Mechanism of Fragility at BCL2 Gene Minor Breakpoint Cluster Region during t(14;18) Chromosomal Translocation

Mridula Nambiar and Sathees C. Raghavan

From the Department of Biochemistry, Indian Institute of Science, Bangalore-560 012, India

Background: The mechanism of fragility at mcr during t(14;18) translocation is not known.

Results: RAGs nick mcr using a unique mechanism involving the CCACCTCT motif, which is critical for its fragility. Importantly, mcr undergoes synapsis with RSS within cells, which is RAG-dependent.

Conclusion: RAGs are responsible for fragility at mcr.

Significance: The mechanism identified herein may help in understanding how DNA breaks during other translocations.

The t(14;18) translocation in follicular lymphoma is one of the most common chromosomal translocations. Breaks in chromosome 18 are localized at the 3′-UTR of BCL2 gene or downstream and are mainly clustered in either the major breakpoint region or the minor breakpoint cluster region (mcr). The recombination activating gene (RAG) complex induces breaks at IgH locus of chromosome 14, whereas the mechanism of fragility at BCL2 mcr remains unclear. Here, for the first time, we show that RAGs can nick mcr; however, the mechanism is unique. Three independent nicking efficiencies are generated, when both Mg2+ and Mn2+ are present, unlike a single nick during V(D)J recombination. Further, we demonstrate that RAG binding and nicking at the mcr are independent of nonamer, whereas a CCACCTCT motif plays a critical role in its fragility, as shown by sequential mutagenesis. More importantly, we recapitulate the BCL2 mcr translocation and find that mcr can undergo synapsis with a standard recombination signal sequence within the cells, in a RAG-dependent manner. Further, mutation to the CCACCTCT motif abolishes recombination within the cells, indicating its vital role. Hence, our data suggest a novel, physiologically relevant, nonamer-independent mechanism of RAG nicking at mcr, which may be important for generation of chromosomal translocations in humans.

Many chromosomal translocations between genes that encode for antigen receptors of B- or T-cells and proto-oncogenes or transcription factors have been detected in leukemias and lymphomas (1–4). Follicular lymphoma, one of the most common subtypes (~40%) of non-Hodgkin lymphoma, is characteristically associated with t(14;18) translocation (5, 6). It results in increased expression of the BCL2 gene due to its repositioning next to the enhancer of immunoglobulin heavy chain (Igh) locus, which is actively transcribed in B-cells.

V(D)J recombination is a site-specific recombination and is responsible for the generation of antigen receptor diversity in higher eukaryotes (7, 8). The recombination activating gene (RAG)3 complex, comprising RAG1 and RAG2, recognizes the recombination signal sequences (RSS) flanking the V, D, and J subexons on the Igh locus of chromosome 14 (9–11). The RSS consists of conserved heptamer and nonamer sequences interspersed with a nonconserved spacer. Depending on the length of the spacer, RSS can be termed as 12- (12RSS) or 23-signal (23RSS). Normally, 12RSS can recombine only with 23RSS, which is known as the 12/23 rule (10, 12). RAG nicking at the RSS is highly specific, and only a single nick is generated at the 5′ end of the heptamer (CAGAGTG). RAGs bind to the nonamer and direct the site-specific cleavage (13). The nick is further converted to a hairpin by transesterification leading to generation of a double-strand break at the signal joint (14, 15). The hairpins are further opened by DNA protein kinase catalytic subunit-Artemis complex and finally processed by nonhomologous end joining (16, 17).

Many chromosomal translocations are known to occur due to erroneous V(D)J recombination (18–21). During t(14;18) translocation, the break at chromosome 14 is induced by standard V(D)J recombination mechanism. However, if a concomitant break occurs in chromosome 18, misjoining of these products could result in translocation (5, 6, 18). Interestingly, most of the breaks in chromosome 18 occur in the 3′-UTR of the BCL2 gene or downstream of it (supplemental Fig. 1). In 50% of the patients, the breaks on BCL2 gene are localized in a 150-bp region in the 3′-UTR of the third exon known as the major breakpoint region (MBR) (6, 22, 23). In ~5% of the cases, the breakpoints are found in a 561-bp region, ~29 kb downstream of MBR, designated as the minor breakpoint cluster region (mcr) (24–28). Within the mcr, at least 12 breakpoints are clustered toward one end of the region within a span of 20 nucleotides, whereas there are seven breakpoints across the remaining 541 bp (29). Recently, another breakpoint region between the MBR and mcr was discovered, termed the intermediate cluster region (supplemental Fig. 1) (25).

The t(14;18) translocation in follicular lymphoma is one of the most common chromosomal translocations. Breaks in chromosome 18 are localized at the 3′-UTR of BCL2 gene or downstream and are mainly clustered in either the major breakpoint region or the minor breakpoint cluster region (mcr). The recombination activating gene (RAG) complex induces breaks at IgH locus of chromosome 14, whereas the mechanism of fragility at BCL2 mcr remains unclear. Here, for the first time, we show that RAGs can nick mcr; however, the mechanism is unique. Three independent nicking efficiencies are generated, when both Mg2+ and Mn2+ are present, unlike a single nick during V(D)J recombination. Further, we demonstrate that RAG binding and nicking at the mcr are independent of nonamer, whereas a CCACCTCT motif plays a critical role in its fragility, as shown by sequential mutagenesis. More importantly, we recapitulate the BCL2 mcr translocation and find that mcr can undergo synapsis with a standard recombination signal sequence within the cells, in a RAG-dependent manner. Further, mutation to the CCACCTCT motif abolishes recombination within the cells, indicating its vital role. Hence, our data suggest a novel, physiologically relevant, nonamer-independent mechanism of RAG nicking at mcr, which may be important for generation of chromosomal translocations in humans.
Mechanism of Fragility at BCL2 mcr

It has been reported that many genes involved in chromosomal translocations observed in leukemia harbor sequences resembling the RSS, known as cryptic RSS (cRSS) (18). These sequences generally contain at least a CAC, which is very crucial for RAG cleavage (30). It has been shown that such cRSS present at translocation sites are misrecognized by RAGs and undergo cleavage using the V(D)J recombination mechanism (18, 31, 32). The double-strand break thus generated is a prerequisite for chromosomal translocations. Another RAG-mediated mechanism in translocations utilizes the novel property of RAGs being a structure-specific nuclease (33–36). It has been shown that a non-B DNA structure formed at the RSS at a low frequency, by undergoing synapsis within the cells, when RAGs are expressed. In addition, mutation to a novel octameric motif CCACCTCT present in mcr completely abolishes RAG cleavage both in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Oligomers and 5’ End Labeling—The oligomers used are listed (supplemental Table 1). The oligomers were gel-purified as described (37). The 5’ end labeling of the oligomeric DNA was done using T4 polynucleotide kinase with [γ-32P]ATP (38). For details of preparation of oligonucleotide DNA substrates, refer to the supplemental Materials and Methods.

Construction of Episomes—Episomes for transfections, pMN1, pMN3, pMN4, pMN5, pMN6, pMN13, pMN15, pMN27, pMN28, and pMN29 were constructed as described in the supplemental Materials and Methods.

Cell Lines and Culture—Human cell lines, 293T (kidney) was grown in DMEM with L-glutamine, whereas REH and Nalm6 (pre-B) were grown in RPMI 1640 as per standard protocol.

RAG Expression and Purification—Human GST and MBP core RAG1 (384–1008 amino acids; cRAG1) and core RAG2 (1–383 amino acids; cRAG2) proteins were purified as described (35, 36). The protein expression was checked by Western blotting by using the appropriate antibodies (Santa Cruz Biotechnology) (supplemental Fig. 2, A and B). The activity of the proteins was tested by RAG nicking on 12RSS.

RAG Cleavage Assay—The substrate DNA containing standard RSS sequence, BCL2 mcr, mcr mutants, LMO2, BCL1, or SCL were incubated with cRAGs for 1 h at 37 °C in a buffer containing 25 mM MOPS (pH 7.0), 30 mM KCl, 30 mM potassium glutamate, and 5 mM MgCl2, supplemented with MnCl2 as specified in the figure legends. In control reactions, buffer alone was used. In the experiment where synthesis of mcr and RSS was studied, purified high mobility group protein B1 (100 nM) was added to the reaction. Reactions were terminated, and the products were resolved on 12–15% denaturing polyacrylamide gels. The bands were dried and exposed to a PhosphorImager screen. Bands of interest were excised and subjected to image analysis using Image J software.

End Labeling—The oligomeric DNA was end labeled by using [γ-32P]ATP end labeling kit (Ambion). Reaction mixtures were incubated with RAGs in a reaction containing 5 mM MgCl2 for 1 h. The products were resolved on a 12% denaturing PAGE. The signals were detected after drying the gel as described above.

In Vivo Recombination Assay—The recombination assay was performed as described earlier (31). REH or Nalm6 cells were transfected with appropriate episomal substrates by electroporation and cultured for 48 h at 37 °C. The plasmid substrates were recovered by using the rapid alkaline lysis method and used for transforming Escherichia coli. The transformation mixture was plated on ampicillin (A) and chloramphenicol-ampicillin (CA) LB agar plates. The recombination frequencies (R) were calculated using the equation (CA/A) × 100. Each eukaryotic transfection was typically analyzed with multiple E. coli transformations. In the case of 293T cells, pMN4 along with MBP core, full-length, or mutant RAG expression constructs were transfected using the calcium phosphate method as described (33).

Radioactive PCR of Transfection Products and Sequencing of Recombinants—The plasmid DNA harvested after transfection were digested with HindIII (making most of the unrecombined episomes unsuitable for PCR) and subjected to PCR amplification using [γ-32P]ATP end-labeled SGR190 and SGR21. The PCR products were resolved on a 1.6% agarose gel, which was dried and exposed to a PhosphorImager screen. Bands of interest resulting from independent transfections were cut out from the dried gel, and removal of the bands was confirmed by scanning. The DNA from the bands was eluted in 500 mM NaCl, 10 mM Tris, and 1 mM EDTA and purified (39). The recombinant was then PCR-amplified and ligated to a TA vector (Invitrogen), and positive clones were sequenced (SciGenom).

RESULTS

RAG Complex Induces Three Independent Nicks at Heptamer of BCL2 mcr—The majority of the translocation breakpoints spanning the BCL2 mcr in the patients are 2 nt away from a CACCTCT heptamer (24–28). Based on previous studies, we hypothesized that the CAC of the heptamer at the mcr may
facilitate RAG cleavage using cryptic RSS. To test this, we designed an oligomer containing \textit{BCL2} mcr (47-mer, in which CAC sequence was placed at the 18-nt position) that can mimic a 12-signal (indicated as 12MCR) (Fig. 1A, II). The top or bottom strands of 12MCR or standard 12RSS were \[^{32}P\]\textit{ATP}-labeled and incubated with the purified GST cRAGs in a reaction buffer containing 5 mM MgCl\(_2\). Results showed a single nick at the 5' end of CACAGTG in the case of the top strand of 12RSS (Fig. 1B, lanes 1 and 2). There was no cleavage product at the bottom strand of 12RSS (Fig. 1B, lanes 3 and 4). However, we could not detect any RAG cleavage on either strand of 12MCR (Fig. 1B, lanes 5–8), indicating that RAGs were unable to cleave 12MCR under standard nicking conditions.

Because it is known that other divalent cations such as Mn\(^{2+}\) play a role in RAG reactivity, the above results prompted us to test whether the presence of MnCl\(_2\) could facilitate the RAG nicking at the mcr. To this end, we incubated 12MCR with RAGs in a buffer containing MgCl\(_2\) (5 mM) and MnCl\(_2\) (5 mM). Surprisingly, we observed three specific bands due to RAG cleavage on the top strand (Fig. 1C, lanes 5 and 6; marked by arrowheads). Specifically, the RAG nicking resulted in products of 16, 18, and 19 nt, which corresponded to nicks between the G and C (G\_CCACC), C and A (GCC\_ACC), and A and C (GCCA\_CC) of the mcr cRSS (Fig. 1A and C). However, the nick at the 5' end of CAC (GC\_CAC), corresponding to a 17-nt product, was either very weak or nil (Fig. 1C, lane 6).

Because only one nick can be visualized in a \[^{32}P\] end-labeled molecule at a time, the results obtained suggest that RAGs can nick the mcr independently at three different positions, one nick in one molecule within a population. As expected, RAG cleavage at 12RSS resulted in a specific nick at 5' end of the heptamer. Interestingly, irrespective of the pattern of RAG nicks, hairpin formation was observed both in 12RSS and in 12MCR (Fig. 1C, lanes 2 and 6, marked by asterisk). This can explain the bands seen in the bottom strand in both cases as the hairpin formation leads to double-strand breaks in the signal ends (Fig. 1C, lanes 4 and 8). Thus, the above results suggest that mcr is susceptible to RAG nicking.

FIGURE 1. Comparison of RAG cleavage on \textit{BCL2} mcr in presence of Mg\(^{2+}\) and Mn\(^{2+}\). \[^{32}P\]ATP-labeled oligomeric DNA spanning the mcr was incubated with purified GST or MBP cRAGs in a buffer containing Mg\(^{2+}\) or both Mg\(^{2+}\) and Mn\(^{2+}\). Standard 12RSS was used as positive control. Reaction products were resolved on 15% denaturing PAGE and analyzed. A, diagrammatic representation of the oligomeric DNA containing 12RSS (I) and 12MCR (II). B, gel profile showing cRAG (GST) nicking of 12MCR in a buffer containing Mg\(^{2+}\). C, GST cRAG nicking pattern of 12MCR in the presence of Mg\(^{2+}\) and Mn\(^{2+}\). D, gel profile showing cleavage of 12MCR substrate by MBP cRAGs in the presence of Mg\(^{2+}\). E, gel profile showing cleavage of 12MCR by MBP cRAGs in the presence of Mg\(^{2+}\) and Mn\(^{2+}\). Hairpins and RAG nicking products are marked by asterisks and arrows, respectively. M is a 1–nt ladder generated by partial Klenow polymerase digestion of a \[^{32}P\] labeled oligomer. Hairpin markers (M1) and specific molecular weight markers (M2) with the respective sizes are indicated. The sequence of the hairpin marker used for 12RSS is 5'-GATCAGCTGATGCTACGTAGCTATCAGCTGATC-3' and for 12MCR, the sequences are 5'-TCGACTGCTGCAAACGCGTTTGCCAGCAG-3' and 5'-TCGACTGCTGCAAACGCCATGGCGTTTGCAGCAG-3'.

Mechanism of Fragility at BCL2 mcr
To test whether the tag used for protein purification contributed to unusual RAG cleavage at mcr, MBP cRAGs were incubated with 12MCR and 12RSS substrates (Fig. 1A) in the presence of either Mg$^{2+}$/H$^{11001}$ or both Mg$^{2+}$/H$^{11001}$ and Mn$^{2+}$/H$^{11001}$. Results showed that MBP cRAGs were unable to nick the 12MCR in Mg$^{2+}$/H$^{11001}$ (Fig. 1D, lanes 5–8), although nicking at 12RSS was efficient (Fig. 1D, lanes 1–4). However, the addition of 5 mM MnCl$_2$ to MgCl$_2$ buffer resulted in three independent nicks as before at 12MCR (Fig. 1E, lanes 5 and 6). Thus, our results show that the observed RAG nicking at the mcr is an inherent property of RAGs and is unrelated to the tags used.

Because the pattern of RAG nicking seen at the BCL2 mcr was unique, we tested whether these nicks could form hairpins. To test this, prenicked substrates for 12RSS and mcr were synthesized. Upon incubation of RAGs with prenicked 12RSS, we observed the generation of a hairpin product, which was confirmed by the hairpin markers (supplemental Fig. 3A). In the case of mcr, two of three prenicked substrates were able to form the hairpins (the first nick (5’ of the CCAC) and the third nick (between A and C; GCCA ↓ CC)). These results indicate that RAG-induced nicks at the mcr could form hairpins as seen during V(D)J recombination. In addition, we performed dimethyl sulfate modification on the prenicked mcr substrate upon incubation with RAGs. Results showed a mixed pattern of DMS modification in the presence of RAGs, similar to both the specific markers used (HP1 and HP2) and different from the duplex 12MCR substrate, suggesting the formation of multiple hairpins at the mcr (supplemental Fig. 3B, arrows). Primer extension studies using such hairpin structures also confirm formation of such structures (data not shown).

**RAGs Support Nicking at BCL2 mcr at Intracellular Concentrations of MnCl$_2$**—The concentration of Mn$^{2+}$ within the cells is quite low and is estimated to be in micromolar levels (40). Therefore, we were interested in determining the minimum concentration of MnCl$_2$ at which RAGs could nick the BCL2 mcr in vitro. For this, we performed a RAG cleavage assay in the presence of increasing concentrations of MnCl$_2$ along with 5 mM MgCl$_2$. Interestingly, in the case of the top strand, we found the expected three bands from 1 μM MnCl$_2$ onwards, although...
The intensity of the nicks was weak (supplemental Fig. 4, A and B). The hairpin formation could be observed from 1 mM onwards (supplemental Fig. 4A, lanes 5–7), and the corresponding bottom strand cleavage product could also be detected at those concentrations of MnCl₂ (supplemental Fig. 4A, lanes 12–14).
**Mechanism of Fragility at BCL2 mcr**

**RAGs Nick BCL2 mcr Irrespective of Nonamer**—Although we found specific RAG nicking at 12MCR, this cryptic signal does not have an appropriate nonamer (Fig. 2A). Hence, we were interested in testing whether the observed nicking at the mcr is nonamer-independent. Oligomers containing the mcr breakpoint region with CAC, which can mimic a 12RSS, a 23RSS (23MCR, III), or heptamer alone (heptamer MCR, IV) were synthesized (Fig. 2A). Results showed a similar pattern of RAG-specific nicks for all substrates when the top strand was labeled (Fig. 2B, lanes 1, 2, 5, 6, 9, and 10), although the efficiency of the nicking was weaker in the case of heptamer MCR (Fig. 2B). Besides the above three bands, another band of similar intensity was seen in the latter case. However, in none of the other longer substrates was this band observed with such intensity. This suggests that the sequences flanking the mcr heptamer may play some role in dictating the nicking pattern. Interestingly, RAGs
Mechanism of Fragility at BCL2 mcr

FIGURE 6. RAG nicking on LMO2, BCL1, and SCL genes that are associated with chromosomal translocations. A, diagrammatic representation of the oligomeric DNA from LMO2 (XX), BCL1 (XXI), and SCL (XXII) genomic sequences in the physiological orientation containing the cryptic RSS. B, gel picture showing RAG cleavage on top and bottom strands of LMO2 translocation breakpoint region. In the case of LMO2 substrates, RAG cleavage reactions were performed in Mg²⁺-containing buffer in the presence of increasing concentrations of MnCl₂ as indicated. The nicked and the hairpin products are marked by arrows and asterisks, respectively. C, RAG cleavage on oligomeric DNA substrates containing breakpoints of BCL1 and SCL genes. 12RSS was used as a positive control. In all cases, RAG cleavage reaction was performed in a buffer containing 1 mM MnCl₂ in addition to 5 mM MgCl₂. The RAG-specific nick at the 12RSS is marked by an arrowhead. In all panels, M is a 1-nt ladder.

could not nick the heptamer RSS, unlike the heptamer of BCL2 mcr (Fig. 2A, V; 2C, compare lanes 1 and 2 with lanes 3 and 4; supplemental Fig. 5). These results reiterate that BCL2 mcr can be cleaved independent of a nonamer.

An 8-nt CCACCTCT Motif Dictates RAG Nicking at BCL2 mcr—To understand the unique features of the RAG nicking at the mcr, we replaced the nonamer, the heptamer, or both from the mcr backbone with that of the standard RSS (Fig. 3A, VI, VII, and VIII). Upon RAG cleavage, we found that the overall efficiency of cleavage was enhanced by ∼3-fold, when mcr nonamer was replaced with a standard RSS nonamer (Fig. 3B, lanes 3–6). Interestingly, besides the three RAG nicks, an additional band due to a nick at the 5′ end of the CAC (identical to the RSS nicking) was observed (Fig. 3B, lanes 1, 2, 5, and 6). Hairpin formation was about 10-fold higher when compared with 12MCR substrates in this case (Fig. 3B, lanes 3–6). When the mcr heptamer (CACCTCT) was replaced with a heptamer of a standard RSS (CACAGTG) (Fig. 3A, VII), the nicking was observed at the 5′ end of CACAGTG as on a 12RSS (Fig. 3B, lanes 1, 2, 7, and 8). This indicates that the mcr heptamer sequence dictates the unique pattern of nicking, whereas the nonamer does not. When both heptamer and nonamer sequences were replaced with that of the standard signal in the mcr backbone, the nicking occurred exactly at 5′ of the heptamer (Fig. 3B, lanes 9 and 10), although the efficiency of nicking was lower (Fig. 3B, lanes 1, 2, 9, and 10). Overall, these results clearly indicate the indispensability of the mcr heptamer sequence in RAG nicking at the BCL2 mcr.

To study the role of sequences other than the CAC of the heptamer, we mutated one additional nucleotide at a time (Fig. 3A, IX, X, and XI). Results showed that only the wild type mcr heptamer displayed the specific nicking pattern, whereas the cleavage was abrogated when the 4th, 5th, and 6th positions of the heptamer were altered (Fig. 3C, lanes 1–10). The nicking at 5′ to the heptamer was seen only when the entire mcr heptamer was changed to optimal CACAGTG sequence (Fig. 3C, lanes 9–12). Further, to assess the role of CAC on RAG nicking on mcr, its sequences were mutated (Fig. 3A, XII, XIII, and XIV). The results showed that changing the 1st C of CAC did not alter the nicking pattern; however, the cleavage efficiency was markedly lowered (Fig. 3D, lanes 3–6). Other alterations led to abrogation of the RAG nicking at the mcr (Fig. 3D, lanes 7–10). Because one of the RAG nicking positions was the cytosine immediately upstream of the mcr heptamer, we mutated it to thymine, adenine, or guanine (Fig. 3A, XV, XVI, and XVII). Although the RAG nicking at the cytosine was abolished upon mutation, cleavage at the other nucleotides persisted (Fig. 3E). Overall, our data suggest that the mcr heptamer sequence in conjunction with the upstream cytosine plays a critical role in determining the RAG nicking at the BCL2 mcr. Thus, we propose CCACCTCT as the motif responsible for RAG cleavage at the mcr.

To confirm that the 8-nt motif is primarily responsible for the RAG nicking at mcr, its position was shifted downstream on the same backbone or additional nucleotides were introduced just upstream of the motif (Fig. 4A, XVIII and XII). The motif in the new substrates was placed such that the nicks generated by RAGs would occur at 27, 29, and 30 nt. Upon incubation with RAGs, both these substrates showed similar nicking patterns as before (Fig. 4B). Interestingly, in the case of substrate XIX, nicking at the 28th position corresponding to nick at 5′ to CAC (GC ↓ CACC) was also efficient. This could be explained due to the presence of different coding flank sequence upstream of the motif. Thus, results confirm the importance of octameric motif for RAG cleavage at the mcr.

RAGs Can Bind to BCL2 mcr Substrates—RAG binding experiments were performed with standard RSS (12RSS and 23RSS) and mcr substrates (12MCR, 23MCR, and heptamer MCR). In the case of the standard RSS, we could observe RAG binding in both 12RSS and 23RSS (Fig. 5A). Among mcr substrates, the RAG binding was maximum for 23MCR followed by 12MCR (Fig. 5B, lanes 1–4). We could see distinct RAG binding even in the case of heptamer MCR (Fig. 5B, lanes 5 and 6). Specificity of RAG binding at the mcr was studied by adding increasing concentrations of either unlabeled 12MCR or nonspecific double-stranded DNA (AKN46/48) substrate to the reaction. Results showed a dose-dependent reduction in the binding of RAGs to the mcr motif in the presence of unlabeled 12MCR substrate (Fig. 5C). In contrast, the addition of increasing concentrations of a nonspecific substrate, which does not
contain the mcr motif, did not lead to the reduction of the RAG-bound fraction at the mcr (Fig. 5D). Hence, these results confirm the specificity of the RAG binding to the BCL2 mcr.

RAGs Cannot Nick BCL1 and SCL Breakpoint Regions Despite Presence of CAC—Many genes involved in chromosomal translocations associated with leukemia and lymphoma possess cRSS. Despite this, not all such sequences are cleaved by RAGs (31, 32). Because LMO2, a gene associated with t(11;14) translocation in T-cell leukemia, has been shown to support V(D)J recombination (31), we were interested in testing its nicking under conditions used for mcr. The results showed that LMO2 could efficiently nick 5′/H11032 to the heptamer, similar to...
12RSS in Mg$^{2+}$ (Fig. 6, A and B, lanes 8 and 9). A concentration-dependent enhancement in RAG nicking specifically 5' to the heptamer was observed with increasing concentrations of MnCl$_2$ (Fig. 6B, lanes 10–14). Besides, hairpin formation was also observed (Fig. 6B, lanes 10–14), suggesting that LMO2 followed a standard nick-hairpin mechanism even in Mn$^{2+}$. Further, when translocation breakpoint junctions of BCL1 and SCL, which possess CAC, were tested for RAG nicking, results showed no RAG-specific nicks, even in the presence of MnCl$_2$ (Fig. 6, A and C, lanes 3–6). Thus, it appears that the mechanism of RAG nicking at the BCL2 mcr is unique.

Recently, it was reported that a significant number of chromosomal translocations occur at CpG sites (29, 41). The BCL2 mcr has also a CpG immediately upstream of the CCACCTCT, where the majority of the breakpoints from patients are clustered. RAG cleavage studies using oligomeric DNA, mimicking the intermediates of CpG methylation, showed that RAG nicking at the mcr due to CpG mechanism can coexist with the alternate three nick mechanism (supplemental Fig. 6). For details, refer to supplemental Results.

RAG Cleavage at mcr Occurs in Vivo at Very Low Frequency When Paired with an RSS—To detect whether mcr can recombine with an RSS inside cells, a recombination assay was performed as described (Fig. 7A) (31). First, either the genomic fragment or the oligomeric DNA sequences harboring BCL2 mcr were cloned into the appropriate episomes, coupled with either optimal 12RSS or optimal 23RSS (Fig. 7B). REH, a pre-B-cell line expressing RAG proteins, was transfected with episomal constructs and harvested after 48 h. Upon transformation of E. coli with transfection products, we could not observe any chloramphenicol-ampicillin double-resistant colonies, although the number of colonies on ampicillin was high (Fig. 7C). This suggests that the recombination frequency of mcr could be extremely low to be detected by this assay. Transfection products were further screened for rare recombinants by radioactive PCR using [$\gamma$-32P]ATP-labeled primer (Fig. 7D). A recombinant obtained between standard RSS from an inde-
FIGURE 8. Sequencing of breakpoint junctions of mcr and standard RSS following transfection into mammalian cells. A, sequences of the junctions obtained after transfection of pGG51, containing standard recombination signal sequences. The gray triangle represents the standard 12RSS, whereas the black triangle represents the 23RSS. The anticipated positions of breaks are indicated by arrows, and the novel insertions are underlined. The deleted sequences from the coding region are depicted by lowercase.

B, schematic representation and sequences of the junctions obtained upon transfection of episomes, containing an mcr fragment and a standard 12RSS. The mcr is represented by a dashed triangle, and the positions of anticipated breaks are indicated by arrows. The 526- and 120-bp region between the cryptic mcr or the standard RSS and the transcription terminator, respectively, is marked. The deleted sequences from the coding region of both the mcr and the standard RSS are depicted by lowercase. The transcription terminator is indicated by STOP in a box. C, sequences of the breakpoint junctions after recombination between mcr and standard signals. Nucleotide sequence of the clones containing breaks at or upstream of the mcr cryptic heptamer is shown. The strand at the top among three represents the mcr sequence, whereas the lowest strand is the sequence from the episomal backbone adjacent to the standard 12RSS. The middle strand depicts the sequence of the recombinant clone. The sequence alignment between the regions common between either the mcr or the 12RSS with that of the recombinant is marked by vertical lines. The dashed box represents the microhomology region utilized for the repair of the breaks within the cells.
Mechanism of Fragility at BCL2 mcr

pendent transfection of pGG51 was used as a positive control, giving an amplification product of 310 bp (Fig. 7, E–G).

In the case of episomes, pSCR102, pSCR104, and pSCR105, a predominant band (250–280 bp) corresponding to recombination between mcr and RSS was observed consistently (Fig. 7E). In the case of pMN4, a product of ~320 bp was seen after transfection (Fig. 7F, lanes 4–6). We have also transfected the episomes containing 12RSS (pMN28), 23RSS (pMN27), or mcr (pMN29) alone in the same plasmid backbone, as negative controls (Fig. 7, F and G). Upon radioactive PCR, none of these constructs showed the presence of the recombinant band, unlike when the mcr was paired with an RSS (Fig. 7, E–G). The frequency of mcr recombination was calculated by normalizing the intensity of the product obtained in the control and found to be less than/equal to 1 in 1011 molecules (supplemental Table 2). These results suggest that the BCL2 mcr can synapse with a standard RSS within the cells during the recombination process.

Experiments were also performed to test the synopsis of mcr and RSS, using purified cRAGs on radiolabeled 12MCR and cold 23RSS in trans, in the presence of HMGB1, a protein known to facilitate synopsis during recombination (Fig. 7H). Results showed that with increasing concentrations of partner 23RSS in the presence of HMGB1, there was a significant increase in the RAG cleavage efficiency at the mcr, confirming the synopsis between MCR and RSS (Fig. 7, H and J). However, at highest concentrations of unlabeled cold RSS (10 and 12 nM), the RAG cleavage percentage at the mcr decreases due to competition with the partner standard RSS, which reduces the cleavage at the mcr. Standard 12RSS paired with 23RSS was used as a control (Fig. 7H).

The bands resulting due to recombination between mcr and RSS were eluted, PCR-amplified, cloned, and sequenced. Results showed that the bands observed were indeed due to joining of breaks generated at the mcr and standard RSS (Fig. 8 and data not shown). Most of the recombinants showed extensive processing of the ends, unlike those between 12RSS and 23RSS (Fig. 8A). Interestingly, in the case of pMN4, breaks at the mcr were clustered around the cryptic heptamer and not in the remaining 560-bp sequence (Fig. 8B). Closer analysis showed that the joining of these breaks occurred with typical features of nonhomologous end joining; however, some molecules utilized 1–13-nt microhomology for joining (Fig. 8C). Extensive processing was observed prior to joining at the coding sequence of 12RSS (Fig. 8B). Consistent to in vitro studies on mcr, the majority of the clones showed breakpoints at or upstream of the cryptic heptamer (Fig. 8C). Importantly, we noted at least two independent junctions containing breaks exactly at the C present upstream of the CACCTCT sequence (Fig. 8C, Clones 2 and 3). One clone harboring breakpoint exactly at the CpG upstream of mcr heptamer was also observed (Fig. 8C, Clone 1). Another five clones showed breaks upstream to the heptamer, which could be due to end processing (Fig. 8C, Clones 4–8). Taken together, these results suggest that we could recapitulate the mcr breakage process using an episomal system within the cells.

To decipher the role of mcr in the observed recombination process, episomal constructs were made by introducing muta-

| No. of transfections | No. of radioactive PCR | PCR positive for recombination |
|----------------------|------------------------|-------------------------------|
| Wild Type            | 9                      | 30                            | 20                            |
| Mutant I             | 9                      | 30                            | 0                             |
| Mutant II            | 9                      | 30                            | 0                             |

FIGURE 9. Intracellular recombination assay using mutant mcr constructs or mutant RAGs. Appropriate episomal DNA substrates were transfected into REH cells or 293T cells, and DNA was harvested, HinfI-digested, subjected to radioactive PCR, and electrophoresed on agarose gel. A, summary of the number of transfections performed in REH cells and positive recombinants obtained after PCR amplification for the wild type (pMN5) and mutant episomes (pMN15 and pMN13). B, gel profiles showing PCR products of the individual transfection products derived from REH. Representative ethidium bromide-stained gels (top) and autoradiogram of the exposed dried gels (middle) are shown. Lanes 1–3, 4–6, and 7–9 represent PCR amplification of wild type or mutant transfection products in triplicates. The bottom panel represents a loading control, in which PCR amplification of ampicillin resistance gene is done. C, recombination assay using episomal constructs harboring mcr paired with standard 23RSS in 293T cells following overexpression of RAGs. Episome containing the BCL2 mcr sequence was transfected along with wild type (lanes 1 and 2) or active site mutant (D600A RAG1) (lanes 3 and 4) RAG constructs. The gel profiles of the ethidium bromide-stained gel (top) and the autoradiogram (bottom) are shown. P is a positive control. W is a water control, and M is a 2-log DNA ladder. For other details, refer to the legend for Fig. 7.
observed recombination. To verify the role of RAGs in the mcr recombination, we also cotransfected pMN4 along with wild type and D600A (RAG1) mutant RAG overexpression vectors in 293T cells, which do not express native RAGs. Upon radioactive PCR of transfection products, we observed the presence of weak but distinct bands corresponding to the mcr recombination in the case of wild type RAGs as opposed to mutant RAGs (Fig. 9C). Hence, overall our results suggest that mcr undergoes synapsis with an RSS in a RAG-dependent manner during its recombination.

DISCUSSION

We find that RAGs induce three independent nicks at the BCL2 mcr at physiological concentrations of Mg²⁺ and Mn²⁺, in a nonamer-independent manner (Fig. 10). Studies by pairing mcr to a single RSS (12 or 23) or paired RSS (12 and 23) in a recombination assay showed that mcr can undergo synapsis with RSS in the presence of RAGs, which was further confirmed by in vitro experiments. Thus, it is possible that the mcr translocation follows a nick-hairpin mechanism and that the hairpin formed can be processed by nuclease and then joins to partner RSS flanking the J₄ segment (Fig. 10). Our results also suggest that besides classical nonhomologous end joining, at least in a fraction of recombinants, mcr and partner RSS could join by using microhomology-mediated end joining; however, this needs to be tested further.

Previously, it was shown that breaks at the BCL2 MBR and IgH locus were two independent events, wherein the recombination occurs due to a mistake in V(D)J recombination (33). However, this does not appear to be the case in mcr. We were unable to find recombinants when mcr was placed along with a pair of 12- and 23-signals, suggesting that the mechanism of joining at the mcr is different from that of MBR. Moreover, our study shows that mcr fragility is sequence-dependent and not based on non-B DNA structure as seen in the case of MBR (33, 37, 42).

The mechanism of RAG cleavage at the BCL2 mcr differs from the standard V(D)J mechanism in the following ways. Firstly, we find that unlike the standard mechanism, where RAGs nick exactly 5’ to the heptamer, specific nicks of equal efficiency are generated at three different positions in mcr (Fig. 10). This cleavage deviates sharply from the standard pattern and implies an alternate mechanism operating at the mcr. Previously, it was shown that the RAG cleavage at a cRSS in one of the VH segments occurred two bases downstream of the standard nick site (43). However, such a nick could not form a hairpin, and double-strand breaks were formed due to a “nick-nick” mechanism. Therefore, we tested the ability of all three RAG-
induced nicks at the mcr to form hairpins. Interestingly, we noticed that two of the nicks could form hairpins in independent molecules (Fig. 10). Previous studies have indicated that the coding flank sequences affect hairpin formation when A or G was present at 5’ of heptamer (13).

Another major difference between RAG cleavage at the mcr and other cRSS was with respect to the nonamer sequence. The presence of a consensus nonamer has been shown to be essential for RAG binding and directing the nick at 5’ of the heptamer (10). However, we found efficient RAG binding and nicking despite the absence of a canonical nonamer, suggesting that mcr fragility could be independent of nonamer. Interestingly, upon providing the mcr heptamer with standard nonamer, the three nicks persisted, although the efficiency improved. Conversely, when the mcr heptamer was replaced with a standard sequence in the mcr backbone, the cleavage pattern changed from multiple nicks to a single, specific nick, identical to that of RSS. These results strongly suggest the importance of heptamer sequence for RAG cleavage at BCL2 mcr. The presence of a perfect nonamer sequence can only enhance the cleavage efficiency. The nonamer independence of the RAG cleavage observed at the mcr could also be explained by the requirement of Mn$^{2+}$ in these reactions. Previous studies have shown that Mn$^{2+}$ can compromise the need for the nonamer during RAG cleavage (44). Besides, nonamer-independent recombination has also been speculated to occur at endogenous loci at a low level (45).

Sequential mutation of each nucleotide of the heptamer further demonstrated that the mcr heptamer sequence is critical for the observed RAG nicking. Mutation of any nucleotide except the first C of CACCTCT led to a complete abrogation of the observed RAG nicking pattern. Besides, we showed that the cytosine present immediately upstream of CACCTCT is also crucial for the pattern of the observed RAG nicking, suggesting that a CCACCTCT motif is important for RAG cleavage.

Why would RAG cleavage at the mcr be preferred only in the presence of Mn$^{2+}$ when the intracellular concentration of Mg$^{2+}$ is many fold higher? Mutations in RAGs can dictate the choice and affinity for the divalent ion used for cleavage. One such interesting RAG1 mutation is the E719C, which leads to enhanced RAG activity only in the presence of Mn$^{2+}$, whereas there is a loss of the same in Mg$^{2+}$, in B-cell negative SCID patients (46). This can be explained on the basis of the metal ion binding property of cysteine residue. In addition, it is well known that the coordination of sulfur by Mn$^{2+}$ is stronger than the coordination of sulfur by Mg$^{2+}$. Thus, it is possible that presence of such RAG mutations in follicular lymphoma patients can result in the observed RAG cleavage at a cRSS-like BCL2 mcr under the influence of Mn$^{2+}$. This might further explain the lower incidence of patients harboring the t(14;18) translocations at the mcr locus. However, this interesting circumstantial correlation needs to be investigated further.

Another interesting question is why only some CAC-containing sequences get recognized and cleaved by RAGs, whereas other cRSS do not. As discussed above, the sequence of the heptamer could be critical. Studies have also shown the effect of coding flank sequences to have a role in determining the efficiency of RAG cleavage (47, 48). Inside a cell, we can envisage the occurrence of many such CAC sequences interspersed throughout the genome. However, it is also possible that the accessibility of RAGs to such cryptic sites could be restricted due to chromatin organization (49).

Acknowledgments—We thank Dr. B. Choudhary, V. Kari, M. Nishana, A. K. Naik, M. Srivastava, and members of the S. C. Raghavan laboratory for discussions, help, and comments on the manuscript. We thank Dr. P. Swanson for providing MBP RAG constructs.

REFERENCES

1. Rowley, J. D. (2001) Chromosome translocations: dangerous liaisons revisited. Nat. Rev. Cancer 1, 245–250
2. Rabbits, T. H. (1994) Chromosomal translocations in human cancer. Nature 372, 143–149
3. Nambiar, M., Kari, V., and Raghavan, S. C. (2008) Chromosomal translocations at the mcr locus. Biochim. Biophys. Acta 1786, 139–152
4. Korsmeyer, S. J. (1992) Mechanism of the t(14;18) chromosomal translocation: structural analysis of both derivative 14 and 18 reciprocal partners. Proc. Natl. Acad. Sci. U.S.A. 89, 2364–2400
5. Cleary, M. L., and Sklar, J. (1985) Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc. Natl. Acad. Sci. U.S.A. 82, 7439–7443
6. Lewis, S. M. (1994) The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. Adv. Immunol. 56, 27–150
7. Schatz, D. G., and Baltimore, D. (2004) Uncovering the V(D)J recombinase. Cell 116, 5103–5106
8. Schatz, D. G., Oettinger, M. A., and Baltimore, D. (1989) The V(D)J recombinase activating gene, RAG-1. Cell 59, 1035–1048
9. Gellert, M. (2002) V(D)J recombination: RAG proteins, repair factors, and regulation. Annu. Rev. Biochem. 71, 101–132
10. Swanson, P. C. (2004) The bounty of RAGs: recombination signal complexes and reaction outcomes. Immuno. Rev. 200, 90–114
11. Ramsden, D. A., McClane, J. F., van Gent, D. C., and Gellert, M. (1996) Distinct DNA sequence and structure requirements for the two steps of V(D)J recombination signal cleavage. EMBO J. 15, 3197–3206
12. Roth, D. B., Menetski, J. P., Nakajima, P. B., Bosma, M. J., and Gellert, M. (1999) Double-strand signal sequence breaks in V(D)J recombination are blunt, 5’-phosphorylated, RAG-dependent, and cell cycle-regulated. Genes Dev. 7, 2520–2532
13. Lieber, M. R., Yu, K., and Raghavan, S. C. (2006) Roles of nonhomologous DNA end joining, V(D)J recombination, and class switch recombination in chromosomal translocations. DNA Repair 5, 1234–1245
14. Heffernan, M. L., and Tomkinson, A. E. (2005) Mechanism of DNA double-strand break repair by non-homologous end joining. DNA Repair 4, 639–648
15. Nambiar, M., and Raghavan, S. C. (2011) How does DNA break during chromosomal translocations? Nucleic Acids Res. 39, 5813–5825
16. Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E., and Croce, C. M. (1985) The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science 229, 1390–1393
17. Haluska, F. G., Finver, S., Tsujimoto, Y., and Croce, C. M. (1986) The t(8;14) chromosomal translocation occurring in B-cell malignancies results from mistakes in VDJ joining. Nature 324, 158–161

Mechanism of Fragility at BCL2 mcr
Mechanism of Fragility at BCL2 mcr

21. Küppers, R., and Dalla-Favera, R. (2001) Mechanisms of chromosomal translocations in B-cell lymphomas. Oncogene 20, 5580–5594
22. Jäger, U., Böcksör, S., Le, T., Mitterbauer, G., Bolz, I., Chott, A., Kneba, M., Mannhalter, C., and Nadel, B. (2000) Follicular lymphomas’ BCL-2/IgH junctions contain templated nucleotide insertions: novel insights into the mechanism of t(14;18) translocation. Blood 95, 3520–3529
23. Raghavan, S. C., and Lieber, M. R. (2004) Chromosomal translocations and non-B DNA structures in the human genome. Cell Cycle 3, 762–768
24. Ngan, B. Y., Nourse, J., and Cleary, M. L. (1989) Detection of chromosomal translocation t(14;18) within the minor cluster region of bcl-2 by polymerase chain reaction and direct genomic sequencing of the enzymatically amplified DNA in follicular lymphomas. Blood 73, 1759–1762
25. Albinger-Hegyi, A., Hochreutener, B., Abdou, M. T., Hegyi, I., Dours-Zimmermann, M. T., Kurrer, M. O., Heitz, P. U., and Zimmermann, D. R. (2002) High frequency of t(14;18)-translocation breakpoints outside of major breakpoint and minor cluster regions in follicular lymphomas: improved polymerase chain reaction protocols for their detection. Am. J. Pathol. 160, 823–832
26. Galli, N., Cleary, M. L., and Sklar, J. (1987) Human follicular lymphomas: identification of a second t(14;18) breakpoint cluster region. Haematol. Blood Transfus. 31, 167–171
27. Buchonnet, G., Jardin, F., Jean, N., Bertrand, P., Parmentier, F., Tison, S., Lepretre, S., Contentin, N., Lenain, P., Stamatoullas-Bastard, A., Tilly, H., and Bastard, C. (2002) Distribution of BCL2 breakpoints in follicular lymphoma and correlation with clinical features: specific subtypes or same disease? Leukemia 16, 1852–1856
28. Weinberg, O. K., Ai, W. Z., Mariappan, M. R., Shum, C., Levy, R., and Arber, D. A. (2007) “Minor” BCL2 breakpoints in follicular lymphoma: frequency and correlation with grade and disease presentation in 236 cases. J. Mol. Diagn. 9, 530–537
29. Tsai, A. G., Lu, H., Raghavan, S. C., Muschen, M., Hsieh, C. L., and Lieber, M. R. (2008) Human chromosomal translocations at CpG sites and a theoretical basis for their lineage and stage specificity. Cell 135, 1130–1142
30. Lewis, S. M., Agard, E., Suh, S., and Czysz, L. (1997) Cryptic signals and the fidelity of V(D)J joining. Mol. Cell. Biol. 17, 3125–3136
31. Raghavan, S. C., Kirsch, I. R., and Lieber, M. R. (2001) Analysis of the V(D)J recombination efficiency at lymphoid chromosomal translocation breakpoints. J. Biol. Chem. 276, 29126–29133
32. Marculescu, R., Le, T., Simon, P., Jaeger, U., and Nadel, B. (2002) V(D)J-mediated translocations in lymphoid neoplasms: a functional assessment of genomic instability by cryptic sites. J. Exp. Med. 195, 85–98
33. Raghavan, S. C., Swanson, P. C., Wu, X., Hsieh, C. L., and Lieber, M. R. (2004) A non-B-DNA structure at the Bcl-2 major breakpoint region is cleaved by the RAG complex. Nature 428, 88–93
34. Raghavan, S. C., Hsieh, C. L., and Lieber, M. R. (2005) Both V(D)J coding ends but neither signal end can recombine at the bcl-2 major breakpoint region, and the rejoining is ligase IV-dependent. Mol. Cell. Biol. 25, 6475–6484
35. Raghavan, S. C., Swanson, P. C., Ma, Y., and Lieber, M. R. (2005) Double-strand break formation by the RAG complex at the bcl-2 major breakpoint region and at other non-B DNA structures in vitro. Mol. Cell. Biol. 25, 5904–5919
36. Naik, A. K., Lieber, M. R., and Raghavan, S. C. (2010) Cytosines, but not purines, determine recombination activating gene (RAG)-induced breaks on heteroduplex DNA structures: implications for genomic instability. J. Biol. Chem. 285, 7587–7597
37. Nambar, M., Goldsmith, G., Moorthy, B. T., Lieber, M. R., Joshi, M. V., Choudhary, B., Hosur, R. V., and Raghavan, S. C. (2011) Formation of a G-quadruplex at the BCL2 major breakpoint region of the t(14;18) translocation in follicular lymphoma. Nucleic Acids Res. 39, 936–948
38. Sharma, S., Choudhary, B., and Raghavan, S. C. (2011) Efficiency of non-homologous DNA end joining varies among somatic tissues, despite similarity in mechanism. Cell. Mol. Life Sci 68, 661–676
39. Kumar, T. S., Kari, V., Choudhary, B., Nambar, M., Akila, T. S., and Raghavan, S. C. (2010) Anti-apoptotic protein BCL2 down-regulates DNA end joining in cancer cells. J. Biol. Chem. 285, 32657–32670
40. Sigel, A., and Sigel, H. (eds) (2000) Manganese and Its Role in Biological Process, Vol. 37, Marcel Dekker, New York
41. Tsai, A. G., Yoda, A., Weinstock, D. M., and Lieber, M. R. (2010) t(X;14)(p22;q32)/t(Y;14)(p11;q32) CRLF2-IGH translocations from human B-lineage ALLs involve CpG-type breaks at CRLF2, but CRLF2/P2RY8 intrachromosomal deletions do not. Blood 116, 1993–1994
42. Raghavan, S. C., Chastain, P., Lee, J. S., Hegde, B. G., Houston, S., Langen, R., Hsieh, C. L., Haworth, I. S., and Lieber, M. R. (2005) Evidence for a triplex DNA conformation at the bcl-2 major breakpoint region of the t(14;18) translocation. J. Biol. Chem. 280, 22749–22760
43. Rahman, N. S., Godderz, L. J., Stray, S. J., Capra, J. D., and Rodgers, K. K. (2006) DNA cleavage of a cryptic recombination signal sequence by RAG1 and RAG2: implications for partial V(H) gene replacement. J. Biol. Chem. 281, 12370–12380
44. Santagata, S., Aidinis, V., and Spanopoulou, E. (1998) The effect of Me2+ cofactors at the initial stages of V(D)J recombination. J. Biol. Chem. 273, 16325–16331
45. Reth, M. G., Jackson, S., and Alt, F. W. (1986) VHDJH formation and DJH recombination during pre-B differentiation: non-random usage of gene segments. EMBO J. 5, 2131–2138
46. Li, W., Chang, F. C., and Desiderio, S. (2001) Rag-1 mutations associated with B-cell-negative scid dissociate the nicking and transesterification steps of V(D)J recombination. Mol. Cell. Biol. 21, 3935–3946
47. Gerstein, R. M., and Lieber, M. R. (1993) Coding end sequence can markedly affect the initiation of V(D)J recombination. Genes Dev. 7, 1459–1469
48. Boubnov, N. V., Wills, Z. P., and Weaver, D. T. (1995) Coding sequence composition flanking either signal element alters V(D)J recombination efficiency. Nucleic Acids Res. 23, 1060–1067
49. Yancopoulos, G. D., and Alt, F. W. (1985) Developmentally controlled and tissue-specific expression of unrearranged VH gene segments. Cell 40, 271–281

MARCH 16, 2012 • VOLUME 287 • NUMBER 12

JOURNAL OF BIOLOGICAL CHEMISTRY

8701