Evidence for Physical Interaction between the Immunoglobulin Heavy Chain Variable Region and the 3′ Regulatory Region

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B cell-specific expression of immunoglobulin heavy chain (IgH) genes utilizes two cis regulatory regions, the intronic enhancer (Eµ), located in the Jµ-Cµ intron, and a complex regulatory region that lies 3′ to the IgH gene cluster, 3′ RR. We hypothesized that the 3′ RR is involved in IgH gene transcription in plasma cells via physical interaction between distal 3′ RR enhancers and target VµH sequences, with loop formation by intervening DNA. In support of this hypothesis we report sequence data at DNA recombination breakpoints as evidence for loop formation preceding DNA inversion in a plasma cell line. In addition, using the chromosome conformation capture technique, physical interactions between VµH and 3′ RR were analyzed directly and detected in MPC11 plasma cells and variants and normal splenic B cells but not detected in splenic T cells or in non-B cells. VµH-3′ RR interactions were present in the absence of Eµ, but when the hs1,2 enhancer was replaced by a NeoR gene in a variant cell line lacking Eµ, H chain expression was lost, and interactions between VµH and 3′ RR and among the 3′ RR regulators themselves were severely disrupted. In addition, the chromosome conformation capture technique detected interactions between the myc promoter and 3′ RR elements in MPC11, which like other plasmacytomas contains a reciprocal translocation between the c-myc and the IgH locus. In sum, our data support a hypothesis that cis VµH-3′ RR and myc-3′ RR interactions involve physical interactions between these DNA elements.

During B cell development, antibody heavy chain genes undergo sequential DNA recombination and mutation events. These include construction of the variable region gene (VDJ joining), which occurs very early in B cell development, synthesis of membrane Ig, class switch recombination, and somatic hypermutation at the mature B cell stage, and secretion of IgH from plasma cells. Key cis regulators of B cell-specific expression of immunoglobulin heavy chain (IgH) genes are VµH promoters, I region promoters located upstream of each constant region gene, and two sets of enhancers (the intronic enhancer (Eµ) located in the Jµ-Cµ intron and a complex regulatory region that lies 3′ of the IgH gene locus (3′ RR) (1)). The murine 3′ RR contains four enhancers arrayed in two separate structural and functional units (2) together with a recently identified downstream extension, which contains additional DNase I hypersensitive sites, binding sites for CCCTC-binding factor (CTCF), and insulator sequences (3).

Initial insight into the contribution of 3′ RR enhancers to high levels of IgH gene expression in plasma cells came from analysis of cell lines containing deletions of intronic or 3′ RR enhancers. These data include 1) maintenance of IgH chain expression in plasma cell lines at high levels despite deletion of the intronic enhancer (4, 2) ~90% reduction in IgH expression in a plasma cell line that has a deletion of 3′ RR regulators extending from hs3a to hs4 (5, 6, and 3) complete loss of H chain expression from a plasma cell line that has a combination of a deletion of the intronic enhancer and substitution of the hs1,2 enhancer by the NeoR gene (7).

Knock-out mouse models have provided insight into the contribution of 3′ enhancers to CSR; a combined deletion of hs3b and hs4 affects CSR to all isotypes except IgG1 (8), whereas a deletion of all 3′ IgH sequences from a BAC transgenic mouse affected CSR to all isotypes (9). However, the contribution of the entire endogenous 3′ RR to heavy chain expression in plasma cells has not been addressed by knock-out models, and no phenotype was detected upon examination of other 3′ RR deletions in cell lines, including a combined deletion of hs3a and hs1,2 (10) and a specific deletion of hs4 (11).

These various observations suggest a potential physical interaction between distal 3′ RR enhancers and target IgH sequences, with intervening DNA sequences forming loops. The distances involved are as much as ~40–180 kb. Previous experiments identified a plasma cell line in which DNA rearrangements detected by genomic Southern analysis were con-

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sistent with an inversion of sequences extending from V_{H4} to 3’ RR (12). Here we report DNA sequence analysis of recombination breakpoints that explains this inversion by recombination between the V_{H4} gene and the 3’ RR. To determine whether V_{H4}-3’ RR interactions regularly occur, we have implemented the chromosome conformation capture (3C) technique, which captures transient interactions between otherwise linearly distant DNA sequences. This method has been used to analyze the effects of enhancers on target promoters in the β-globin and T_{H12} cytokine loci (13–15). Of particular pertinence to the regulation of antibody gene regulation is a recent study that has shown 3C interactions between DNA segments containing the κ intronic and 3’ enhancers and the expressed V_κ gene both in the MPC11 plasmacytoma cell line and in normal splenic B cells (16). This interaction likely reflects the impact of intronic and distal enhancers on transcriptional activation of κ expression.

The 3C experiments reported here have confirmed interactions in cis between V_{H1} sequences and 3’ RR enhancers in the MPC11 plasma cell line and in normal resting B cells. Interactions in cis between the myc exon 2 promoter and 3’ RR enhancers were also detected in MPC11, which like other plasmacytoma cell lines contains a reciprocal chromosomal translocation that brings the myc oncogene under the control of IgH regulatory sequences. Analysis of MPC11 variants with deletions of endogenous intronic or 3’ enhancers has addressed the role of specific V_{H1}-3’ RR interactions in heavy chain expression.

**EXPERIMENTAL PROCEDURES**

Cells—The mouse plasmacytoma cell line, MPC11 (BALB/c, IgG2b, κ), synthesizes a 55-kDa heavy chain and has a single J_{H1} region and intronic enhancer associated with the expressed y2b chain (17). The IgH unexpressed allele has undergone a reciprocal chromosomal translocation with the myc oncogene (18). 9921 cells were derived from MPC11 via an intermediate (9.7.1) (19). These cells synthesize IgG2a instead of the parental IgG2b as a result of a deletion that juxtaposes the MPC11-rearranged V_{H1} gene to switch y2a sequences (17). In the course of the DNA recombination, the intronic enhancer and cy2b sequences were deleted. B48 cells, obtained from Laurel Eckhardt, Hunter College, The City University of New York, were derived from 9921, differing only in the substitution of the NeoR gene for the hs1,2 enhancer on the expressed allele. H chain expression is completely absent from B48 cells (7). F5.5 cells were derived from MPC11 via an intermediate (ICR4.68) (20). Sequence analysis of F5.5 cDNA showed that the expressed 50-kDa γ2b H chain resulted from a single base insertion leading to a premature termination (20). Genomic Southern analysis of F5.5 revealed a second J_{H1}-associated map and a Ce rearrangement (20). EL-4 cells are a T lymphoma; the IgH locus is in germline configuration (21). The mouse erythroleukemia cell line is derived from DBA mice, and the IgH locus is in germline configuration. All cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin. All cells were grown at 37 °C in an atmosphere of 5% CO₂.

Resting B cells were isolated from spleens of C57BL/6 mice. Total splenocytes were depleted of CD43⁺-activated B cells and non-B cells by incubation with anti-CD43 microbeads (Miltenyi Biotec), washing with cold phosphate-buffered saline containing 3% fetal bovine serum, and passage through AutoMACS (Miltenyi Biotec) according to the manufacturer’s instructions. The purity of resting B cells, as detected by FACScan (BD Biosciences), was ~95% B220⁺. Splenic T cells (~95% CD3⁺) were isolated using a pan-T cell isolation kit (Miltenyi Biotec). The protocol for these studies was approved by the Animal Institute Committee of the Albert Einstein College of Medicine.

**Isolation and Sequence Determination of the VDJ-3’ RR and Ce Rearrangement Breakpoints from F5.5**—Genomic Southern analysis of F5.5 identified an 8.1-kb BglIII fragment that hybridized to both J_{H1} and 3’ RR sequences (12). This fragment was isolated from a Charon 40 phage library prepared from F5.5. A 1.4-kb BamHI/BglIII fragment containing the inversion breakpoint was subjected to partial DNA sequence analysis. A 10.1-kb SpeI fragment containing the rearranged Ce gene (12) was cloned into Charon 40 Xbal-digested arms. An 800-bp SacI/KpnI fragment containing the Ce rearrangement breakpoint (12) provided the template for initial DNA sequence analysis. Preliminary sequences obtained from clones of the breakpoints located the DNA segments involved in these rearrangements. Primers designed to analyze genomic sequences at the inversion breakpoints were selected accordingly. The first set of primers (3’ RR/VDJ-forward, F, gaactgtgaggtgcttc; 3’ RR/VDJ-reverse, R, tcagagccttcagagc) amplified a ~500-bp segment from VDJ to 3’ RR. The second set (VDJ/Ce_{m}-F, ccttagatgactagg; VDJ/Ce_{m}-R, tcagactcttcagagcc) amplified an ~800-bp fragment from the VDJ to Ce region. The expressed V_{H1} gene of F5.5 was sequenced using the primers VDJ/Ce_{m}-F2 and 3’ RR/VDJ-F as above, found to be identical to that previously shown for MPC11 variants, 9.7.1 (GenBank accession number M17056), 9921 (GenBank accession number M17058), and 11.9.3 (GenBank accession number M17059) (22) and provided the foundation for identification of the VDJ inversion breakpoints in F5.5.

Overall, 10 pmol of each primer, 2 units of HiFi Taq polymerase, and 100 ng of purified F5.5 DNA were used in a standard 50-µl PCR reaction. The PCR reaction was performed using 1.5 min of extension time, 68 °C annealing temperature, and 30–35 cycles in a ThermoHybaid PCR machine. PCR products were examined on a 0.8% agarose gel and purified using the QIAquick PCR purification kit. DNA was sequenced in the Albert Einstein College of Medicine sequencing facility using the primers used for PCR amplification. Sequences were analyzed via BLAST; Ce and 3’ RR sequences were compared with 1295v germline sequences contained in two BAC clones, i.e. e (GenBank accession number AJ851868) and 3’ RR (GenBank accession number AF450245), respectively. MPC11 and its variants, like F5.5, derive from BALB/c, which is more similar to 1295v than to the C57Bl/6 strain selected for the mouse genome project.

**3C Assay**—Except for minor modifications, the procedure was performed according to the method of Splinter et al. (23). Briefly, 10⁷ cells were subjected to formaldehyde cross-linking for 10 min at room temperature with tumbling, after which glycine was added to quench the formaldehyde. Nuclei were prepared, and non-cross-linked protein was removed from chromatin DNA by incubation with SDS. Triton X-100 was
added to sequester the SDS, after which samples were digested with 2000 units of HindIII overnight at 37 °C with shaking. The digest was terminated with the addition of SDS and incubation at 65 °C. Digested nuclei (10⁶ nuclei) were then diluted (~1:15) with 1× T4 DNA ligase buffer to a concentration of ~3 ng of DNA/μl, and SDS was sequestered by Triton X-100. Ligation was promoted by T4 DNA ligase and terminated with the addition of proteinase K, and cross-links were reversed by incubation at 65 °C overnight. Samples were treated with RNaseA, and DNA was purified by phenol:chloroform extraction and isopropanol precipitation. The concentration of dsDNA was determined using picogreen ( Molecular probes kit) by the FLUOstar OPTIMA fluorescence reader (BMG Lab Technologies).

One hundred ng of ligated DNA samples were analyzed by 32 cycles of PCR (94 °C/30 s, 60 °C/20 s, and 72 °C/30 s) in a 25-μl reaction volume. Analysis of JH13 association with hs1,2 and hs5–7 was determined using 62 °C as the annealing temperature. Taq polymerase was obtained from New England Biolabs. PCR products were separated by 2% agarose gel. Gel pictures were captured with Gene Snap Software and then analyzed by Gene Tools Software (SYNGENE GENE genius BioImaging System).

To exclude the biased restriction digestion of one site over another in different cell types, we checked the digestion efficiency of each designated HindIII cleavage site by PCR (27 cycles of 94 °C/30 s, 58 °C/20 s, and 72 °C/30 s) using primers flanking the cut sites (supplemental Table S1) and 100 ng of template DNA extracted from undigested and HindIII-digested nuclei. Restriction digestion efficiencies were calculated by comparing PCR signals from digested and undigested samples and ranged from 66 to 89%, comparable with other studies (16, 24). The identity of PCR fragments was confirmed by sequence determination.

To correct for differences in amplification efficiency between different primer sets, we generated an equimolar mixture of two BACs, namely BAC199M11 (AF450245, containing all the 3′ RR elements: HS3a, HS1, HS3b, HS4, and HS5,6,7) and RP109B20 (AC079180, extending from DH segments through to the first exon of γ2a), a plasmid (M11-myc 3′) containing the MPC11 myc translocation breakpoint with switch γ2a sequences (18), two calreticulin fragments that contain the selected HindIII restriction sites (Ensembl: ENSMUST 0000003912), and a pGK-NeoR PCR fragment containing a HindIII cleavage site (7). The control template, containing all possible ligation products, was then subjected to HindIII digestion followed by random ligation with DNA ligase. Primers for 3C analysis were upstream (forward) of HindIII cleavage sites (supplemental Table S1).

Variations in efficiency of cross-linking, restriction digestion, ligation, and amplification in different cell types were normalized via the cross-linking signal derived from two non-adjacent DNA fragments of the calreticulin housekeeping gene (fragments A and B, supplemental Table S1) as used by others (13). Reverse transcription-PCR analysis showed that calreticulin transcription levels were at similar levels in the cell lines we used and, independently, at comparable levels in primary B and T cells.

3C experiments were performed on three independent cell preparations of each source using multiple independent PCR reactions. Means and S.D. were calculated, and Student’s t test was conducted to determine the statistical significance of the differences between two samples.

RESULTS

Inversion/Deletion in F5.5 Is Indicative of V_{H}3′ RR Interaction—The results of genomic Southern analysis of F5.5, a murine myeloma cell line derived from MPC11, are consistent with an inversion between the expressed V_{H}gene and the 3′ RR (12, 20). A likely mechanism to account for this inversion is physical interaction between these distally located segments accompanied by DNA breaks and rejoining (Fig. 1A). Such an interaction would bring the expressed V_{H} gene into contact with distally located 3′ RR sequences that have been predicted to influence its expression.

To obtain additional evidence for this model by identifying the sequences at the breakpoints, we compared DNA rearrangements in F5.5 with wild-type genomic sequences of the 3′ RR (GenBank™ accession number AF450245 (25)). The VDJ-3′ RR inversion breakpoint in F5.5 cells was contained in an 8.1-kb BglII fragment, whereas the predicted reciprocal Cε breakpoint was analyzed from a 10.1-kb SpeI fragment. These fragments were initially isolated from phage libraries, confirmed by Southern analysis, subcloned, and then subjected to partial sequence analysis (data not shown). We completed the sequences of the inversion breakpoints on F5.5 genomic DNA using a PCR strategy (Fig. 1A and supplemental Figs. S1A and S1B). Consistent with an inversion model, VDJ sequences located on the 5′ to 3′ non-coding strand were associated with the 5′ to 3′ coding strand of the 3′ RR to generate the VDJ-3′ RR breakpoint (supplemental Fig. S1A). Sequence analysis of the Cε rearrangement showed that VDJ 5′ coding sequences were joined through a 50-bp junction of 3′ RR sequences to Cε membrane exon 1 sequences, both from the non-coding strand (supplemental Fig. S1A). This implied that in addition to the inversion, there was an internal ~40-kb deletion that began in Cε membrane exon 1 and continued through Cα and enhancers hs3a and hs1,2 of the 3′ RR (Fig. 1B). The 3′ end point of this deletion, marked by a TGGG motif in 3′ RR sequences, was located ~6 kb downstream of hs1,2.

The DNA sequences of the inversion breakpoints provide direct evidence for the reciprocal recombination between expressed VDJ and 3′ RR sequences (Fig. 1C). The VDJ breakpoint occurred immediately after the codon for N14 (numbering from the N terminus of the mature MPC11 H chain, GenBank™ accession number AAA38329), whereas the 3′ RR breakpoint occurred ~midway between hs1,2 and hs3b enhancers. Sequences at the breakpoints (VDJ: 5′ to 3′ coding strand depicted) match their respective 3′ RR and V_{H} MPC11 counterparts except for a three-base pair insertion (AGA) in the 3′ RR segment immediately upstream of the crossover point. Upstream of the VDJ-3′ RR breakpoint, there is sequence identity (10/12 bases) between germline V_{H} MPC11 and 3′ RR sequences, whereas downstream sequences show little homology. The crossover point itself is within a CCT triplet contained in both V_{H} MPC11 and 3′ RR at the 3′ limit of the region of homology. The V_{H} MPC11 breakpoint, including the CCT, is a palindrome (AGGCCT). The inversion breakpoint is located only 50 bp downstream of the 3′ RR deletion end point. These
Interaction between the IgH Variable Region and 3′ RR

A. Variable Region

| 5′ | VDJ | Eu | γ2b | Eu | y2b | hs1.2 | hs4 | 3′ |
|---|----|----|-----|----|-----|-------|-----|----|
| 80 kb |  |  |  |  |  |  |  | 28 kb |

Deletion

Inversion

B. Variable

CTAGACCTCCAGGACATCTGGTTAAGAGGTTGAGCCG

catacactgagggagcgcgggtaattacagctgtagga

F5.5.1/AJ851868 (C5 RR+e)

AF450245 (3′ RR+e)

AJ851868 (Ce)

AF450245 (3′ RR)

C. 3′ RR

| VH | 5′ | gGACCGCTGAGCTGAAACCCAGGATATGCAGATTCGTC | 3′ |
|---|----|-------------------------------------------|----|
| F5.5 (VH/3′ RR+e) | 5′ | ttcctacctggagtacggttgccgacctgagatgtgc | 3′ |

D. Inversion

Vh3′ RR-e break point

Jh-3′ RR breakpoint

hs4, hs3b

hs1,2

Data imply that the inversion of DNA sequences extending from V_{14} to 3′ RR in F5.5 has occurred through recombination after physical interaction between V_{14} and 3′ RR sequences.

3C Assay—Dynamic interactions between the 3′ RR and its predicted V_{14} target sequence might generate only a transient intervening loop without any detectable downstream DNA rearrangements. To detect these predicted V_{14}-3′ RR interactions, we have implemented the 3C technique (Fig. 2). The MPC11 cell line and several of its variants provide unique resources for analysis of interactions between the 3′ RR elements and their predicted V_{14} gene target sequences. MPC11 has only one J_{H1} region and a single intronic enhancer, both of which are located on the single expressed IgH locus (17). Hence, J_{H1} sequences used as a probe or primer source can exclusively track the single IgH expressed allele in MPC11 and its variants. As in other plasmacytomas, the second IgH locus in MPC11 has undergone reciprocal translocation with the myc gene (18), and the translocated IgH:myc allele has been amplified to three copies (26).

In the 3C method, formaldehyde is added to live cells to fix DNA-protein interactions that may accompany long-range regulation involving linearly distant chromatin fragments. Restriction digestion of chromatin in fixed nuclei followed by ligation in dilute solution captures these interactions in a covalent form, which provides a template for PCR amplification. HindIII was chosen based on the position of its restriction sites with respect to IgH regulatory elements (Fig. 2A), its enzymatic activity in the presence of SDS, and its being subject to inactivation at 65 °C. Because chromatin limits the accessibility of restriction enzymes to their specific sites (Fig. 2B), we compared the relative efficiency of HindIII cleavage in the cell sources we analyzed. Individual sites were cleaved at comparable levels ranging from 66 to 89% in both EL-4 and MPC11 and in
other cells we examined (data not shown) that were similar to levels reported in other studies (16).

Relative primer pair efficiency was measured (Fig. 2C, control template, first lane) using a mixture of naked DNA from BACs and plasmids in equimolar amounts as templates for all possible interactions between these otherwise linearly distant fragments. The mixture was subjected to restriction enzyme cleavage and random DNA ligation. Interactions between restriction fragments in MPC11 cells were not observed unless DNA ligase was added (Fig. 2C). Individual primer pairs showed differences in amplification efficiency that were normalized to the signal generated by interactions between two non-adjacent fragments affiliated with the calreticulin housekeeping gene. As shown by others (13), we observed that calreticulin was expressed at similar levels in the various cell sources we have analyzed (data not shown). 3C analysis used DNA amounts in the linear range of the assay, as shown for CalR (Fig. 2D) and for V_{H}-hs1,2, V_{H}-hs3b,4, and V_{H}-hs5–7 (supplemental Fig. S2, A–C). The 3C signal obtained from cells was normalized to the endogenous calreticulin signal (Fig. 2E).

**Interactions between Expressed V_{H} and 3′ RR in MPC11—** We specifically wanted to test whether 3′ RR regulators could interact with sequences at the 5′ end of the expression unit, namely the expressed VDJ gene and/or the intronic enhancer, E_{H}. The expressed MPC11 VDJ_{H2} gene (Fig. 2A) is located within a 3.7-kb HindIII restriction fragment, which begins upstream of the VH promoter and terminates downstream of JH3, whereas unrearranged germline J H2 and J H3 genes are contained in a 0.8-kb HindIII fragment. A primer for JH3 sequences tracks the expressed VH gene from MPC11 and corresponding JH2 and JH3 germline sequences in non-B cells, such as EL-4. Interactions between VDJ-associated fragments and 3′ RR regulators were substantially stronger in MPC11 than in EL-4 (Fig. 3, A and B). In MPC11, the fragment containing the expressed VDJ gene interacted with 3′ RR regulators, namely, hs3a, hs1,2, hs3b–hs4, and hs5–7, whereas the analogous unrearranged germline fragment from EL-4 T cells (or from mouse erythroleukemia cells, data not shown) showed only low levels of interactions with 3′ RR fragments (Fig. 3B). Other studies have also shown similar background bands in cell sources selected to be biological controls (16, 27). Under our
there were no interactions between J_{H3} and the CalR gene, which is located on a separate chromosome (Fig. 2C and 3B). These observations suggest that physical interactions between the 5’ end of the IgH transcription unit and the 3’ RR are specific to the IgH locus and to the plasma cell line that we have analyzed.

The Intronic Enhancer Is Dispensable for V_{H}-3’ RR Interaction—Immediately downstream and adjacent to the HindIII fragment containing the expressed V_{H} gene in MPC11 is a 2.2-kb HindIII fragment that contains J_{H4} and E_{\mu}. Specific interactions between V_{H} and E_{\mu} could not be analyzed because the HindIII fragments that contain these elements are adjacent. In a flexible chromatin context, adjacent fragments are especially subject to random collisions, resulting in nonspecific interactions even when monitored with unidirectional primers (27). We therefore chose to analyze the contribution of the intronic enhancer to 3C interactions by assaying 9921 (Fig. 3A). The IgG2a expression levels in 9921 (which lacks the intronic enhancer) are comparable not only to levels of IgG2b in MPC11 but also to levels of IgG2a expressed in MPC11 switch variants that retain the intronic enhancer (17, 29). These data have contributed to the general hypothesis that high levels of IgH expression in murine plasma cells that occur in the absence of the intronic enhancer depend on the 3’ RR (30). In fact, 3C experiments in 9921 cells (Fig. 3C) have detected interactions between the expressed V_{H} gene and each of the three HindIII fragments containing 3’ RR enhancers at the same general levels as shown in MPC11. These observations were confirmed for hs3a and hs5–7 with primers designed in the opposite orientation (supplemental Fig. S3, A and B). The binding of V_{H} to hs3a, although slightly reduced, is significantly higher than in EL-4 T cells. These experiments show that E_{\mu} is not required for V_{H}-3’ RR interactions that occur in a plasma cell line synthesizing large levels of antibody and suggest that 3’ RR interactions with the expressed V_{H} gene may be important for H chain expression.

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* Z. Ju, unpublished observations.
Replacement of hs1,2 on the Expressed IgH Allele by NeoR Disrupts V1-H3’ RR Interactions—The B48 cell line (Fig. 2A) differs from 9921 only by a substitution of the NeoR gene for hs1,2 on the expressed H chain allele; this entirely cripples heavy chain expression (7). To determine whether the NeoR substitution for hs1,2 affects interactions between the VH gene and the 3’ RR, we carried out 3C on B48 cells. These experiments (Fig. 3D) showed a significantly enhanced interaction between the expressed VH gene and hs3a; this was coupled with a severe reduction in interactions between VH and 3’ RR elements downstream of the NeoR insertion, including hs1,2 downstream sequences, hs3b-4 and hs5–7 fragments, to the same background levels as observed in EL-4 thymoma cells. Interaction of the VH gene with hs3a but not with hs 5–7 in B48 cells was also observed with primers of the opposite orientation designed for sequences near the upstream HindIII sites of hs3a and hs5–7 (supplemental Fig. S3C). These same primers detected interaction between the VH gene and both hs3a and hs 5–7 in MPC11 and 9921 cells. Repetitive sequences near the 5’ HindIII sites of hs1,2 and of hs3b-4 precluded the design of primers for these specific segments. Our experiments showed that the retention of interaction of VH with hs3a but not with downstream 3’ RR elements in B48 was unlikely to derive from a bias in primer design or orientation. These experiments provide evidence for the contributions of 3’ RR regulators to H chain expression.

3’ RR Elements Interact with Each Other and with Eu—3C experiments in MPC11 and 9921 detected interactions between the upstream expressed VH, 3’ RR fragment and each of the fragments containing 3’ RR enhancers. We, therefore, wanted to determine whether the 3’ RR regulators interacted with each other. Experiments showed that hs3a interacted at similar levels with the other 3’ regulators as well as with Eu in MPC11 cells, but not in EL-4 T cells, and the interaction did not extend to the hole gene (Fig. 4). Similar observations were detected for normal splenic B cells and T cells, respectively (data not shown). It should be noted that we cannot distinguish the interactions between 3’ regulators that may occur on the non-IgH expressed alleles that are involved in the reciprocal myc chromosomal translocation from those occurring on the IgH expressed allele. These observations of 3’ RR interactions in B cells are relevant to our studies of B48 where, using the NeoR gene as an anchor (Fig. 3, E and F), we observed its strong interaction only with hs3a but not with any of the downstream 3’ RR enhancers nor with the upstream expressed VH gene. These findings suggested that a closed loop between NeoR and hs3a precluded additional 3’ RR interactions. However, we cannot eliminate the possibility that nonspecific 3C interactions that are known to be able to occur between adjacent or nearby restriction fragments (27) may contribute at least in part to the NeoR interaction with hs3a. The replacement of hs1,2 (5.1 kb) by the NeoR gene (1.9 kb) results in a shorter distance between NeoR and hs3a (~7.5 kb) than between hs1,2 and hs3a (~10.3 kb) in cells with a germline configuration, and both distances are shorter than the distance between NeoR (or hs1,2) and hs3b (13.7 kb). In addition, between hs3a and the NeoR gene, there is a single intervening ~5-kb HindIII fragment, whereas between NeoR and hs3b, there are four HindIII fragments. Both features would favor nonspecific hs3a-NeoR interactions. We can conclude, however, that the NeoR replacement of hs1,2 has severely disrupted VH interactions with downstream 3’ RR elements.

Detection of Interactions between myc Promoter and 3’ RR—The chromosomal translocation breakpoint in MPC11 joins myc exon 2 to switch y2a sequences (18), and various experiments have suggested that myc is under the control of the 3’ RR (31). 3C experiments (Fig. 5) showed that the myc promoter interacted with the 3’ RR regulators. In accord with the detection of only cis interactions by the 3C assay conditions, no interaction was detected with the intronic enhancer, which is located only on the IgH expressed, non-translocated chromosome.

FIGURE 4. Graphical presentation of 3C interactions with Eu and 3’ RR elements in MPC11 and EL-4 using hs3a as an anchor primer (denoted by a triangle).

FIGURE 5. Graphical presentation of 3C interactions with 3’ RR elements using a myc primer as an anchor (denoted as a triangle) in MPC11 compared with EL-4. IgH expressed and translocated alleles in MPC11 are depicted. The lack of interaction with Eu indicates that the 3C signals are derived in cis. No signals were detected in EL-4, as indicated by the small open squares.
Interaction between the IgH Variable Region and 3’ RR

**DISCUSSION**

The results we report here document physical interaction between the expressed IgH V_{H} gene and 3’ RR sequences by two approaches, 1) characterization of an inversion of the expressed IgH locus in the F5.5 plasmacytoma cell line and 2) 3C analysis of the MPC11 plasmacytoma cell line that is the parent of F5.5, MPC11 variants that contain targeted deletions of IgH regulatory elements, and normal splenic B cells. The distance separating expressed VDJ and 3’ RR sequences is ~80 kb in MPC11 and F5.5, the two γ2b, κ-producing cell lines that we have studied, and ~180 kb in IgM-producing splenic B cells.

Sequence analysis of the F5.5 inversion/deletion breakpoints provides evidence for interaction between the expressed V_{H} gene and the 3’ RR (see model, Fig. 1D). The inversion can be explained by reciprocal recombination between VDJ coding sequences immediately downstream of the codon for N-14, i.e. the 14th amino acid from the N terminus of the mature MPC11 IgH protein, and 3’ RR sequences at a position located ~halfway between hs1,2 and hs3b. DNA interactions in F5.5 could be facilitated by the microhomology observed upstream of the inversion breakpoint between V_{H} MPC11, a member of the populous IGHV J558 family, and 3’ RR sequences (22). The inverted allele in F5.5 also contains an ~40-kb deletion that encompasses Cα together with two of the 3’ RR enhancers, i.e. hs3a and hs1,2. A similar deletion has been characterized in the 70Z/3 pre-B cell line (10), suggesting that specific IgH DNA segments may regularly interact with each other.

The mechanisms that result in DNA breaks and recombination in F5.5 are not known. Motifs in the vicinity of the inversion and deletion breakpoints include AGCC, a known activation-induced cytidine deaminase hotspot (32) (Fig. 1), GAGCT “switch-like” motifs, and TGGG. However, IgH segments that contain F5.5 breakpoints are not known to undergo recombination associated with CSR, and there is no additional evidence to support the involvement of activation-induced cytidine deaminase or CSR machinery. The inversion in F5.5 is most simply explained as an unusual sequel to ongoing physical interaction between VDJ and 3’ RR sequences that is predicted to occur regularly as part of the transcriptional regulation of the IgH locus in plasma cells.

To test this prediction, we implemented 3C analysis on a progressive series of MPC11 variants. Our studies detected interactions between fragments mapping to the 5’ end of the MPC11 IgH transcription unit (V_{H}, J_{H}, and E_{H}) with enhancers and insulators at the 3’ end of the entire IgH locus (3’ RR and hs5–7). As shown in Fig. 3, we found that 5’ fragments could bind to several different 3’ fragments. We do not know, however, whether in individual cells these interactions occur in single pairs or in varying constellations of multiple interactions; 3C analysis captures the totality of interactions that occur in the population of cells analyzed and does not discriminate subpopulations.

Our observations have not only revealed loop formation between V_{H} and 3’ RR regulators but also underscored its intimate association with H chain expression (see the model in Fig. 7). In accord with previous studies in which the deletion of the endogenous intronic enhancer (9921) had no effect on levels of IgH expression, the studies reported here found essentially no modifications in 3C interactions between V_{H} and 3’ RR regulators, except for a slight reduction in interaction between V_{H} and hs3a. In contrast, examination of B48, which upon substitution of hs1,2 by the Neo^R gene suffered complete loss of H chain expression, revealed an accompanying disruption of the loop structures present in MPC11 and 9921. In B48 the hs3a enhancer was the focus of increased interaction with upstream V_{H} sequences on the one hand and downstream Neo^R sequences on the other, coupled with a loss of interactions between V_{H} and 3’ RR regulators downstream of the Neo^R insertion. As discussed under “Results,” we cannot eliminate the possibility that the deletion of genomic sequences during the construction of B48 may have contributed a nonspecific component to the hs3a-Neo^R signal. In any event it seems unlikely that hs3a would interact with both V_{H} and Neo^R at the same time because there was no detectable interaction between V_{H} and Neo^R. One explanation of these findings would then be a competition between V_{H} and Neo^R promoters for the hs3a enhancer. These data show that the enhanced interaction detected between the expressed V_{H} gene and hs3a is inadequate to support any H chain expression in the absence of the intronic enhancer, instead suggesting that interactions of V_{H} with hs1,2 and/or with other downstream enhancers are essential.
Interaction between the IgH Variable Region and 3' RR

Whether long-range interactions are similar at various stages of B cell development and how they might contribute to IgH expression and rearrangements are not known. Studies (34, 35) using fluorescence in situ hybridization have reported loops involving unarranged V_{H} proximal and distal genes and C_{\alpha} genes in both pro-B and, although less frequently, also in CD8^{+} T cells, some of which appear to be related to the onset of VDJ joining. Our observations of 3C interactions between Ig_{H} and 3' RR sequences in plasma cells and resting splenic B cells but only at background levels in splenic T cells, EL-4 thymoma cells, or in mouse erythroblastemia cells imply cell type-specific long-range interactions involving 3' RR sequences. It should be noted that the interactions extend through the 3' RR to the hs5–7 region, which has insulator activity (3). Interactions are not evident with the hole gene located ~30 kb downstream of hs7 or with the CalR gene located on a different chromosome. This implies that interactions between Ig_{H} and/or E_{\mu} with 3' RR elements are limited to the IgH locus in plasma cells and B cells.

Recent studies on the \(\beta\)-globin locus have suggested that interactions between locus regulators and distal target sequences may help target the locus to transcription factories; hence, facilitating gene expression (36). Such physical interaction is consistent with the prediction that the 3' RR is required for high levels of IgH expression in plasma cells (5–7, 37). Similar interactions in normal splenic B cells provide a potential scaffold by which the 3' enhancers can facilitate germline transcription, which is a prerequisite for class switch recombination. Identification of 3C interactions in the \(k\) locus between promoters, intronic, and 3' enhancers (16) invokes a similar mechanism for both H and L expression.

The 3' RR has also been implicated in the dysregulation of the\(myc\) oncogene upon its translocation with the IgH locus in murine plasmacytomas and Burkitt's lymphomas. Involvement of the\(myc\) promoter in cis with the multiple elements of the 3' RR is analogous to the interactions in cis of the V_{H} gene with the 3' RR. This suggests that the 3' RR may work similarly in its influence on target elements within the IgH locus or on other loci, such as those involved in malignant transformation.

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