between the 3' RR/CTCF region and CTCF sites in DFL16.1 (Degner et al., 2011) have been detected both in E2a^{-/-} pre-pro B cells and RAG1^{-/-} pro-B cells. DFL16.1 also interacts with Eμ in the IgH transcription unit at a lower level and in a CTCF-independent manner (Degner et al., 2011). These data raise the possibility that different sets of interactions are present in different subsets of pro-B cells. 3' RR-DFL16.1 interactions have been associated with locus contraction and are predicted to impact on VDJ joining (Degner et al., 2011). We propose that the interaction between J_H and 3' RR is independently regulated and related to the architecture needed for H chain expression.

In the 70Z/3 pre-B cell line where we can distinguish the expressed (VDJ) allele from the unexpressed (DJ) allele, J_H-3'RR interactions are detected only on the expressed allele, and

are, therefore, predicted to be associated with H chain expression. Similar interactions are associated with IgH expression in plasma cells (Ju et al., 2007). The finding of 3' RR-J_H interactions in 63-12 pro-B cells raises the possibility that interactions can occur prior to VDJ joining and provide a structure for supporting subsequent IgH transcriptional expression once VDJ joining has taken place. It should be noted that analyses carried out in cell lines and normal cells may show differences that relate to cellular transformation of cultured cells or to particular stages of differentiation that are captured by the culture adaptation process.

Collectively, these studies identify several different loop interactions involving the 3' RR as an anchor. 3' RR-DFL16.1 interactions associated with locus contraction and potentially with VDJ joining are detected in pro-B cells, while 3' RR-J_H interactions associated with heavy chain transcription are detected in the 63-12 Abelson-virus transformed pro-B cell line, and in pre-B and plasma cells. Interactions with I/switch regions are important for class switch recombination (Chatterjee et al., 2011; Wuerffel et al., 2007; Yan et al., 2011). The identification of proteins that facilitate these interactions is in progress.

Acknowledgments

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Highlights

>J_H-3'RR interactions are essential for IgH expression in plasma cells. >These interactions can be independent of CTCF or Oct-2/OBF-1. > J_H-3'RR interactions on the expressed allele in a pre-B cell line. >J_H3-3'RR interactions detected in Abelson virus-transformed pro-B cell line. >J_H3-3'RR interactions not detected in RAG$^{-/-}$ or Pax5$^{-/-}$ pro-B cells.
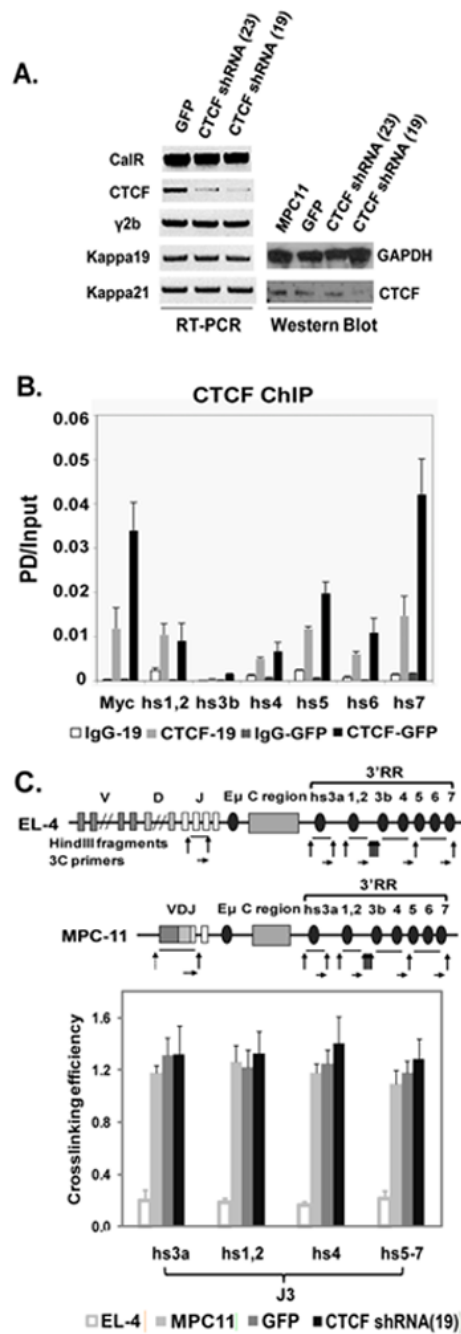
Fig. 1. Effect of CTCF on Ig expression and 3C interactions in MPC11 plasma cells
A. CTCF mRNA (RT-PCR) and protein levels (Western blot) in CTCF shRNA treated
MPC11 plasma cells, with GFP- infected MPC11 cell lines as a control. MPC11 H (γ2b) and
L(kappa 19 and 21) chain expression were unchanged. Calreticulin (CalR) housekeeping
gene and GAPDH were used for normalization of mRNA and protein levels, respectively. B.
Effect of CTCF KD on ChIP analysis of CTCF binding to 3' RR enhancers and insulator
region. CTCF binding, as measured by PD (pulldown)/input, was reduced by lentiviral
shRNA, as shown in the gray bars. Note that the predominant CTCF binding sites are in
hs5-7 (Chatterjee et al., 2011). The 5' end of the c-myc gene was used as a positive control.
Levels of binding with non-CTCF specific IgG are also shown. C. Effect of CTCF KD on

3C interactions involving J_H3 and 3' RR. Schematic maps of immunoglobulin heavy chain (IgH) loci in EL-4 T cells and MPC11 plasma cells used for 3C analysis. Vertical arrows indicate HindIII cleavage sites. Horizontal arrows indicate the primers for 3C assay. 3C interactions between J3 and 3' RR elements were analyzed in EL-4 T cells, MPC11 cells, and MPC11 cells infected with GFP or CTCF shRNA 19. No changes were observed with CTCF KD.
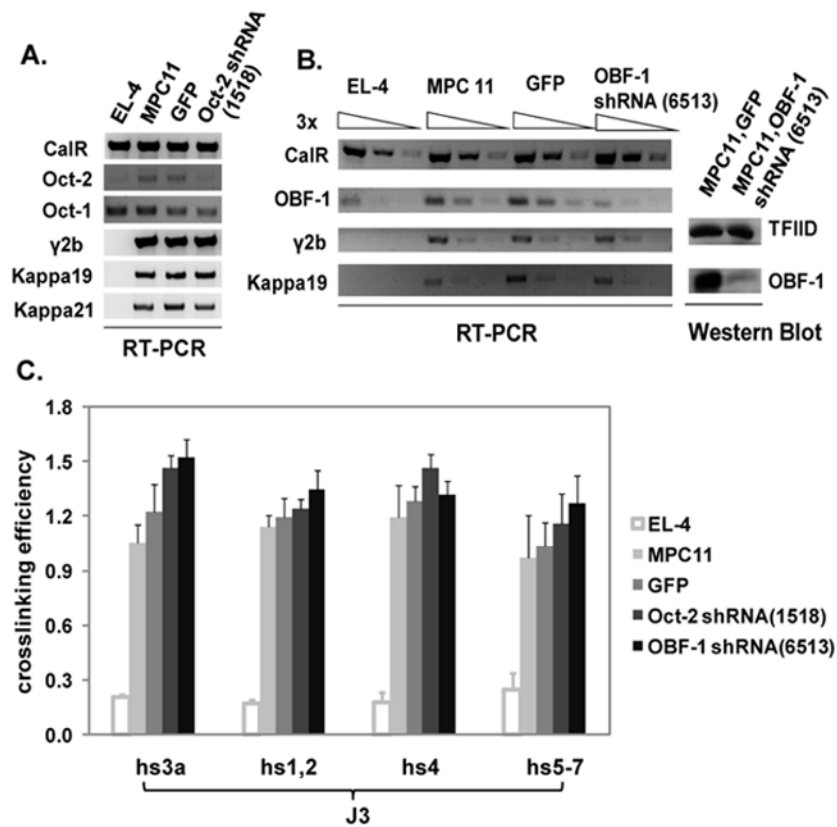
Fig. 2. Effect of Oct-2 and OBF-1 on Ig expression and 3C interactions in MPC11 plasma cell line

A. RT-PCR detection of Oct-2 mRNA levels in GFP or Oct-2 shRNA-infected MPC11 cells. The levels of Oct-1 expression in the GFP- or Oct-2 shRNA treated cells are equivalent and both reduced as compared to the untreated MPC 11 cells. The levels of MPC11 H (γ2b) and L(kappa 19 and 21) chain expression were unchanged. Calreticulin (CalR) housekeeping gene was used for normalization of mRNA levels. EL-4 T cells were used for comparison. B. OBF-1 mRNA and protein levels were assessed in OBF-1 shRNA-treated MPC11 cells by RT-PCR (by three-fold dilutions) and Western blot, respectively. TFIID was used for protein normalization. C. 3C interactions were measured in EL-4 T cells, MPC11 cells, and GFP or Oct-2/OBF-1 shRNA infected MPC11 cells. No differences in interactions were observed with the KDs.
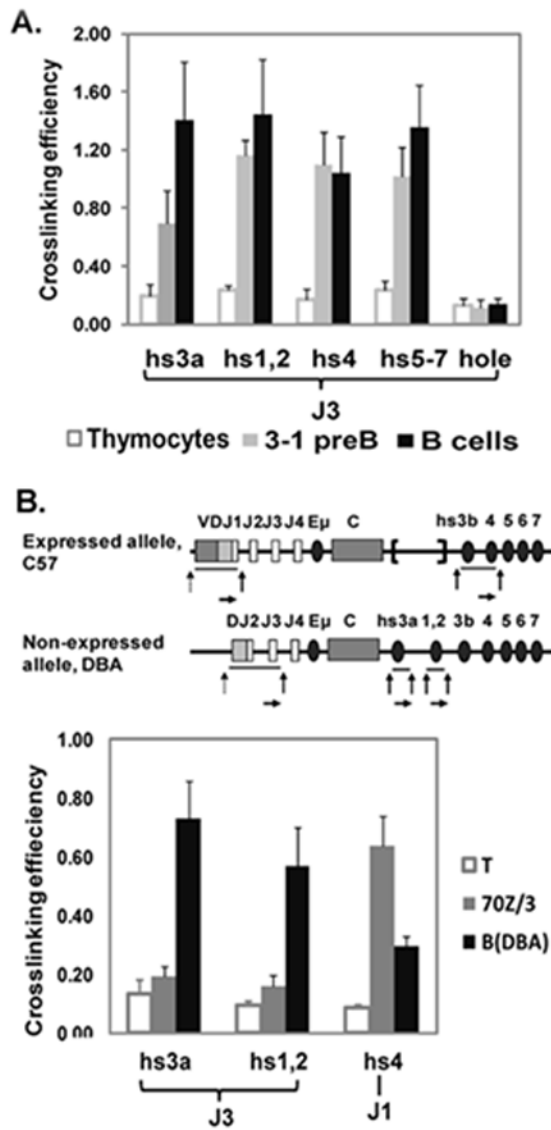
Fig. 3. Analysis of 3C interactions in pre-B cell lines
A. J_H3-3'RR interactions were analyzed in thymocytes, 3-1 pre-B cells, and splenic B cells. *Hole*, the nearest non-Ig gene downstream of the 3' RR, was used as a negative control. B. A schematic description of DJ/VDJ rearrangements on expressed (C57BL/6) and non-expressed (DBA) IgH alleles in 70Z/3 pre-B cell line. The J1 and hs4 primers were paired to detect 3C interaction only on the expressed allele. J3 and hs3a/hs1,2 were paired to detect 3C interactions only on the non-expressed allele. 3C interactions were measured in T cells from DBA mice (negative control), 70Z/3 pre-B cells, and DBA splenic B cells (positive control). Specific interactions were observed on the expressed allele of 70Z/3 pre-B cells.
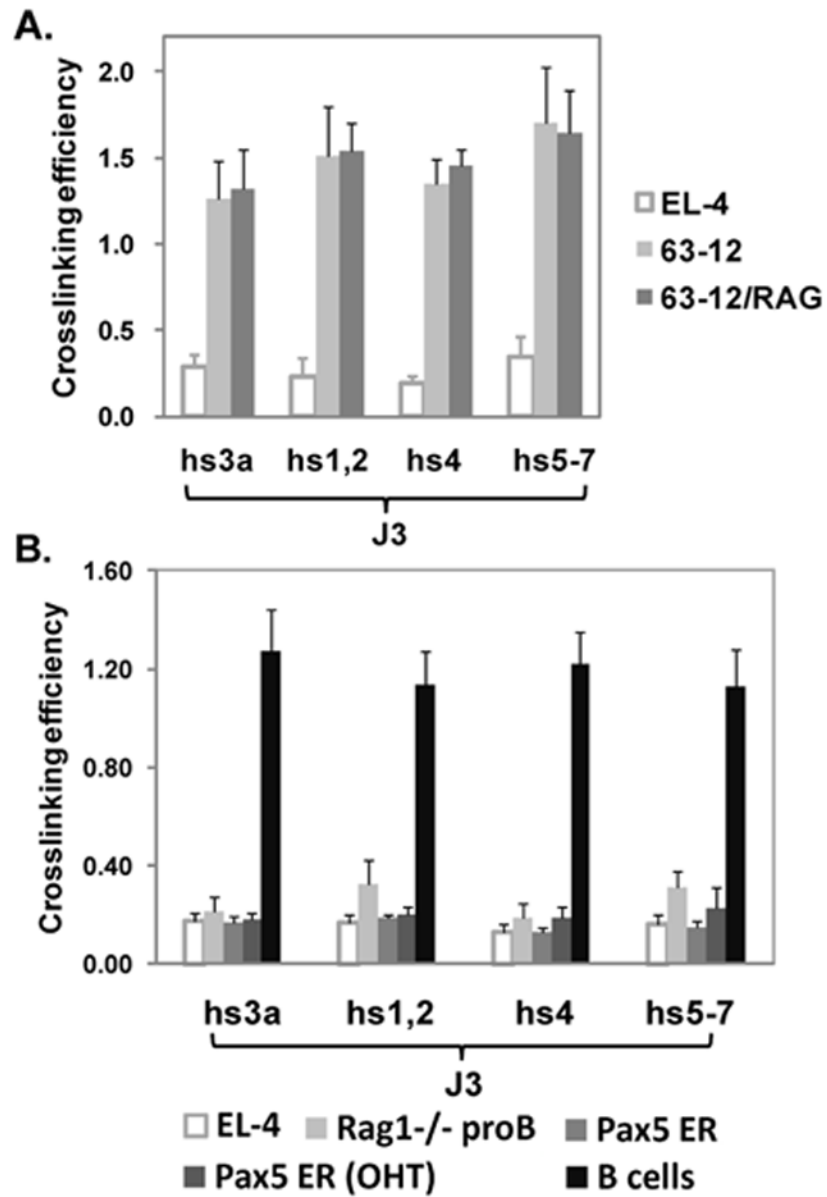
Fig. 4. Analysis of J_H-3' RR interactions involving the 3' RR in pro-B cells
A. 3C interactions in 63-12 RAG2 $^{-/-}$ pro-B cell line, and in 63-12 cells reconstituted with RAG2 protein. B. J_H3-3'RR interactions measured in EL-4 T cells, RAG1$^{-/-}$ pro-B cells, Pax5 ER (B1.1 cells), Pax5 ER (OHT), and B cells. Interactions were observed in B cells.

Table I

Primer sequences

RT-PCR primers:

CalR F: 5' AAGCCAAGATGCCCGATTTTAC 3'

CalR R: 5' CCTTTATCTTCTTGGGTGGCAG 3'

Oct-2 F: 5' ATTGTCCCAAGCTTCTAGCAGTCT 3'

Oct-2 R: 5' GTTCAAGAAGAGAGGCGAGGTTAC 3'

OBF-1F: 5' GAAAGCACGCCCAGTCACATT 3'

OBF-1R: 5' GCTCCTTGACTCGAACACCCT 3'

Oct-1F: 5' AACGCCAGTCTTGCTGCTATG 3'

OCT-1R: 5' AGGGTTCAGGAACAGAGGTGC 3'

J558 F: 5' CACTGTGCAAGA GGGATTTACTAC 3'

γ2b R: 5' CATCCTCGCTCACATCCACC 3'

Igκ V19-17: 5' GGATTTCACTTTCACCATCAGCA 3'

Igκ V21-5: 5' AGGTTCAGTGGCAGTGGGTCT 3'

Igκ CR: 5' TAGAAGGGAAGATAGGATGGAGC 3'

3C primers:

J1: 5' TTCTTTCTGCACATTCCATTCTGA 3'

J3 : 5' AGATTCAACCCCT TTGTCCCA 3'

hs3a : 5' AGTGTTAGGTGTTGTCCTCGTCAT 3'

hs1,2: 5' GAGAACAAGTA AGAAAGCCCAGC 3'

hs4 : 5' ACCCAACCTGTGTCCCTAGAG 3'

hs7: 5' CTCCACA ATGACCACAG CGTAG 3'

hole: 5' TCACTCTCCCTGCTGATAGACAAC 3'

CalRA: 5' GTGTAGTAGGTTGAAGAATGGGCA 3'

CalRB: 5' CCCAAACCACCACTACCATTACA 3'