Dynamic Changes in Binding of Immunoglobulin Heavy Chain 3’ Regulatory Region to Protein Factors during Class Switching

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The 3’ regulatory region (3’ RR) of the Igh locus works at long distances on variable region (Vδ) and switch region (I) region promoters to initiate germ line (non-coding) transcription (GT) and promote class switch recombination (CSR). The 3’ RR contains multiple elements, including enhancers (hs3a, hs1.2, hs3b, and hs4) and a proposed insulator region containing CTCF (CCCTC-binding factor) binding sites, i.e. hs5/6/7 and the downstream region (“3”). Notably, deletion of each individual enhancer (hs3a-hs4) has no significant phenotypic consequence, suggesting that the 3’ RR has considerable structural flexibility in its function. To better understand how the 3’ RR functions, we identified transcription factor binding sites and used chromatin immunoprecipitation (ChIP) assays to monitor their occupancy in splenic B cells that initiate GT and undergo CSR (LPS±IL4), are deficient in GT and CSR (p50−/−), or do not undergo CSR despite efficient GT (anti-IgM+IL4). Like 3’ RR enhancers, hs5–7 and the 38 region were observed to contain multiple Pax5 binding sites (in addition to multiple CTCF sites). We found that the Pax5 binding profile to the 3’ RR dynamically changed during CSR independent of the specific isotype to which switching was induced, and binding focused on hs1.2, hs4, and hs7. CTCF-associated and CTCF-independent cohesin interactions were also identified. Our observations are consistent with a scaffold model in which a platform of active protein complexes capable of facilitating GT and CSR can be formed by varying constellations of 3’ RR elements.

The immunoglobulin heavy chain (Igh)3 locus is subject to multiple processes that markedly increase the number of different antibody gene coding sequences (reviewed in Ref. 1). These include the random DNA recombination of V (variable), D (diversity) and J (joining) sequences to form multiple variable region coding segments and class switch recombination (CSR) that juxtaposes assembled variable region genes to different constant region (C4) genes to provide distinct biological functions associated with specific antigen-binding specificities. In addition, somatic hypermutation of variable region genes augments genetic diversity. These processes are regulated by cis-acting elements, including locally acting promoters, and by long distance regulators, such as the intronic enhancer (Eμ) and the 3’ regulatory region (3’ RR). Knockout mice models identified Eμ as a major regulator of VDJ recombination (2–5) and showed that the 3’ RR influenced germ line (non-coding) transcription (GT) of individual C4 genes (6–11). GT is initiated from a promoter upstream of each C4 gene and extends through switch and C4 sequences, generating a single-stranded DNA substrate for activation-induced cytidine deaminase (AID), a protein critical for CSR (12). Long-range physical interactions between an element of the 3’ RR and I region promoters that drive GT have been shown in cells stimulated by LPS to undergo CSR by use of a chromosome conformation capture (3C) assay (13). Studies have also implicated the 3’ RR in the switch recombination process itself and in lgh expression at distances up to ~200 kb at later stages of B cell development (6, 11). A contribution of the 3’ RR to somatic hypermutation has been reported additionally (10). Here, we address mechanisms that enable the 3’ RR to function at long distances during GT and CSR.

In contrast to the ~1 kb Eμ, the 3’ RR is substantially longer and more complex (see schematic, Fig. 1A). It extends ~40 kb and contains multiple DNase I hypersensitive (hs) sites indicative of regulatory elements, some of which correspond to four enhancers (hs3a, hs1.2, hs3b, and hs4) (reviewed in Refs. 14, 15). Mice with targeted deletions of each of the individual 3’ RR enhancers (hs3a, hs1.2, hs3b, or hs4) were indistinguishable in CSR from wild-type controls (8, 16–19). In contrast, a combined deletion of all four 3’ RR enhancers eliminated CSR to all isotypes (11), whereas a smaller deletion of hs3b and hs4 affected CSR to all isotypes except for IgG1 (7). GT of individual C4 genes was affected in each of these combined deletions, indicating a role for the 3’ RR in this activity. The contrasting results from individual or combined deletions imply that individual steps in GT and CSR can be carried out by different constellations of 3’ RR hs sites. Hence, there must be consider-
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able structural and functional flexibility in the use of 3′ RR enhancers. Nonetheless, to our surprise, no significant changes in 3′ RR histone modifications during switching have been reported (20, 21).

Additional elements of the 3′ RR located downstream of the hs4 enhancer, indicated by hs sites 5–7 (20), have been predicted to mark a terminus of B cell-specific regulation of the Igh locus and potentially to insulate and protect downstream (and other) genes from the DNA recombination and mutation events of the Igh locus (20). In accord with the observation that CREC binds to all mammalian insulators (22), we found that hs5–7 contains a number of CREC binding sites, segments of which displayed insulator but not enhancer activity (20). A region 4 kb further downstream of hs7, termed “38” (23), also contained CREC binding sites based on a ChiP assay (20). Although the role of the CREC-binding region in the context of the endogenous 3′ RR has not yet been determined, a 3C assay has shown that all 3′ elements, i.e. those associated with the enhancers and with the predicted insulators, form loops with each other and with Eμ in a plasma cell line and in resting B cells (13, 23). The interaction of the 3′ RR with the promoters (I region) that drive GT through switch regions in cells stimulated by LPS (13, 23) was reported but used only hs1.2 as “bait.” Mutations of 3′ RR elements that resulted in loss of Igh expression in plasma cells or loss of CSR in mice were associated with deficiencies in loop formation (13, 23), implicating loop interactions as critical for 3′ RR activity.

Previous analysis of transcriptional regulation by reporter assays showed that 3′ RR enhancers hs1.2 and hs4 are regulated by a common set of factors, including Pax5, octamer-binding proteins, a G-rich binding protein, and Nkx2, each of which can serve as an activator or repressor of enhancer activity (24–26), in part depending on the cell line and the contribution of other cofactors (25). In other experiments, Pax5 was found to maintain B cell identity throughout B cell development by activating B cell-specific genes (27) and repressing non-B cell genes (28).

In this report, we used ChiP analysis to reveal the dynamic occupancy of various protein binding sites at 3′ RR enhancers and in the CREC-binding region during GT and CSR promoted by LPS+IL4 stimulation. We compared these findings to cells stimulated by anti-IgM+IL4 that are deficient in CSR and to p50−/− cells that are deficient in GT and switching to multiple isotypes. Our observations have suggested that the entire 3′ RR is “poised” in resting B cells to drive GT and CSR upon appropriate stimulation. We propose here that a set of shared binding sites, including Pax5, contributes to a scaffold structure that is essential to successive steps in CSR and accommodates deletions of individual enhancers.

EXPERIMENTAL PROCEDURES

Cell Preparation and Culture Conditions—Splenic B cells were isolated from spleens of 6- to 8-week-old wild-type or p50−/− C57BL/6 mice (The Jackson Laboratory) as described previously (23). Briefly, activated B and non-B cells were depleted from total splenocytes by using anti-CD43 magnetic microbeads (Miltenyi Biotec). The resulting population of naive B cells was > 95% B220+ as determined by FACS analysis. Resting wild-type B cells were stimulated with 50 µg/ml of LPS (Calbiochem) with or without 25 ng/ml of IL4 at 106 cells/ml concentration. In some experiments, B cells were stimulated with 10 µg/ml of anti-IgM (goat anti-mouse IgM Fab’2 (Jackson ImmunoResearch Laboratories, Inc.)) plus IL4. p50−/− cells, and wild-type control B cells were stimulated with 50 µg/ml LPS + 3 ng/ml of anti-δ dextran (Fina Biolabs), conditions similar to those used to stimulate CSR in normal B cells (21). Splenic T cells (95% CD3ε+) were isolated using the Pan T cell isolation kit (Miltenyi Biotec).

Assay of GT and CSR—To monitor GT, TRIZol was used to isolate RNA from cells stimulated for 48 h. This was followed by chloroform extraction and isopropanol precipitation. The pellet was washed with 70% ethanol, dried, and resuspended in RNase-free distilled water. cDNA was synthesized with SuperScript III First-Strand (Invitrogen). DNA was quantitated by real-time PCR in the Einstein real-time facility using Power SYBR Green master mix (Applied Biosystems, Inc., catalog no. 4367659). Triplicate samples of 1:10 dilution were normalized to the calretulin housekeeping gene using the 2(ΔΔC(T)) method as described (29). FACS analysis on cells stimulated for 96 h was carried out with goat anti-mouse IgM-Cy5 (Jackson ImmunoResearch Laboratories, Inc.), goat anti-mouse IgG1-FITC (Southern Biotech), or goat anti-mouse IgG3-FITC (Southern Biotech), using FACSScan. This study received institutional review board approval for mouse studies.

Electrophoretic Mobility Shift Assay—Probes were generated by PCR (primer sequences are available upon request), restriction digestion, or oligonucleotide annealing and labeled with [γ-32P]ATP in the presence of T4 polynucleotide kinase (Roche). Nuclear extracts were made from 63–12 pro-B, A2B, and MPC11 plasma cell lines, as described previously (30). Purified probes were incubated with 10 µg of nuclear extracts in the presence of poly(dI-dC) at 37 °C for 1 h. For supershift analysis, antibody was added to the reaction mix before addition of any probe and incubated for 20 min at room temperature. The reaction mixture was then subjected to electrophoresis in a 5% acrylamide gel, dried in a gel drier (Hoefer Scientific Instruments), and exposed to Kodak Biomax MS film at –20 °C overnight.

Cloning of Hs7 and 38 in pGL2Vh and Luciferase Assay—The hs7 region (32.6–34.5 kb of BAC199M11, GenBank™ accession number AF450245) and 38 region (37.48–39.8 kb of BAC199M11, GenBank™ accession number AF450245) were PCR-amplified from the BAC and cloned using a TOPO TA cloning kit (Invitrogen) according to the manufacturer’s protocol. Clones were then digested with SmaI, and a 1.9-kb hs7 fragment and a 2.3-kb 38 fragment were ligated with SmaI-digested pGL2-Vh vector (vector provided by Laurel Eckhardt, Hunter College) at a position upstream of the VH gene. The clones were initially checked for orientation by restriction digestion and further confirmed by sequencing. Test B cell lines were electroporated with pGL2Vh-hs7F/R or with pGL2Vh-
38kbF/R together with the pGL2Vh plasmid and a Renilla luciferase construct. After 2 days, cells were lysed in 100 μl Tropix lysis buffer, and the lysates were measured for firefly luciferase and Renilla luciferase activity according to the manufacturer’s protocol (Promega dual luciferase reporter assay system). Positive controls included QM293, a vector containing E/H9262 and a luciferase reporter (31) and pGL3Vh-hs4 (32).

**ChIP**—ChIP assays were performed and quantitated as described previously (20). Briefly, 5 × 10^7 to 10^8 cells were fixed in 40 ml of RPMI medium for 10 min at room temperature by addition of 4 ml of fixation solution (11% formaldehyde, 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES (pH 8.0)). The fixation reaction was stopped with 0.125 M glycine, and cells were washed twice with cold PBS. Cells were lysed on ice for 10 min with lysis buffer 1 (50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 0.25% Triton X-100), and the cell pellet was then resuspended in lysis buffer 2 (1 mM EDTA, 0.5 mM EGTA, 10 mM Tris HCl, and 200 mM NaCl) at room temperature for 10 min. Sonication of nuclei from 4 × 10^7 cells/ml in lysis buffer 3 (0.06% SDS, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris-HCl (pH 8.0)) was done by Sonic Dismembrator Model 500 (Fisher Scientific) at 44% amplitude at 4 °C. 200- to 1000-bp fragments were generated by 16 min sonication in a dry ice–ethanol bath. Precleared chromatin from ~4 × 10^7 cells was taken for each pull-down. ChIPs were done with 4 μg of antibodies to Pax5 (custom polyclonal rabbit anti-Pax5 antibody (33)), CTCF (Upstate, 07–729), and Rad21 (Abcam, ab992). 4 μg of rabbit IgG (sc-2027) was used as a negative control. 30 μl of salmon sperm protein A+G agarose beads (Upstate) were used to immunoprecipitate the antibody–chromatin complex. After this complex was washed for 5 min four times at 4 °C, bound chromatin was eluted by 1% SDS in TE buffer.
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(10 mM Tris-HCl, 1 mM EDTA, final pH = 8.0) buffer at 37 °C, and protein-DNA cross-links were reversed overnight with NaCl at 65 °C. Chromatin was then digested with proteinase-K, and DNA was extracted with a Qiagen PCR purification kit (catalog no. 28104). DNA was diluted to a concentration of 4.95 ng (3000 copies)/ml, and a standard curve of C_T versus 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 described previously (20). For assay of ChIP, a 2% dilution of chromatin was used as input, and each pull-down and corresponding input were analyzed in triplicate and calculated for copy number. The results were plotted on the y axis as copy numbers of pull-down (PD)/copy numbers in the input (PD/input). ChIP experiments were normalized by comparison to a positive control whenever possible.

3C—The 3C assay was done with resting or 48-h stimulated splenic B cells as described previously (23). Briefly, 1 × 10⁷ cells were fixed in 2% formaldehyde solution for 10 min at room temperature and then lysed with lysis buffer. Nuclei from which non-cross-linked protein was removed by SDS, with SDS then sequestered by Triton X-100, were digested overnight at 37 °C with 1500 units of HindIII (New England Biolabs) with gentle shaking. The digestion was terminated in the presence of SDS at 65 °C. Digested nuclei (10⁶) were ligated with 400 units of New England Biolabs T4 DNA ligase for 4 h at 16 °C, and cross-links were reversed. Samples were then treated with proteinase K and RNase A, and DNA was purified by phenol-chloroform extraction and isopropanol precipitation. Ligated DNA samples were analyzed by 32 cycles of PCR amplification (94 °C/30 s, 55 °C/30 s, and 72 °C/30 s) with 100 ng of DNA in a 25-μl reaction system. Primers for the 3C assay (23) are in supplemental Table 1. A control template to normalize for PCR efficiency was formed by mixing BACs that contain sequences of interest with two calreticulin (CalR) fragments (separated by an intermediate fragment) in equal molar ratios, after which these were subjected to HindIII digestion and, subsequently, DNA ligation. PCR products were separated by electrophoresis on a 2% agarose gel. Gel pictures were taken with Gene Snap software and analyzed by Gene Tools software (SYNGENE GENE Genius BioImaging system). SLIG and SCaR are the band intensity signals (S) between two HindIII fragments in the Igh locus, and two HindIII fragments in the CalR locus, respectively. Cross-linking efficiency was defined as (S_IgH/SCaR) in the selected cell type/(S_LigH/SCaR) in the control template.

RESULTS

Hs7 and 38 Contain Multiple Pax5 and CTCF Binding Sites—Evaluation of the 3’ RR for its biological activity has predominantly focused on four enhancers: hs3a, hs1.2, hs3b, and hs4. Additional hs sites were identified downstream of hs4 as candidates for a functional terminus of the lgh locus and predicted to contain binding sites for the insulator-associated protein CTCF (Fig. 1A). In fact, EMSA experiments of >50 overlapping DNA fragments in an ~12-kb region beginning at hs4 and extending downstream identified multiple sites that bound to recombinant CTCF (20). To determine whether there were other, non-CTCF, sites in this region, we carried out EMSA with the same set of DNA fragments described above using nuclear extracts from B cell lines of different developmental stages. We also analyzed the 38 region, which was shown by ChIP to bind to CTCF (20). In addition to the band corresponding to CTCF, an additional band was identified by EMSA when DNA fragments from hs7 and the 38 region were incubated with nuclear extracts from pro-B, pre-B, and B cell lines but not myeloma cell lines. This band was predicted to reflect Pax5 binding because, like the EMSA pattern, Pax5 is also expressed in pro-B, pre-B, and B cells but not in plasma cells. Five overlapping probes (probes 24–28) of the hs7 region were cloned in both forward (hs7F) and reverse (hs7R) orientations upstream of a Vh promoter in a pGL2 vector. B, luciferase reporter assay was carried out in pro-B (63–12), B (A20), and plasma (S194) cell lines for hs7 and C, in B (A20) and plasma (S194) cell lines for the 38 region. The activity of the luciferase plasmid was normalized with Renilla. QM293, a luciferase reporter vector containing Eμ, served as a positive control in each reporter assay, with increase in enhancer activity compared with the empty vector of ~4–16-fold (data not plotted here). In some assays, pGL3Vh-hs4 was also used as a positive control.

FIGURE 2. Hs7 and 38 have repressor activity in B cells. A, hs7 and 38 were cloned in both forward (hs7F) and reverse (hs7R) orientations upstream of a Vh promoter in a pGL2 vector. B, luciferase reporter assay was carried out in pro-B (63–12), B (A20), and plasma (S194) cell lines for hs7 and C, in B (A20) and plasma (S194) cell lines for the 38 region. The activity of the luciferase plasmid was normalized with Renilla. QM293, a luciferase reporter vector containing Eμ, served as a positive control in each reporter assay, with increase in enhancer activity compared with the empty vector of ~4–16-fold (data not plotted here). In some assays, pGL3Vh-hs4 was also used as a positive control.
that was included in each reporter assay (4–16× increase in activity), hs7 showed no enhancer activity (Fig. 2B). Instead, both hs7F and hs7R orientations showed repression of luciferase activity in pro-B and B cells (both Pax5+) but not in plasma cells (Pax5-), where the hs7 region showed no effect compared with the PGL2Vh vector (Fig. 2B). The 38 region cloned in the pGL2-Vh vector showed repressor activity in both the Pax5+ A20 B cell line and Pax5- S194 cell line (Fig. 2C). Hence, both hs7 and the 38 region have the potential to serve as repressors throughout B cell differentiation until the plasma cell stage. However, hs7 had repressor activity only in Pax5+ cell lines, whereas 38 showed repressor activity in plasma cells as well, suggesting that hs7 and the 38 region have different in vivo functions.

**Pax5 Binding to the 3’ RR Is Regulated during CSR**—The identification of Pax5 sites in all 3’ RR elements prompted our studies on the interaction of Pax5 with the 3’ RR during CSR. ChIP analysis of the 3’ RR in normal splenic B cells stimulated to undergo CSR revealed dynamic changes in Pax5 binding throughout this process (Fig. 3). Interestingly, although all 3’ RR enhancers and the hs5–7/38 region have Pax5 binding sites, Pax5 binds predominantly to hs4 in resting B cells (Fig. 3, A and B). This binding was reduced after 48 h of LPS stimulation when GT levels are high and was then restored at 96 h when CSR has mainly occurred (Fig. 3A). Concomitant with reduction in Pax5 binding to hs4 48 h after LPS stimulation, ChIP analysis detected acquisition of or increases in Pax5 binding to the hs1.2 enhancer and to hs7 (Fig. 3A). Relative to Pax5 binding to the promoter of its CD19 target gene (34), changes in Pax5 binding to the 3’ RR appear to be specific for this regulatory region. Levels of Pax5 protein were reduced during CSR (supplemental Fig. 1A), as similarly shown by others in CH12 B cells (35), but Pax5 levels showed no direct association with alterations in 3’ RR binding profiles and hence could not account for these changes.

Because different isotypes result from stimulation with LPS (IgG3 and IgG2b) or LPS + IL4 (IgG1 and IgE), we also analyzed Pax5 binding to the 3’ RR in cells stimulated with LPS + IL4 (supplemental Fig. 1B). Similar to LPS stimulation, we found comparable changes in Pax5 binding to hs4 under LPS + IL4 stimulation conditions. These results show that the Pax5 binding profile to the 3’ RR dynamically changes during CSR but is independent of the specific isotype to which switching is induced.

**The Pax5 Binding Profile Is Changed When CSR Is Blocked**—To identify whether Pax5 binding profiles were associated with GT or CSR, we compared ChIP profiles of selected proteins in wild-type mice with cells deficient in steps in CSR. B cells stimulated with anti-IgM + IL4, like B cells stimulated with LPS + IL4, proliferate extensively and initiate GT (supplemental Fig. 1C). Nonetheless, cells stimulated by anti-IgM + IL4 alone or even together with LPS

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**Figure 3. Pax5 binding to 3’ RR in B cells is associated with CSR.** ChIP of 3’ RR of splenic B cells stimulated by LPS, which carry out GT and CSR (A), and anti-IgM + IL4, which carry out GT but not CSR (supplemental Fig. 1) (B). The black bars are Pax5 binding at 3’ RR, and the gray bars are IgG controls. The dashed lines represent the highest signal from IgG control, and any signal below that line is considered background. A ChIP analysis was done in unstimulated B cells (top panel) and after 48 h (center panel) or 96 h (bottom panel) of stimulation. Quantitation was carried out as described under “Experimental Procedures.” The CD19 promoter region, a known target of Pax5, was used as a positive control (clear bar) for Pax5 binding. PD/input for CD19 was considered as 100. (CD19 signal as calculated by real-time PCR analyses from LPS-stimulated Pax5 ChIP: 0 h = 0.02, 48 h = 0.03, 96 h = 0.13; from anti-IgM + IL4 stimulated cells: 0 h = 0.025, 48 h = 0.04, 96 h = 0.008). The level of Pax5 binding to the CD19 promoter at 96 h was reduced in cells stimulated with anti-IgM + IL4 as compared with LPS, in accord with reduced levels of Pax5 expression. Hs4 is highlighted to demarcate the palindromic enhancers (hs3a-hs3b) from CTCF binding sites (hs5/6/7 and 38-kb region).
either fail to undergo CSR or have seriously reduced CSR levels (36, 37) (supplemental Fig. 1D). We reasoned that ChIP analysis of the 3' RR upon anti-IgM + IL4 stimulation might reveal those protein binding patterns specifically involved in events that take place after GT. Using this approach, we found that similar to cells that both initiate GT and undergo CSR after stimulation by LPS±IL4, Pax5 binding shifted from hs4 to hs1.2 and hs7 48 h after stimulation by anti-IgM + IL4 (Fig. 3B) (or LPS + anti-IgM + IL4, data not shown). However, Pax5 binding to hs4 was not restored at 96 h in cells stimulated with anti-IgM + IL4 (Fig. 3B). This reduction in Pax5 binding to hs4 was not due to the decrease in Pax5 expression seen at 96 h because a similar decrease was seen in cells stimulated by LPS for 96 h (where Pax5 binding to hs4 was restored) (supplemental Fig. 1A). Importantly, our analysis was done by comparison to CD19 as a Pax5 target gene (38, 39). Hence, the lack of or severe reduction in Pax5 binding to hs4 at 96 h was correlated with the inability to achieve CSR subsequent to GT.

**Alteration of Pax5 Binding to the 3' RR in p50−/− Splenic B Cells**—Mice deficient in the p50 component of NFκB, i.e. p50−/− mice, show a deficiency in CSR to the isotypes measured (γ3, γ1, ε, and α) except IgG1 (40). Except for IgA, for which GT is detected, this deficiency in CSR to γ3 and ε is associated with a reduction in isotype-specific GT (40). These observations were the foundation for previous experiments that showed that NFκB, and especially p50-containing complexes, bound to 3' RR enhancers and contributed to their transcriptional regulation (25). We therefore predicted that p50−/− mice would provide a model system for analysis of transcriptional factor association with the 3' RR in the absence of normal GT. Splenic B cells from p50−/− mice do not respond to LPS stimulation alone, but they do proliferate in response to anti-δ dextran and even more vigorously to anti-δ dextran + LPS (40). Wild-type B cells stimulated with anti-δ dextran + LPS produce GT and undergo CSR to γ3 and γ2b (21), and ChIP analysis (Fig. 4) showed the same reduction in Pax5 binding to hs4 as found 48 h after stimulation with LPS±IL4. However, p50−/− splenic B cells stimulated for 48 h with anti-δ dextran + LPS maintained Pax5 binding to hs4. This suggests that for the 3' RR to support normal GT, Pax5 must preferentially shift binding away from hs4 while maintaining binding to CD19, a process that may directly or indirectly require NFκB.

**CTCF-associated and CTCF-independent Cohesin Binding during CSR**—Further analysis of protein interactions involving the hs5–7 and 38 region and other 3' RR elements naturally focused on occupancy of CTCF binding sites. As reported in resting and 48 h-stimulated cells (20), and here extended to 96 h after LPS stimulation (Fig. 5A), the binding of CTCF predominantly to hs5–7 remained mostly unchanged during this entire stimulation period. Similar CTCF binding occurs in cells stimulated with anti-IgM + IL4 (supplemental Fig. 2A). At 48 h post-stimulation, when GT is occurring and Pax5 moves away from hs4, both Pax5 and CTCF bind to hs7, but for the most part, Pax5 and CTCF binding are independent (compare Figs. 3A, 5A and supplemental Fig. 2A).

CTCF has been shown to recruit cohesin to ~70% of its genome-wide binding sites (41, 42), and, accordingly, the cohesin subunit Rad21 bound preferentially to the CTCF-binding hs7 region throughout stimulation with LPS (Fig. 5B) or anti-IgM + IL4 (supplemental Fig. 3B). However, Rad21 also bound at hs1.2, independently of CTCF, in resting B cells, and after stimulation by LPS (Fig. 5B) or with anti-IgM + IL4 (supplemental Fig. 2). At this same 48-h time point, Pax5 binding to hs1.2 is also detected.

**Interactions Involving the 3' RR, Eμ, and Switch Regions during Switch Simulation**—The shift in binding of Pax5 from hs4 (0 h) to hs1.2/hs7 (48 h) and back to hs4 (96 h) in cells that undergo GT and CSR might be facilitated if these elements were in physical proximity. In fact, 3C analysis identified interaction among the various 3' hs sites in resting B cells and in a plasma cell line (23). To determine whether interactions among 3' RR elements were sustained during CSR, we carried out a 3C assay on B cells stimulated by LPS±IL4. As contrasted to background interactions in splenic T cells (23), we found strong interaction among the 3' regulators, including hs7, in resting B cells and maintenance of these interactions upon stimulation (Fig. 6A), potentially providing a stable platform for their work together during CSR. As reported previously (13, 23), these interactions extended in resting B cells to Eμ (Fig. 6B) and to the specific I region promoters that drive GT (Fig. 6C). Modest interactions between hs3a and hole (Tmem121), i.e. the nearest downstream non-Igh gene (Fig. 6A), compared with the levels of interactions between hs3a and Eμ (Fig. 6B), Iγ3 and Iγ1 (Fig. 6C) show the specificity of particular interactions involving 3' RR elements. Compared with the relatively unchanged interactions among 3' RR elements upon stimulation of GT, 3' RR-Eμ
interactions are somewhat enhanced, whereas specific 3’ RR-I region interactions, e.g. to I/H9253 in response to LPS and to I/H92531 in response to LPS/IL4, are substantially increased (Fig. 6, B and C).

DISCUSSION
As described in the introduction, the 3’ RR is essential both for initiation of expression of GT of Igh switch sequences that is required for subsequent CSR and for the expression of post-switch IgH transcripts and secretion of immunoglobulin of all isotypes (11). Although it is known that there is physical interaction through looping between the 3’ RR and I regions that promote GT, there is little to no phenotypic impact on CSR of individual KO’s of any of the 3’ enhancers, implying compensatory flexibility throughout the 3’ RR structure. Here we report that dynamic changes in binding of various proteins to the 3’ RR accompany successive steps in CSR (summarized in Fig. 7).

We have identified Pax5, CTCF and cohesin (Rad21) as key binding proteins of the 3’ RR. Importantly, we have shown that the hs7 and 38 regions contain substantial numbers of CTCF and Pax5 binding sites. The existence of at least two independent Pax5 sites and at least two independent CTCF sites in the probe 28 fragment of hs7 raises the possibility that other fragments in this region similarly have multiple Pax5 and CTCF sites. Collectively, these observations predict that the hs4 downstream region has a high density of CTCF and Pax5 sites. On the basis of their content of CTCF sites, the hs5–7 and 38 regions are predicted to be insulators of the Igh locus, isolating it from non-Igh genes located downstream or elsewhere. Repressor and insulator activity analyzed by reporter assays of hs7 and 38 (Ref. 20 and this study) are in accord with this scenario, which might have an impact on downstream genes. In addition, the presence of Pax5 sites in these “insulator” segments identifies a functional protein binding site shared with “enhancer” hs sites (3a-4) and predicts that this insulator region (hs5–7 and 38) may work together with upstream enhancer sequences as part of an active structure.

In 3’ RR enhancer sequences located upstream of the CTCF binding region, i.e. hs3a-hs4, we have discovered protein-binding patterns that shift in concert with the different phases of class switch recombination. We found that Pax5 is bound to hs4 in resting B cells, is absent from hs4 at 48 h when GT are being synthesized in response to stimulation with LPS, and again is bound to hs4 at 96 h when CSR is essentially complete. In the absence of p50, Pax5 does not leave hs4 at 48 h, and neither GT nor CSR to specific isotypes takes place normally. Upon anti-IgM + IL-4 stimulation, the occupancy of Pax5 does shift from hs4 to hs1.2 at 48 h, and GT is normal, but the binding of Pax5 to hs4 does not return at 96 h, and CSR itself is blocked. Together, these findings suggest that not only does Pax5 play an active role in the two phases of CSR (GT and DNA recombination), but also the site at which it binds in the 3’ RR is important.

FIGURE 5. CTCF and cohesin (Rad21) binding at 3’ RR in LPS-stimulated cells. CTCF (A) and Rad21 (B) ChIP assays were done at three different time points during LPS stimulation. From top to bottom, shown are unstimulated B cells (top panel), 48 h (center panel), and 96 h (bottom panel) after stimulation. Myc was used as a positive control (clear bar) for CTCF. PD/input for myc was considered as 100. A segment of chromosome 9 (Chr9) was used as a positive control (clear bar) for Rad21 ChIP. PD/input for Chr9 was considered as 100. The dashed lines represent the highest signal from IgG control, and any signal below that line is considered background. The black bars are CTCF or Rad21 binding at 3’ RR, and the gray bars are IgG control. (PD/input for Myc in LPS stimulated CTCF ChIP: 0 h = 0.09, 48 h = 0.022, 96 h = 0.08. PD/input for Chr9 in LPS stimulated Rad21 ChIPs: 0 h = 0.017, 48 h = 0.017, 96 h = 0.016). Hs4 is highlighted to demarcate the palindromic enhancers (hs3a-hs3b) from CTCF binding sites (hs5/6/7 and 38-kb region).
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Figure 6. Physical interaction between individual 3’ RR segments and between these 3’ RR elements and either Eμ or I-switch regions in resting cells (clear bars) and stimulated B cells (48 h LPS, gray bars or LPS+IL4, black bars). A 3C assay was done with hs3a, Eμ, Iγ3, or Iγ1 as anchor primers for interaction with hs3a, hs1.2, hs4, or hs7. Interaction of hs3a with the nearest non-Igh gene, hole (Tmem121), shows background levels of interactions. This experiment shows that interactions among 3’ RR elements that occur in resting B cells are maintained at 48 h after stimulation with LPS (A). Interactions of 3’ RR elements with Eμ are modestly enhanced upon stimulation for GT and CSR (B), and interactions of 3’ RR elements with I-switch regions are markedly enhanced when GT and CSR are stimulated (C). The graph plots the results from two independent experiments.

Figure 7. Summary of protein binding to hs1.2, hs4, and hs7 during GT and CSR. Pax5 binding to 3’ RR differs in cells stimulated with LPS (left panel) or anti-IgM+IL4 (GT but no CSR) (center panel) and also in p50−/− cells (deficiencies in GT and CSR) (right panel). CTCF binds preferentially to hs7 throughout stimulation. Rad21 binding is similar in cells stimulated with LPS or with anti-IgM+IL4 and was not assessed in p50−/− cells.

shown that Pax5 and NFκB contribute to concerted repression of hs1.2 and activation of hs4. From these studies, we infer that NFκB and Pax5 are transcriptional regulators of the 3’ RR in the context of the Igh locus.

Prior studies showed reduced serum IgG levels in mice with a B cell-specific KO of Pax5 (43). It was suggested that this was due to reduced proliferation in response to LPS, but the findings presented here and others published recently (21, 44) prompt reconsideration of that explanation. These studies have reported Pax5 binding to hs4 in resting B cells. In addition, they have shown that PTIP (Pax-interaction with transcription-activation domain protein-1), a Pax-interacting protein, engages with switch sequences as an essential part of CSR and is instrumental in the role of Pax5 in promoting looping between the 3’ RR and I regions. To what degree these looping mechanisms facilitate AID recruitment to both the switch regions and the 3’ RR (45) is not yet known.

Our studies show that Pax5 binding to the 3’ RR may be regulated, directly or indirectly, by p50-containing NFκB complexes, as Pax5 binding to hs4 is retained at 48 h after stimulation of p50−/− B cells as compared with wild-type cells. It is noteworthy that p50−/− cells are deficient in normal GT and in CSR (46), suggesting that the shift in Pax5 binding away from hs4 and back again are requirements for normal GT and CSR. It is of interest that the NFκB and Pax5 binding sites are located 20 bp apart in hs4 (25), raising the possibility that engagement of one of these factors might affect occupancy of the other.

Interestingly, in addition to a relatively unchanged pattern of binding of CTCF and the cohesin subunit Rad21 to the predominant hs7 CTCF-binding site, Rad21 binds to hs1.2 in an apparently CTCF-independent manner. Recent studies have shown alterations in binding of Pax5 and cohesin as they affect stage- and cell type-specific regulation of the VH genes (47, 48) and other examples of CTCF-independent cohesin binding (49, 50).

Curiously, protein binding to the 3’ RR focuses on individual hs sites. For example, initial changes in protein binding to the 3’ RR occurred at hs4 and involved Pax5, whereas hs1.2 was the primary binding site for temporal alterations involving Pax5 and Rad21 (Fig. 7). As assessed by EMSA competition experiments, the major Pax5 binding site in hs4 was of higher affinity than either of two sites in hs1.2 (25). However, these relative binding affinities do not account for the shifts in association. Data, including those presented here, imply that loop interactions among all the 3’ hs sites are formed in resting B cells and are critical for 3’ RR activity (Fig. 6). We propose that interac-

to its function in these processes. Our previous studies have identified roles for p50 and Pax5 in transcriptional regulation of hs1.2 (24, 26) and hs4 (25). Mutagenesis experiments have
tions among the individual 3’ RR enhancers create a strong scaffold for an active module(s) of protein factors. The interactions among the 3’ RR enhancers themselves and their subsequent interactions with specific I regions coincident with GT appear to be maintained at similar levels, regardless of varying efficiencies in GT and CSR to specific isotopes. Such a scaffold formed by interactions with multiple alternative binding sites could accommodate a shift of Pax5 from hs4 to hs1.2 and hs7 and back again to hs4 as part of a mechanism to support GT and CSR. The Pax-interacting protein PTIP, which is essential for GT and CSR to specific isotopes, has been shown to differentially contribute to the various stages of CSR, from chromatin marks in resting B cells to GT and, separately, to CSR (21, 44). Deletions of individual hs elements could also easily be accommodated through interactions with remaining functional binding sites. Although a temporal and spatial set of protein interactions with 3’ RR elements has been described here for wild-type splenic B cells stimulated by LPS±IL4 to undergo CSR, different interactions that account for functional compensation are expected to be present in mice with deletions of 3’ RR elements or trans-regulators. The complexity of regulation of the 3’ RR and its multiple backups are likely to reflect requirements critical for its role in normal functioning of the Igh locus together with protection of other genomic segments from the mechanistic deleterious intrusions of Igh.

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