Stability and Strand Asymmetry in the Non-B DNA Structure at the bcl-2 Major Breakpoint Region*

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The t(14;18) translocation involving the Ig heavy chain locus and the BCL-2 gene is the single most common chromosomal translocation in human cancer. Recently we reported in vitro and in vivo chemical probing data indicating that the 150-bp major breakpoint region (Mbr), which contains three breakage subregions (hotspots) (known as peaks I, II, and III), has single-stranded character and hence a non-B DNA conformation. Although we could document the non-B DNA structure formation at the bcl-2 Mbr, the structural studies were limited to chemical probing. Therefore, in the present study, we used multiple methods including circular dichroism to detect the non-B DNA at the bcl-2 Mbr. We established a new gel shift method to detect the altered structure at neutral pH on shorter DNA fragments containing the bcl-2 Mbr and analyzed the fine structural features. We found that the single-stranded region in the non-B DNA structure observed is stable for days and is asymmetric with respect to the Watson and Crick strands. It could be detected by oligomer probing, a bisulfite modification assay, or a P1 nuclease assay. We provide evidence that two different non-B conformations exist at peak I in addition to the single one observed at peak III. Finally we used mutagenesis and base analogue incorporation to show that the non-B DNA structure formation requires Hoogsteen pairing. These findings place major constraints on the location and nature of the non-B conformations assumed at peaks I and III of the bcl-2 Mbr.

The t(14;18) translocation involving the Ig heavy chain locus and the BCL-2 gene is the single most common chromosomal translocation in human cancer. It is the most important of all lymphoid cell translocations because follicular lymphomas account for nearly half of all non-Hodgkin’s lymphomas. Analyses of the breakpoint regions have shown that the BCL-2 gene on chromosome 18 is joined to the \( J_b \) segment of the immunoglobulin heavy chain locus on chromosome 14, while the reciprocal junction consists in fusion of a \( D_h \) segment from the IgH locus on chromosome 14 with the remaining 3’ BCL-2 untranslated region on chromosome 18 (1–9).

The major breakpoint region (Mbr)\(^1\) on chromosome 18 is in the 3’ untranslated region of the BCL-2 gene (3, 5, 10–13). The bcl-2 Mbr is distinctive among breakpoint regions (14). Nearly 80% of t(14;18) events occur in the 150-bp bcl-2 Mbr (11). The breakpoints are not at a specific nucleotide but rather are dispersed over a 150-bp region with relatively sharp boundaries (11, 12, 15). Within the 150 bp, there are three peaks of breakage, each about 15–20 bp in size (12). The characteristic precision and frequency profile within the bcl-2 Mbr strongly suggest some strong local regional specificity to the bcl-2 Mbr (14).

We and others have previously provided experimental evidence that the RAG complex can mistakenly cleave at pseudosignals in lymphoid translocations involving LMO2, Tgα-1, and SIL (16–19) but not at the bcl-2 Mbr (15, 18). Instead the 150-bp bcl-2 Mbr assumes a non-B form DNA structure within human cells at 20–30% of alleles (15). The structure contains distinctive regions of single-strandedness that correspond well to the regions of translocation frequency among patients. We were able to reproduce the single-stranded character of the bcl-2 Mbr on human minichromosomes, indicating that this distinctive property does not rely on the sequence of the surrounding DNA. We were also able to reproduce the single-stranded character when the Mbr was carried on prokaryotic plasmids (replicated in Escherichia coli), indicating that eukaryotic proteins are not necessary to generate the altered DNA structure. We further showed that RAGs are the nuclease responsible for the bcl-2 translocation (15).

In the present study we showed that the bcl-2 Mbr adopts a non-B DNA conformation at neutral pH that is sufficiently stable to discern by gel electrophoresis. The conformation is stable for days, and Mg\(^{2+}\) and spermidine stabilize it. We used single-stranded DNA oligonucleotides to probe which regions of the structure are available for hydrogen bonding, and these probing provided an independent demonstration of the asymmetry in single-strandedness between the top and bottom strands. Substitution of 7-deazaadenine or 7-deazaguanine for dA or dG, respectively, blocked the ability to form the non-B structure, indicating the importance of Hoogsteen hydrogen bonding. A 3-bp exchange between the top and bottom strands of the duplex reduced the non-B structure formation, which is also consistent with Hoogsteen hydrogen bonding. Circular dichroism documented the structure by yet another method. These studies confirmed the non-B structure, provided new information about its longevity, and provided higher resolution information about its asymmetry. Based on this, we conclude that the structure requires Hoogsteen hydrogen bonding, and we can begin to consider the types of possible non-B DNA conformations that form at the bcl-2 Mbr.

Experimental Procedures

Enzymes, Chemicals, and Reagents—Sodium bisulfite and other chemical reagents were purchased from Sigma. Restriction enzymes were purchased from New England BioLabs. Dideoxynucleotides were synthesized at the University of Southern California Keck School of Medicine facility. Labeled nucleotides were purchased from PerkinElmer Life Sciences. The primers were synthesized by a commercial supplier (Integrated DNA Technologies). The major breakpoint region (Mbr) was synthesized at the University of Southern California Keck School of Medicine facility.

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\(^1\) The abbreviations used are: Mbr, major breakpoint region; ss, single-stranded; nt, nucleotide(s).

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and DNA-modifying enzymes were obtained from New England Biolabs (Beverly, MA). Radioisotope-labeled nucleotides were purchased from PerkinElmer Life Sciences.

PCR Amplification of Shorter DNA Fragments with bcl-2 Mbr or Control Regions—The fragments of interest were PCR-amplified from the genomic DNA or from plasmid DNA containing the bcl-2 Mbr with appropriate primers and using standard PCR conditions (Fig. 1). The 231-bp control regions were amplified from the ampicillin or chloramphenicol-treated DNA with appropriate primers using standard PCR conditions. The 85- and 270-bp fragments were “sewn” together by a PCR polymerase in a volume of 10 μl. Amplification was carried out for 35 cycles. The annealing temperature used was standardized according to the primer used in each specific PCR. The PCR products were resolved on an agarose gel, and the correct sized fragments were recovered using a Gene Clean kit (BIO101, Vista, CA). In the case of the 3-bp mutant bcl-2 Mbr PCR fragment, the PCR was generated by using pSCR41 as the amplification template (see below).

To generate deazaadenine- or deazaguanine-containing PCR fragments, the PCRs were carried out in the presence of 200 μM dCTP, 200 μM dTTP, 40 μM dATP, 40 μM dGTP, 160 μM deazaadenine, and 160 μM deazaguanine.

Self-stranded DNA (ssDNA) was obtained using primer extension with a 248-bp PCR fragment containing bcl-2 Mbr as the template. Primer extension reaction was performed by mixing the following on ice: DNA sample (10 ng), 1× Thermo polymerase buffer (1×, 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.8), and 0.1% Triton X-100), 4 μM MgSO₄, 4 μM dNTPs, 0.25 μM (γ-32P)ATP end-labeled oligonucleotide, and 0.4 unit of exon (5′)-polymerase (New England Biolabs, Beverly, MA) after layering with mineral oil. Linear amplification products were used for mutagenesis: SCR89, 5′-CCTACTGGTACCTTCTTCCTGGC-3′; SCR90, 5′-CCCTGTTGACAATTAATCATCG-3′; SCR89, 5′-GGGAAGCCCTCCTGCCCTGCGG-3′; SCR90, 5′-GGCCCAAGGCTCCCTGCTCCGGC-3′; SCR114, 5′-GGAGGAGGCCCAGAGCCCTC-3′; SCR115, 5′-AGGAGGCTCTGGAAGCCACATGGAAGA-3′; SCR117, 5′-GATCTATTCCCTGCTGCTTCCGGC-3′; SCR118, 5′-GCACATTGAACTTCACTTACCCGG-3′; SCR119, 5′-GAATATAGGAGGCTCCCTGCGG-3′; and SCR90, 3′-GCCTCGCTGCTCCGGC-3′.

The oligomers were purified using 8–15% denaturing polyacrylamide gel electrophoresis. The complementary oligomers (or DNA strands) were annealed in 100 mM NaCl and 1 mM EDTA by heating in a beaker of boiling water for 10 min followed by slow cooling as indicated.

Gel Shift Assay—The gel-purified PCR fragments of interest were applied in 6% denaturing polyacrylamide gel and reactions were then terminated by adding the stop dye. The linear amplified PCR products were run on a native 10% polyacrylamide gel in a volume of 10 μl. Amplification was carried out for 35 cycles. The reaction products were resolved on an agarose gel, and the correct sized fragments were recovered using a Gene Clean kit (BIO101). Purified PCR products were cloned using the TOPO-TA cloning kit (Invitrogen). Plasmid DNA from each clone of interest was purified using the Gene Elute plasmid miniprep kit (Sigma). Sequencing reactions were carried out using the SequiTHERM Excel II sequencing kit (Epigenetics) and MWG thermal cycler model Primus 96 Plus (MWG Biotech, High Point, NC). Oligo-primed sites are subject to bisulfite conversion, and, as a result, some molecules lose the ability to serve as PCR templates. However, this source of background conversion is rare and random in location.

Mutagenesis at the bcl-2 Mbr—Mutagenesis was carried out using a standard PCR protocol with mutant sequence primers (20, 23). To make a CCC to GGG mutation just upstream of peak 1, two oligomers carrying the mutated sequences were designed (SCR89 and SCR90) from the chosen region of the bcl-2 Mbr. SCR89 was in a PCR with XW9 to generate an 85-bp fragment. SCR90 was used in a PCR with XW8 to generate a 270-bp fragment. The 85- and 270-bp PCR products overlap precisely in the 53-bp zone containing the intended, introduced mutation. The 85- and 270-bp fragments were “sewn” together by a PCR containing both fragments and the outermost primers (XW8 and -9) to generate a larger (322-bp) fragment that contains the introduced mutation. After SaII digestion and purification, the PCR fragment was cloned into the SalI site of the pSCR33. The resulting plasmid is pSCR41. The presence of the mutation was further verified by sequencing of the entire Mbr and the surrounding region. A 231-bp fragment containing the mutated Mbr was PCR-amplified, gel-purified, and used for the gel shift assay as described above.

Oligomer Annealing Assay—For the oligomer annealing assay, the end-labeled DNA (10 nm) containing the non-B structure was incubated with different concentrations of oligomers in a buffer containing 50 mM Tris (pH 7.2), 10 mM MgCl₂, 100 mM NaCl, and 10% sucrose for 4 h at 22 °C. The following oligomer concentrations were used for annealing experiments: 1 nm, 10 nm, 100 nm, 1 μM, and 10 μM. Then the products were resolved on a 6% native polyacrylamide gel. The gel was dried and exposed to a PhosphorImager screen, and the signal was detected using PhosphorImager 445Si (Amersham Biosciences) and analyzed with ImageQuant software (Version 5.0).

P1 Nuclease Assay—For the P1 nuclease assay, 10 nm substrate DNA (or ssDNA) was incubated with the following concentration of P1 nuclease in NEB buffer 2 (10 mM Tris (pH 7.9), 10 mM MgCl₂, 50 mM NaCl, and 1 mM dithiothreitol) at 37 °C for 20 min. The reaction products were mixed with 1× DNA dye and immediately loaded onto a native polyacrylamide gel (6%) as described above. P1 concentrations used for the study are 0.0001, 0.001, 0.01, 0.1, 0.2, 0.4, 1, and 3 units or as described in the figure legend. The gel was dried and exposed to a PhosphorImager screen, and images were obtained as described above.
RESULTS

The bcl-2 Mbr Non-B Structure Is Stable—We have shown previously that a non-B DNA structure forms at the bcl-2 Mbr (15). However, such a structure could conceivably be either intermittent or stable. If intermittent, it might change conformation between B form and non-B form DNA rapidly. To determine whether the Mbr non-B structure is stable, we permitted various lengths of DNA containing the Mbr to form the structure under different conditions (Fig. 1). Some of the conditions favor the non-B structure, and others do not based on our previous analyses (15). We then resolved the DNA molecules using native PAGE. If the exchange rate between the B and non-B form of the DNA was on the order of seconds or minutes, then it would not be possible to distinguish them based on their movement through a gel matrix. If the exchange rate was on the order of hours or longer, then separation might be possible.

The [γ-32P]ATP end-labeled PCR fragment (231 bp) was incubated at 37 °C overnight in a buffer containing 50 mM Tris (pH 7.2), 10 mM MgCl2, 100 mM NaCl, and 100 μM spermidine (buffer B) to facilitate the structure formation or in a buffer of 10 mM Tris (pH 7.2) (buffer A). The samples were then resolved by 6% native PAGE. If the exchange rate between the B and non-B form of the DNA was on the order of seconds or minutes, then it would not be possible to distinguish them based on their movement through a gel matrix. If the exchange rate was on the order of hours or longer, then separation might be possible.

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Samples stored at 4 °C for over 2 days or at 22 °C for 14 h also maintained the non-B structure. Based on these studies, we conclude that the non-B DNA structure is stable for hours to days. The non-B structure is favored by Mg2+.

The bcl-2 Mbr Non-B Structure Is Dependent on DNA Sequence and on Specific Types of Hydrogen Bonding—Would alterations in DNA sequence affect the non-B structure? Because we find maximal single-strandedness at peaks I and III of the Mbr (15), the structure formation must involve sequences at or adjacent to these peaks. Therefore, we mutated CCC–GGG on the top strand and vice versa on the bottom strand by using a standard mutagenesis protocol (see “Experimental Procedures”). Such a change would not alter B form...
DNA, but it may alter any of a number of non-B DNA structures because in most such alternative configurations a purine base must form Hoogsteen hydrogen bonds with one strand while simultaneously forming Watson-Crick hydrogen bonds with another strand (and pyrimidines cannot do this). An end-labeled 231-bp PCR fragment containing the Mbr (mutant bcl-2 Mbr) was then studied in the gel shift assay. When buffer A was supplemented with Tris, MgCl2, NaCl, and spermidine to the concentrations equivalent to buffer B (buffer B = 50 mM Tris-HCl (pH 7.2), 10 mM MgCl2, 100 mM NaCl, and 100 μM spermidine), then the shifted species is more distinct (lanes 5 and 6). Lanes 7–12 are treated identically to lanes 5 and 6 with the following modifications. Lanes 9 and 10, 7-deazaadenine and 7-deazaguanine incorporation in the PCR step (see “Experimental Procedures”) indicated by “DEAZA.” Lanes 11 and 12, samples equivalent to those in lanes 5 and 6 were phenol/chloroform-extracted and ethanol-precipitated. The sample was resuspended in 10 mM Tris (pH 7.2), 10 mM EDTA to chelate the Mg2+ buffer, and the solution was heated at 80 °C for 5 min. M designates the markers, which were prepared by end-labeling of the 50-bp DNA ladder.

The Surrounding DNA Length Influences the Non-B DNA Structure at the Mbr—We were interested in determining the length and sequence effects of the DNA surrounding the bcl-2 Mbr. We PCR-amplified DNA fragments of various lengths (130, 231, 248, 263, and 339 bp) containing the Mbr region (Fig. 1). The gel-purified fragments were [γ-32P]ATP end-labeled and incubated in the Mg2+ buffer as described above and resolved by native PAGE. The results showed that in the case of the 263-bp fragment, we could see a new shifted species upon incubation (Fig. 3A, lanes 5 and 6), suggesting the presence of an altered DNA structure. As in the case of the 231-bp fragment, we could destroy the structure by incubating at 80 °C in the presence of EDTA (Fig. 3A, lanes 3 and 4). Similar results were obtained when a 248-bp fragment or a 339-bp fragment was used for the gel shift assay (data not shown). However, when a similar experiment was performed with the 130-bp fragment, there was no band with a shifted mobility compared with duplex DNA (Fig. 3B). The sequences from all three peaks of the Mbr were present in the 130-bp fragment, although the region just upstream of peak I was not included (Fig. 1). This suggests that the sequences just upstream of peak I are critical for the structure formation.

Control experiments were performed by amplifying PCR fragments (231 bp) either from the amplicillin or the chloramphenicol gene. We did not find any shift in the mobility of the duplex DNA bands in these cases when using the same incubation conditions as described above (Fig. 3, C and D, compare lanes 1 and 2 with lanes 3 and 4), indicating that the observed structure formation is specific to the bcl-2 Mbr fragment.

Detection of a Non-B DNA Structure by the Bisulfite Modification Assay—Since we could detect an altered DNA structure on shorter DNA fragments at the bcl-2 Mbr by a gel shift assay, we wondered whether we could detect the single-strandedness, if any, using the bisulfite modification assay on such shorter DNA fragments. Nucleophilic attack by the bisulfite anion can only occur from above or below the plane of the cytosine ring, and this is blocked by stacking in duplex DNA but much less so in single-stranded DNA. The reaction generates uracil wherever there is an unstacked cytosine. Upon PCR amplification and sequencing the bisulfite-converted cytosine can be read as thymine, and this allows the detection of single-stranded regions (15). For this purpose, we PCR-amplified a 200-bp segment containing the bcl-2 Mbr. We then denatured the purified DNA, slowly reannealed the strands of this 200-bp fragment, and subjected it to bisulfite treatment as described earlier (15, 22). The bisulfite modification assay results showed that, even in this case, the non-B structure formed. Here the conversion frequency was 10.1% (Fig. 4A), and 7% of the molecules (27.5% C to T conversion frequency) adopted the single-stranded character within the Mbr (Fig. 4B). In this case, the single-strandedness resided primarily at peak I and downstream of it (Fig. 4C). Importantly, when the same PCR fragment was subjected to the bisulfite modification immediately after the PCR step, no single-strandedness was detected. The overall conversion fre-
quency was reduced to 3.7%, which is comparable to the background conversion (Fig. 4A). This demonstrates that increased bisulfite conversion is in no way intrinsic to this region.

Like the 200-bp PCR fragment, a similar bisulfite conversion frequency was obtained when a 248-bp PCR fragment containing the bcl-2 Mbr was allowed to form the structure by incubating in 50 mM Tris (pH 7.2), 10 mM MgCl₂, 100 mM NaCl, and 100 μM spermidine at 37 °C for 16 h. In this case also, 7.1% of the molecules could form the structure (Fig. 4B), and we found single-strandedness at peak I or upstream of it (Fig. 4D). These data indicate that some non-B structure formation can take place even on a PCR fragment as short as 200 bp, although the frequency of molecules adopting the non-B structure was much lower than compared with purified plasmid DNA, minichromosomes, or chromosomal DNA (15). Because we used acidic pH (5.2) for bisulfite treatment, could the pH of the reaction itself have caused the structure formation? We did not find bisulfite conversion on control PCR fragments. Moreover, by gel shift assays, we saw the Mbr structure formation at pH 7.2 (Fig. 2). These data indicate that some non-B structure formation can take place even on a PCR fragment as short as 200 bp, although the frequency of molecules adopting the non-B structure was much lower than compared with purified plasmid DNA, minichromosomes, or chromosomal DNA (15). Because we used acidic pH (5.2) for bisulfite treatment, could the pH of the reaction itself have caused the structure formation? We did not find bisulfite conversion on control PCR fragments. Moreover, by gel shift assays, we saw the Mbr structure formation at pH 7.2 (Fig. 2). In addition, in earlier studies, we have seen an amount of bisulfite reactivity at pH 6.5 that is quite similar to that at pH 5.2 (15).

Since we saw a lack of structure formation by the gel shift assay on the 231-bp fragment when dNTP analogues (deazaadenine and deazaguanine) were used, we examined the deazaadenine- and deazaguanine-containing PCR products with the bisulfite modification assay. Only a 4.3% bisulfite frequency, which is equivalent to background conversion frequency, was observed at the Mbr region (Fig. 4A). Moreover, among such background level molecules, we could not find any molecules with continuous stretches of conversion (Fig. 4B). This result further suggests that the non-B DNA structure formation at bcl-2 Mbr requires Hoogsteen pairing.

Analysis of the bcl-2 Mbr by Circular Dichroism—Spectroscopic methods of analysis might be expected to show some difference between the duplex Mbr conformation and the non-B form. Such an approach is more complicated than it might first seem because only 10–15% of the molecules form the non-B structure based on the gel and bisulfite studies. Moreover only about 10–30% of the bases in such molecules might participate in the non-B structure; the remainder (lateral portions) of these molecules are in duplex conformation based on bisulfite modification analysis. This means that only about 3% of the bases in a given population of molecules might be base-paired in a manner that deviates from B form DNA. Such a small fraction is most likely to give only a marginal spectroscopic difference.

Despite the anticipated difficulties, we carried out CD analysis using the 231-bp PCR fragment containing the bcl-2 Mbr, the 231-bp fragment containing the 3-bp mutation at the Mbr, a control DNA region of 231-bp length after incubating in a buffer containing 50 mM Tris (pH 7.2), 10 mM MgCl₂, 100 mM sodium acetate, and 100 μM spermidine. The CD spectra were obtained using a Jasco J-810 Spectropolarimeter. UV wavelengths covered were from 210 to 310 nm.
Compared with control and mutant PCR fragment spectra, the spectra of the bcl-2 Mbr were slightly different (Fig. 5). There was a difference in the position of absorption peaks in the 270–275 nm range. This difference was verified and was highly reproducible (data not shown). In addition, spectra due to the Mbr sequences were different in amplitude as well.
Hence, we found a small but consistent increase in absorbance at 275 nm for sequences that include the Mbr relative to control sequences. As was perhaps obvious from the data above and elsewhere (15), the CD spectra in the present study indicated that the non-B structure formation was quite inconsistent with even small amounts of Z-DNA. (Z-DNA has a negative ellipticity at 275 nm for sequences that include the Mbr relative to control DNA.) Hence, we found a small but consistent increase in absorbance at 275 nm and a positive ellipticity at -265 nm with a crossover point at ~280 nm (25, 26.).

Identification of the Single-stranded Region of the bcl-2 Mbr by Probing with Oligomers—Since we could distinguish the non-B form DNA from duplex DNA by PAGE, we tested for the annealing of oligomers to possible unpaired regions at various positions along the bcl-2 Mbr. This would help us to predict the nature of the structure formed and the nucleotide locations. We designed different oligomers of 25–30 nt in length covering the Mbr region (Fig. 6A). The oligomers were then incubated with end-labeled Mbr fragments (231 or 248 bp) possessing the non-B structure in a buffer containing 50 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, 100 mM NaCl, and 100 μM spermidine by incubating for 4 h at 37 °C. MBR1 and MBR2 are two independent DNA preparations and CD spectra sets of bcl-2 Mbr sequences. Mut1 represents the CD spectra of the 231-bp mutant Mbr sequences (see Fig. 2 legend for details of mutation). Con160161 is the CD spectrum of a control DNA fragment of size 231 bp that does not contain the Mbr deg. degrees.

Although most of the oligomers caused no mobility change in the shifted 231-bp fragment, two oligomers (SCR95 and SCR113) converted the shifted species to a slower migrating form, which we refer to as the supershifted species (Fig. 6B). The slower migration of the supershifted species was presumably due to the higher mass attributable to the annealed oligomer. Importantly there was no difference in the position of the duplex band throughout these studies (Fig. 6B). The oligomer annealing was further confirmed by performing a titration of concentrations of oligomers SCR95 and SCR113. For this purpose, different concentrations of oligomers (1 nM, 10 nM, 100 nM, 1 μM, and 10 μM) were incubated with 10 nM substrate DNA as above. Results showed that in the case of SCR95, there was no supershift at the lowest (1 nM) oligomer concentration (Fig. 6C). At 10 nM, a portion of the original shifted band was supershifted to a new position. However, some shifted species still remained at the original position. At 100 nM and higher SCR95 concentrations, all of the shifted band migrated at the supershifted position (Fig. 6C). Comparable results were obtained with the other oligomer (SCR113) (Fig. 6C). Thus, titration experiments with the oligomers SCR95 and SCR113 demonstrated the gradual change from the shifted band to the supershifted band. These results indicate that, in the case of the 231-bp fragment, at two positions (downstream of peak I and at peak III), a considerable length of the DNA (~20 nt) is single-stranded.

Another interesting feature of this experiment is that although SCR95 and SCR113 annealed with the shifted species, the oligomers designed to hybridize to the opposite strand (SCR112 and -115, respectively) did not anneal with the shifted species (Fig. 6B). This indicates that although one strand is single-stranded, the other strand is paired. Hence the non-B structure forming at the Mbr does not simply represent melting apart of the two strands.

In another set of oligomer annealing studies, a 248-bp DNA fragment containing the bcl-2 Mbr was examined. Interestingly, in contrast to the 231-bp fragment, here we observed annealing of the oligomer upstream of peak I (Fig. 7A), matching well with the predominant region of single-strandedness seen in genomic DNA at the Mbr based on bisulfite sequencing methods (15). Specifically oligomer probes (SCR93, SCR94, or SCR114) designed to anneal immediately upstream of peak I caused disappearance of the shifted band. In this case, the supershifted band migrated at the lower edge of the duplex band and could not be discerned (Fig. 7A). (The difference in mobility of the supershifted band relative to the duplex in Figs. 6B and 7A was due to the difference in length (231 versus 248 bp.).) Therefore, these studies using the 231- or 248-bp fragment with the bcl-2 Mbr indicated that, at peak I, there are at least two major conformations of non-B structure that exist. The structure formation at the 248-bp fragment represents the majority of the single-strandedness that we noted by bisulfite modification analysis. The structure associated with the 231-bp fragment represents a less frequent conformation (10–15% of molecules) based on bisulfite sequencing.

Similar oligomer annealing experiments with the 263-bp fragment showed single-strandedness upstream and downstream of peak I and at peak III (Table I and data not shown), while in the case of the 339-bp fragment, sites of annealing (single-strandedness) were at peak III and downstream of peak I (Table I and data not shown). These results suggest that the length of DNA (but not the sequence) surrounding the Mbr may influence how much a given linear fragment favors one of these two major non-B structures that form at peak I (see “Discussion”).

Asymmetric Nature of Single-strandedness—Since we could detect the single-strandedness in the shifted DNA species, we performed a P1 nuclease assay on this DNA. The substrate DNA (231 bp) was incubated with increasing concentrations of...
FIG. 6. Oligomer screening to detect single-stranded regions on shifted non-B form DNA on a 231-bp DNA fragment. A, the exact sequence positions of the different oligomers (oligonucleotides) used are depicted. The 150-bp Mbr sequences are shown in two rows, and the point of continuation is indicated by a hyphen. The sequence of the oligomer is indicated by the long arrow either above or below the respective sequences. The breakpoint peaks are indicated by three lines (peaks I, II, and III, respectively) between the top and bottom strand sequences. B, the oligomers (1 µM) were incubated with a 231-bp PCR fragment containing an altered structure in a buffer containing 50 mM Tris-HCl (pH 7.2), 10 mM MgCl₂,
annealing was demonstrated by performing a titration of concentrations of oligomers SCR95 and SCR113. For this purpose, different concentrations of the oligomer. Oligomers were incubated as above. The shifted and supershifted bands are indicated by arrows. Oligomers showing supershifts are indicated by a star (*) . B, the P1 sensitivity at the non-B structure of the 248-bp PCR fragment was examined. For the P1 assay, DNA was incubated with the indicated concentrations of P1 nuclease at 37 °C for 20 min. The reaction products were immediately loaded on a native polyacrylamide gel, and the species were resolved. The shifted band generated due to structure formation and the new bands resulting from the P1 sensitivity are indicated by arrows.

**TABLE I**

Comparison of structure formation among the various DNA fragments

The size of the DNA fragment used is indicated in the first column. The two major conformations of structure existing at peak I are indicated as I-α or I-β. The presence or absence of the structure is indicated with + or − signs.

| Size (bp) | Source  | Structure | Peak I | Peak II |
|----------|---------|-----------|--------|---------|
| 231      | Mbr     | I-α       | +      | −       |
| 263      | Mbr     | I-β       | +      | −       |
| 248      | Mbr     | I-α       | −      | +       |
| 248      | Mbr     | I-β       | +      | −       |
| 250      | Mbr     | I-α       | −      | +       |
| 260      | Mbr     | I-β       | +      | −       |
| 263      | Mbr     | I-α       | −      | +       |
| 339      | Mbr     | I-β       | +      | −       |

* The structure formation was detected by bisulfite modification assay.

imply that the P1 is generating nicks on both strands. We interpret this to mean that the P1 is nicking anywhere within the large region of single-strandedness on the top strand and at more focal unpaired nucleotides on the bottom strand. (Some DNA degradation observed with 1 and 3 units of the P1 on duplex DNA is due to nonspecific cleavage at higher concentrations of the enzyme.) These results indicate that there are specific single-stranded regions present on the non-B DNA but not on the duplex DNA.

The P1 nuclease assay was also performed on the 248-bp fragment. In this case also, only the shifted band was sensitive to P1 at the optimal concentration (0.1 unit) (Fig. 7B). This suggests that, similar to the 231-bp fragment, here also at least some unpaired nucleotides are present on both strands, although the majority of the single-strandedness is on the top strand consistent with the oligomer annealing assay (Fig. 7A).

On a 6% native PAGE, we compared the mobility of the shifted band on a 248-bp Mbr fragment with the mobility of the top and bottom strand alone (248-nt ssDNA) derived from the same region. Results showed that the mobility of the ssDNA was quite different compared with the mobility shift caused by the non-B DNA structure on the bcl-2 Mbr fragment (Fig. 8B, compare lanes 5 and 6 with lanes 7 and 8, and data not shown). Because we saw a specific pattern of P1 nuclease cleavage on the bcl-2 Mbr shifted band resulting in the generation of new fragments (Figs. 7B and 8A), we adopted the same strategy to compare the cleavability of the ssDNA with that of the shifted band of Mbr. Results showed that, at 0.001 unit of P1, the
248-nt ssDNA was cut into many fragments, suggesting that it is cleaved at many sites. At 0.01 unit of P1 or higher, the ssDNA completely disappeared, indicating that the ssDNA was degraded by the single-strand-specific nuclease (Fig. 8B). However, in the case of the shifted band of the Mbr, P1 cleaved away the single-stranded regions and generated a band of faster mobility (Fig. 8B, lanes 1, 2, and 3, arrowhead), and this species persisted at P1 concentrations that were 10–40-fold higher (Fig. 8B and see above).

Therefore, the P1 nuclease study, in conjunction with the oligomer annealing assay on DNA fragments of various sizes, revealed that at peak I and/or peak III, one of the strands is unpaired for at least 20 nucleotides (Fig. 9). The other strand has less single-strandedness perhaps because it is hydrogen bonded (Watson-Crick or Hoogsteen) with some other region of the Mbr (Fig. 10). Thus, our results using chemical and physical methods demonstrated that a considerable amount of single-strandedness exists at peaks I and III of the bcl-2 Mbr, suggesting the existence of a stable non-B DNA structure at that region in vitro.

DISCUSSION

Here we document that the non-B DNA structure formed at the bcl-2 Mbr is stable and is detectable using a gel shift assay at pH 7.2. The single-stranded regions of this non-B DNA structure could be documented by methods involving the bisulfite modification assay, an oligomer annealing assay, a P1 nuclease assay, and circular dichroism. Hence we have shown three new methods for analysis of the non-B structure beyond the bisulfite modification assay described by us previously (15).

Features of the Non-B DNA Structure—The DNA fragments containing the 150-bp Mbr (200, 231, 248, 263, or 339 bp) could form a non-B DNA structure upon incubation in a Mg$^{2+}$/H$_{100}$ buffer at pH 7.2. This non-B structure was eliminated if EDTA was present to chelate divalent cations and was enhanced by 10 mM Mg$^{2+}$/H$_{100}$ and spermidine. Incorporation of 7-deazaguanine and 7-deazaadenosine, which do not affect Watson-Crick base pairing but prevent Hoogsteen base pairing (24, 25), abolished the structure formation. Exchange of a GGG sequence on the top strand with a CCC on the bottom strand of peak I of the bcl-2 Mbr also markedly reduced the structure. Such an exchange would not be expected in a Watson-Crick B form helix. Therefore, all of the different lines of evidence further confirm that the band of altered mobility is indeed non-B DNA and is present in about 10–15% of molecules when it is formed in vitro.

Could the shifted band that we saw be due to mutations induced during the PCR amplification? We did not see the structure formation on control PCR fragments (Fig. 3, C and D). Moreover, if this were the case, the PCR-induced mutations should be random, and a specific 3-nt substitution is unlikely to have an effect. In addition, while utilizing the bisulfite modification assay, we sequenced thousands of molecules of these PCR fragments, and we found only rare mutations. Most importantly, we used DNA fragments containing the bcl-2 Mbr derived from plasmid DNA (by restriction digestion), and we still we saw the shifted band (non-B conformation).

Is the shifted band that we saw attributable to denatured ssDNA? We showed that this was not the case. First, if the shifted band is ssDNA, then one would not expect to see any difference in the presence or absence of Mg$^{2+}$ buffer. Second, if the shifted band is ssDNA, it should not disappear in the
presence of EDTA and heat. Third, a 3-nucleotide mutation or the presence of deazaadenine/deazaguanine would not make any difference in a ssDNA conformation (Fig. 2). Fourth, if the shifted band at the bcl-2 Mbr were due to ssDNA, then there is no reason not to see it on the 130-bp Mbr fragment (Fig. 3 B) or on control DNA fragments (Fig. 3, C and D). Fifth, we saw a specific oligomer annealing pattern at the Mbr; one would rather expect to bind all oligomers to either the top or bottom strand if it were ssDNA (Figs. 6 B, 7 A, and 9). Sixth, P1 nuclease is known to cut the ssDNA into many fragments as we have shown in Fig. 8 B. But the shifted Mbr band was cut by P1 at specific positions, generating distinguishable fragments (Figs. 7 B and 8). Seventh, the regions of single-strandedness detected on these DNA fragments matched reasonably well with the single-stranded regions observed at the bcl-2 Mbr on chromosomal DNA, minichromosome DNA, and plasmid DNA (15). Finally the mobility of the non-B Mbr did not correspond to the mobility of the top or bottom strand run on the same native gel (Fig. 8 B).

Although we know that a non-B DNA structure exists at the Mbr region in vivo and in vitro (15), we do not know the exact nature of the structure. Our studies using oligomer probing on the 231-bp fragment containing bcl-2 Mbr, oligomer SCR113 binds downstream of peak I, indicating single-strandedness at the top strand in this region (Fig. 6 for more details). On the same DNA fragment, oligomer SCR95 binds to peak III, indicating single-strandedness on the top strand of this region. The single-strandedness observed in these cases could be due to I-β and III non-B DNA structure forms (see Fig. 10 C and "Discussion" for details). On a 248-bp DNA fragment, oligomer annealing experiments (oligomers SCR93, SCR94, and SCR114) show that single-strandedness exists upstream of peak I on the top strand (see Fig. 7 A for more details). The bisulfite modification studies also showed single-stranded regions at that location on the same 248-bp DNA fragment (see Fig. 4 for more details). A proposed I-α structure may be responsible for the single-strandedness at this region (see Fig. 10 B and "Discussion" for details). In both panels, the 150-bp Mbr sequences are presented as two rows separated by a hyphen. Polarity of the top and bottom strands are indicated. The peaks I, II, and III are indicated by three horizontal lines between top and bottom strands, peak I being in the top row and II (left) and III (right) being in the bottom row. Single-stranded regions are marked.

![Pattern and location of single-stranded regions observed by oligomer annealing and bisulfite sequencing studies.](image-url)

Fig. 9. Pattern and location of single-stranded regions observed by oligomer annealing and bisulfite sequencing studies. On a 231-bp DNA fragment containing bcl-2 Mbr, oligomer SCR113 binds downstream of peak I, indicating single-strandedness at the top strand in this region (See Fig. 6 for more details). On the same DNA fragment, oligomer SCR95 binds to peak III, indicating single-strandedness on the top strand of this region. The single-strandedness observed in these cases could be due to I-β and III non-B DNA structure forms (see Fig. 10 C and "Discussion" for details). On a 248-bp DNA fragment, oligomer annealing experiments (oligomers SCR93, SCR94, and SCR114) show that single-strandedness exists upstream of peak I on the top strand (see Fig. 7 A for more details). The bisulfite modification studies also showed single-stranded regions at that location on the same 248-bp DNA fragment (see Fig. 4 for more details). A proposed I-α structure may be responsible for the single-strandedness at this region (see Fig. 10 B and "Discussion" for details). In both panels, the 150-bp Mbr sequences are presented as two rows separated by a hyphen. Polarity of the top and bottom strands are indicated. The peaks I, II, and III are indicated by three horizontal lines between top and bottom strands, peak I being in the top row and II (left) and III (right) being in the bottom row. Single-stranded regions are marked.
non-B structure is asymmetric in its single-strandedness (Figs. 6–8). The non-B DNA structure most consistent with these observations is a triplex (H-DNA). In addition, an antibody specific to GC-rich triplexes is able to bind to non-B conformation of the bcl-2 Mbr. Two of the many possibilities for triplex formation at peak I are shown in Fig. 10, B and C. However, higher resolution studies are required to determine the precise hydrogen and Hoogsteen bonding of specific DNA conformations.

Because we could observe the structure formation even on PCR fragments containing the bcl-2 Mbr, what might be the initial nucleation event? We know that in these fragments the structure formation occurs in only 10–15% of the molecules. In mammalian chromosome (20–30%), minichromosomes (40–50%), or supercoiled plasmid DNA (40–50%), the structure formation occurs on a somewhat higher percentage of the molecules. Although only a lower percentage of the molecules form the structure on shorter DNA fragments, the region of structure formation matches reasonably well in all four cases (PCR DNA, mammalian genomic DNA, human minichromosomal DNA, and E. coli supercoiled plasmid DNA) even by using different chemical or physical probing methods. Because it is possible that supercoiling exists in mammalian chromosomes (46), minichromosomes, and plasmid DNA, this may contribute to the nucleation in those cases. However, on a linear fragment, there is no supercoiling, but we found a small fraction of molecules with the non-B structure. The initial nucleation event in this case may be by random bending of the DNA in the following manner (Fig. 10A). Upon random bending, the strand that forms the Hoogsteen pairing is positioned adjacent to the duplex DNA in the presence of divalent cations and spermidine at pH 7.2 whereupon the H-DNA formation takes place. It is known that GC-rich tracts have rapid opening kinetics (47). The fourth strand becomes single-stranded (Fig. 10). This explains the strand asymmetry and single-strandedness detected by oligomer annealing on the 248-bp Mbr fragment (Fig. 8A), the P1 nuclease analysis (Fig. 8B), and the asymmetry of the bisulfite reactivity. Therefore, when there is no supercoiling, 10–15% may be the maximum level of structure formation that forms at equilibrium.

Multiple Conformations at Peak I of the bcl-2 Mbr—Our experiments with oligomer probing on DNA fragments of various lengths containing the bcl-2 Mbr showed that the single-strandedness can exist either upstream or downstream of peak I (Fig. 9). This indicates that at least two discrete conformations of non-B structure are present at peak I that we call conformations I-α (248-bp fragment) and I-β (231-, 200-, and 399-bp fragments) for purposes of discussion (Table I and Fig. 10, B and C). The I-α conformation showed single-strandedness upstream of peak I (Fig. 10B), and the I-β conformation showed single-strandedness downstream of peak I (Fig. 10C). The 263-bp fragment showed annealing of an oligomer both upstream and downstream of peak I on the bottom strand of the Mbr. We interpret this to be a third conformation that occurs least commonly and that has features of both I-α and I-β. These results suggest that, depending on the size of the surrounding DNA, the precise conformation may change between at least two (and perhaps three if including the I-α, I-β form) structures. Although these short linear PCR fragments could recapitulate many features of the chromosomal non-B structure that we have reported in our previous study (15), the length of DNA surrounding the Mbr on these fragments differed from one another and may yield non-B structures that are similar but not identical in all features to that observed in the chromosomal non-B structure.

Fig. 10. Possible nucleation of a triplex DNA at the bcl-2 Mbr. A, the initial nucleation event for a hypothetical triplex at peak I of the Mbr may rely on transient bending and/or breathing near peak I. The transient location of breathing could extend into a larger region of Hoogsteen pairing while simultaneously generating a displaced single-stranded region on the top strand. The polarity of the top and bottom strands are indicated using filled ovals. The precise hydrogen and Hoogsteen bonding of specific DNA conformations match reasonably well in all four cases (PCR DNA, mammalian genomic DNA, human minichromosomal DNA, and E. coli supercoiled plasmid DNA) even by using different chemical or physical probing methods. Because it is possible that supercoiling exists in mammalian chromosomes (46), minichromosomes, and plasmid DNA, this may contribute to the nucleation in those cases. However, on a linear fragment, there is no supercoiling, but we found a small fraction of molecules with the non-B structure. The initial nucleation event in this case may be by random bending of the DNA in the following manner (Fig. 10A). Upon random bending, the strand that forms the Hoogsteen pairing is positioned adjacent to the duplex DNA in the presence of divalent cations and spermidine at pH 7.2 whereupon the H-DNA formation takes place. It is known that GC-rich tracts have rapid opening kinetics (47). The fourth strand becomes single-stranded (Fig. 10). This explains the strand asymmetry and single-strandedness detected by oligomer annealing on the 248-bp Mbr fragment (Fig. 8A), the P1 nuclease analysis (Fig. 8B), and the asymmetry of the bisulfite reactivity. Therefore, when there is no supercoiling, 10–15% may be the maximum level of structure formation that forms at equilibrium.

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**Fig. 10. Possible nucleation of a triplex DNA at the bcl-2 Mbr.** A, the initial nucleation event for a hypothetical triplex at peak I of the Mbr may rely on transient bending and/or breathing near peak I. The transient location of breathing could extend into a larger region of Hoogsteen pairing while simultaneously generating a displaced single-stranded region on the top strand. B, proposed triplex structure (I-α) formation at peak I to explain the single-strandedness upstream of peak I on a 248-bp DNA fragment containing the bcl-2 Mbr. The filled ovals represent Hoogsteen hydrogen bonding. The bottom two strands are simply regular Watson-Crick hydrogen bonded. The top strand is unpaired, accounting for the observed bisulfite reactivity (Fig. 4) and oligomer annealing specifically at that region (Fig. 7). Peak I is indicated by a curved line on the right between the strands. The polarity of the top and bottom strands are indicated using 5’ and 3’. The region of single-strandedness detected by oligomer annealing is marked. C, proposed alternative triplex structure (I-β) at peak I explaining the single-strandedness observed downstream of the peak I on a 231-bp fragment (by oligomer annealing assay, see Fig. 6) or on a 200-bp fragment by bisulfite modification assay (see Fig. 4). Single-stranded regions are represented by unpaired sequences and marked. Hoogsteen base pairing is indicated by filled ovals. The position of peak I is indicated by a curved line between the strands (right).
mosome. The I-α form corresponds to what we observed most commonly in the human genomic DNA for the Mbr non-B structure at peak I. Importantly, certain PCR fragments (231- and 339-bp Mbr) preferred the I-β form at peak I, and the I-β form is detectable in the mammalian genomic DNA, albeit at a frequency that is 5-fold lower than the I-α form (15).

It is interesting to point out that, although we could observe the structure formation on DNA fragments of various sizes, we could not see the altered structure formation when we used a 130-bp fragment for the gel shift assay. As is obvious from the size, although the 130-bp fragment included all three peaks, it did not contain the upstream sequences of peak I. This suggests that just the fragment of DNA containing the three peaks of chromosomal translocation predisposition may not be sufficient for the structure formation. Since we always saw at least one oligomer annealing around each of the peak I and peak III regions, it will be of interest to see how the upstream sequences of peak I influence the structure formation at peak III.

In our previous study, we reported that RAGs are the nuclease responsible for the t(14;18) translocation. Now studies are underway in our laboratory to characterize what features of the enzyme responsible for the t(14;18) translocation.2

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Stability and Strand Asymmetry in the Non-B DNA Structure at the bcl-2 Major Breakpoint Region
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