Mechanism of the Chromosomal Translocation t(14;18) in Lymphoma: Detection of a 45-Kd Breakpoint Binding Protein

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LYMPHOMAS REPRESENT an interesting model for the mechanism of chromosomal translocation because they rearrange their immunoglobulin genes during normal development and are prone to acquire specific translocations associated with this attempt. The most prominent examples are the t(8;14) in Burkitt’s lymphoma and the t(14;18) in follicular lymphoma. The latter translocation moves the BCL-2 proto-oncogene from chromosome 18 into the Ig heavy chain (IgH) locus on chromosome 14. The majority of translocations on 18q21 is extremely well focused on two hotspots: 60% of follicular lymphomas break within only 150 bp in the major breakpoint region (mbr) in exon 3, while 25% break within the minor breakpoint cluster region (mcr) more than 20 kb downstream. The reciprocal translocation event takes place in early B cells at the time of the D to J rearrangement. The breakpoints on the derivative 14 chromosome occur at one of six heavy chain joining (JH) regions, while the breakpoint on the der (18) chromosome occurs at a diversity (DH) segment of the Ig locus. Moreover, N-segments are present at both of these sites. This argues strongly that V(D)J-recombinase is involved in the generation of the breakpoints on chromosome 14. However, the BCL-2 regions on chromosome 18 possess no convincing heptamer-spacer-nonamer motifs surrounding these breakpoints. Thus it is likely that an additional mechanism is involved. The mbr contains several sequence elements that could potentially infer genetic instability or facilitate homologous recombination: (1) A polypurine-polypyrimidine stretch with a potential to form alternative DNA-structures (H-DNA or triplces) is present in both mbr and mcr. These polypurine tracts can act as targets for mammalian endonucleases like endo R1 and endo G12 and are found around other genetic hot spots. Similar repeats of G-rich tetranucleotides (TGGG, TGAG) have been implicated in immunoglobulin class switching. (2) A human Alu direct repeat is part of the purine rich sequence. Alu elements have been found around breakpoints of the c-MYC and BCR genes. (3) The sequence contains a Chi-like octamer similar to the recombination signal in Escherichia coli and the human minisatellite core sequence, which could activate a RecBCD-like mechanism.

This prompted us to investigate the functional implications of these elements on the structural vulnerability and protein binding capacity of the BCL-2 breakpoint regions. We found that the mbr contains a single nuclease-sensitive site and is a preferred target for an endonucleolytic activity present in early B cell extracts. Using gel retardation assays and Southwestern blots we have further identified a 45 Kd protein (termed bp45), which binds to the CHI-like polypurine-polypyrimidine tract within both mbr and mcr as well as to corresponding Ig sequences and may thus play a role in homologous site-specific recombination associated with the development of human lymphomas.

MATERIALS AND METHODS

Preparation of nuclear extracts. Crude nuclear extracts were prepared by the method of Heberlein and Tijan with a few modifications. Cells growing in log-phase were incubated in a hypotonic buffer (1.15 mmol/L Hepes pH 7.6, 10 mmol/L KCl, 5 mmol/L MgCl2, 0.1 mmol/L EDTA, 1 mmol/L DTT, 10 mmol/L Na2S2O3, 1 mmol/L PMSF) for 15 minutes and lysed by several passages through a 25 G hypodermic needle. After centrifugation (850g X 10 minutes, 4°C) the nuclear pellets were resuspended in buffer II (same as buffer I except for a higher KCl concentration, 115 mmol/L) and lysed by adding 10% vol/vol of 4 mol/L (NH4)2SO4, pH 7.8. After centrifugation (100,000g X 70 minutes, 4°C) the supernatants were collected and proteins were precipitated by stepwise addition of (NH4)2SO4 to a final concentration of 50% and a 70-minute spin at 10,000 rpm. The pellets were then resuspended in buffer III (25 mmol/L HEPES pH 7.6, 50 mmol/L KCl, 12.5 mmol/L MgCl2, 0.1 mmol/L EDTA, 1 mmol/L DTT, 10% glycerol) and dialyzed for 2 X 90 minutes. The extracts were quantitated for total protein and frozen in small aliquots at -70°C. The integrity of each extract was controlled by sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis.
Nuclease assays. Nuclei for S1 digestion were prepared as in step one above except that NP-40 (0.5%) was added to the hypotonic buffer instead of PMSF. The pellets were washed in S1 nuclease digestion buffer (30 mmol/L NaCl, 3 mmol/L ZnSO4, 60 mmol/L sodium acetate pH 4.5) twice and digested with 0.1 or 5 µg chromatin (based on DNA) for 60 minutes at 37°C. DNA was isolated, digested with EcoRI and run on agarose gels (10 µg DNA). The Southern blots were hybridized with a 461 bp EcoRI-Rsal fragment from BCL-2 exon III (Fig 1D).

The endogenous nuclease activity from nuclear extracts was tested according to the method of Gottlieb and Muzynka. One microgram of uncut plasmid (pR15.6; Fig 1D) was incubated with crude extracts (5 to 20 µg) for 60 minutes at 37°C in a reaction mixture containing 20 mmol/L Tris-HCl pH 7.5, 5 mmol/L MgCl2 and 1 mmol/L DTT. The samples were digested with proteinase K, extracted with phenol-chloroform, precipitated with ethanol, digested with EcoRI, run on agarose gels, transferred, and hybridized with the 461 bp EcoRI-Rsal probe.

Gel retardation assays. DNA protein binding reactions (25 µL) were carried out in 20 mmol/L Hepes pH 7.9, 75 mmol/L NaCl, 0.5 mmol/L EDTA, 1 mmol/L DTT and 5% glycerol in the presence of 2.5 µg poly (dI-C) (Boehringer Mannheim, West Germany). The samples were preincubated for 10 minutes at 4°C and 10 minutes at RT before the S' end labeled probes (1 × 106 cpm/sample) were added. The binding reaction was then allowed to proceed for 20 minutes at RT. Crude nuclear extracts, 14 pg, were used for each reaction. The 68 bp probe was prepared by SacII digestion of a 222 bp PCR fragment from the breakpoint of SU-DHL-6. The sequence of the oligonucleotides is as follows (upper strand shown): oligo a (5'TTAGAGAGTGTCTAACGTG 3'), oligo b (5'GCGGTTT- TCAACAGACCCCAACGAG 3'), oligo c (5'ATTCCG- CTCTGCCCTCCCTCGC 3' plus EcoRI linker), oligo d (5'GGGAGGAGTGAAAGGAA 3'), oligo e (5'TCGGGGAGG- CGAACAAAA 3').

The reaction products were separated on 6% to 12% polyacrylamide gels, blotted onto Whatman 3MM paper, and exposed to Kodak XAR film.

Western and Southwestern analysis. SDS-polyacrylamide electrophoresis of the nuclear extracts (150 µg/sample) was performed according to Laemmli. The proteins were electrophobted onto Hybond-C membranes (Amersham, Arlington Heights, IL). DNA protein hybridizations were done in the same binding buffer as the gel retardation assays. Poly (dI-C) (50 µg/mL) was used to block unspecific binding. The blots were preincubated shaking gently for 20 minutes at 4°C and 20 minutes at RT before 0.5 to 1 × 106 cpm/mL were added. After hybridization for 120 minutes at RT they were washed 3 × 10 minutes in 1X and 1 × 10 minutes in 0.5X binding buffer. The 68 bp probe was used end labeled without modification. The short oligos were concatenated and labeled by nick translation.

RESULTS

The mbr of BCL-2 is a nuclease S1-sensitive region. In order to investigate the chromatin structure around the mbr we digested isolated nuclei from a B cell line with S1 nuclease. Indeed a single band that maps to the mbr appears on a Southern blot at higher concentrations of S1. This indicates that the mbr is susceptible to chromatin alterations (Fig 1A).

Detection of an mbr-specific nuclease activity in crude nuclear extracts from early B cells. We then tested extracts from early B cells for the existence of endogenous nucleolytic enzymes that could potentially affect the integrity of the mbr in a living cell. A plasmid (pR15.6, Fig 1D) containing a 5.6-
kb EcoRI genomic fragment from BCL-2 exon 3 was used as substrate for an in vitro nuclease assay. Incubation of the supercoiled plasmid with crude nuclear extracts from FLEB 14-8 (pro-B) or REH (pre-B) revealed three major cleavage sites (Fig 1B and C). The major sites are located 0.5 kb, 1.2 kb, and 2.2 kb downstream of the 5′ EcoRI site. The location of the 1.2-kb band coincides with the 150 bp mbr. The 0.5-kb and 2.2-kb bands have not been characterized yet, but the three nucleolytic activities can be separated from each other by Con-A fractionation (not shown). This indicates that various nucleases are present in the crude extracts. Nevertheless, the experiments prove the existence of a specific endonuclease that preferentially cleaves the major site of chromosomal breakage of the BCL-2 gene. S1 nuclease produces a similar pattern indicating that both enzymes may share certain sequences or structural requirements for their substrates (Fig 1C). When compared with a mature B cell line (SU-DHL-6), the pre B cell REH shows a much greater nucleolytic potential (Fig 1E).

Having established the existence of a site-specific nuclease, we next examined the mbr for its capacity to bind proteins that could either make the chromatin accessible to enzymes or be a candidate for a nuclease themselves.

Identification of a protein binding site within the mbr. The fact that the breakpoints in the mbr are tightly clustered suggests that sequences especially at the borders of the mbr are necessary to focus the chromosomal break. Our experiments concentrated on the 5′ border because it contains potential target sequences for endogenous nucleases as well as combinatorial sequences. Using gel retardation assays we defined a 68 bp double-stranded DNA fragment across the 5′ mbr border that forms a major DNA protein complex (Cl) and a minor upper complex (c2) that migrates slower than Cl and is only seen on long exposures with some extract preparations (Fig 2A). Complex formation can be competed with the cold 68-bp fragment at higher concentrations.

When the 68-bp fragment is divided into three oligonucleotides (Fig 2B) the following pattern emerges: the 5′ portion (oligo a) does not bind proteins and is ineffective in competition experiments; the middle portion (oligo b) forms a minor complex migrating with (c2); the 3′ oligo c shows strong binding and forms a major and a minor complex like the

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**Fig 2.** Gel retardation assays with crude nuclear extracts from REH. (A) Probes are indicated below; lane 0, no extract; lanes 1 through 3, 14 μg extract; competition with cold 68-bp fragment: lane 1, 0; lane 2, 10 ng; lane 3, 100 ng. (B) Lanes 1, 3, 5, no extract; lanes 2, 4, 6, 14 μg extract. (C) Oligo c, competition experiment: lane 0, no extract; lanes 1 through 6, 14 μg extract; competitors: lane 2, oligo c 10 ng; lane 3, oligo c 50 ng; lane 4, oligo c 100 ng; lane 5, oligo a 100 ng; lane 6, 262 bp β-actin fragment 100 ng. (D) Sequence of the 5′ mbr border (most 5′ breakpoint reported is indicated by arrow).
Fig 3. Southwestern blots showing a single 45-Kd protein. REH nuclear extracts were probed with the 68-bp fragment (lane 1), the concatenated oligo c (mbr) (lane 2), or the concatenated oligo d (mcr) (lane 3).

Table 1. Sequence Homologies: Definition of an 11-bp Binding Motif (boxed)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Location/BP</th>
</tr>
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<tbody>
<tr>
<td>GGGAGGACGGGG</td>
<td>BCL-2 mbr (oligo c) t(14;18)</td>
</tr>
<tr>
<td>GGGAGGAg1GGG</td>
<td>BCL-2 mcr (oligo d) t(14;18)</td>
</tr>
<tr>
<td>GGGAGGACGGGG</td>
<td>IgDH t(14;18)</td>
</tr>
<tr>
<td>gtag</td>
<td>J-C Intron (oligo e) t(14;18)</td>
</tr>
<tr>
<td>GXAGGAGGG</td>
<td>Human minisatellite core</td>
</tr>
<tr>
<td>tggg</td>
<td>Human Alu direct repeat</td>
</tr>
<tr>
<td>GGGAGGACGaaa</td>
<td>CHI (E. coli)</td>
</tr>
<tr>
<td>GGGAGGAgGGGG</td>
<td>BCR Intron t(9;22)</td>
</tr>
<tr>
<td>GGGAGGcGAa</td>
<td>C-ABL t(9;22)</td>
</tr>
<tr>
<td>GGGAGGctGAa</td>
<td>C-MYC t(2;8)</td>
</tr>
<tr>
<td>GGGAGGcctGAa</td>
<td>BCL-1 t(11;14)</td>
</tr>
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68-bp fragment. The unlabeled oligo c inhibits DNA protein complex formation while oligo a as well as a 262-bp control fragment from β-actin have no effect in cross competition experiments indicating that binding is specific (Fig 2C). The 20 bp sequence of oligo c maps to the homopurine-homopyrimidine stretch (Fig 2D).

A 45-Kd protein binds to the major and minor breakpoint regions of BCL-2 in early B cells. When used as a DNA probe on a Southwestern blot the 68-bp fragment binds to a single 45-Kd protein (Fig 3). The concatamerized oligo c still binds to this protein suggesting that the binding site is contained within 20 bp.

Interestingly, a computer search showed a long stretch of homology between oligo c and a region within the second breakpoint region of BCL-2 (mcr) (Table 1). Indeed, the corresponding oligo d binds to a 45 Kd protein. Moreover, the concatamerized oligos c and d can inhibit the formation of complexes between the 68 bp fragment and nuclear proteins (Fig 4A), providing evidence that the same 45-Kd protein binds to both breakpoint regions of BCL-2.

Fig 4. Complex formation between the 68-bp fragment and REH extracts is inhibited by sequences from both BCL-2 breakpoint regions as well as from the Ig heavy chain locus. (A) competitor, oligo c (mbr): lane 0, no protein; lane 1, 14 μg extract, no competitor; lane 2, 100 ng; competitor, oligo d (mcr): lane 0, no protein; lane 1, no competitor; lane 2, 10 ng; lane 3, 100 ng; lane 4, 200 ng. (B) competitor, oligo e (J-C intron): lane 0, no protein; lane 1, no competitor; lane 2, 10 ng; lane 3, 100 ng; lane 4, 200 ng; competitor, oligo a (control): lane 5, no competitor; lane 6, 10 ng; lane 7, 100 ng; lane 8, 200 ng.
Homologous sequences from the \( \text{IgH} \) locus cross-compete for protein-binding. We also found homologous sequences in the DH region \(^{14}\) and in the JH-CH intron about 100 bp downstream of J6 (M. Seto, personal communication; and ref 35) on chromosome 14 (Table 1). An oligo derived from the latter sequence (oligo c) is also able to compete with the 68-bp fragment (Fig 4B) and binds to a 45-Kd protein on Southwestern blots (not shown).

Binding specificity. The DNA sequence requirements for the binding of the protein were further specified using mutated mbr-oligonucleotides in a competition assay (Fig 5). Mutations downstream of the 11 bp core sequence (boxed in Table 1) do not influence the ability to compete with the mbr (lanes 2 and 3). Interestingly, destruction of both GGGAGG-motifs present in oligo c results in a loss of function, indicating that this sequence is particularly important (lane 4). Moreover, the protein does not simply bind to a poly(dG)-poly(dC) tract (lane 5).

Tissue distribution. Nuclear extracts from cell lines representing various tissues were tested with the 68 bp fragment (Fig 6). As expected, the protein is present in abundance in early B cells, but is also widely distributed, although some cell lines (HL-60) contain very low levels.

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**DISCUSSION**

We have investigated the molecular basis of illegitimate recombination involving the BCL-2 breakpoint regions. Our results show that the mbr is prone to endonucleolytic cleavage as demonstrated by its SI sensitivity. SI nuclease preferentially cleaves homopurine-homopyrimidine tracts that are able to form alternative DNA structures \(^{36}\) such as Z-DNA \(^{37}\) or H-DNA.\(^{38}\) Mammalian nucleases like Endo R,\(^{37}\) Rep68,\(^{38}\) or Endo G\(^{12}\) have been shown to cleave these (dG\(_n\))(dC\(_n\)) sequences. Our data clearly indicate the existence of an endogenous nuclease present in early B cells that cleaves sequences within the mbr. The target of this mammalian nuclease is most likely the homopurine-homopyrimidine stretch at the 5’ border of the mbr, since it produces the same 1.2 kb band (Fig 1C) as SI nuclease in the in vitro assays. In addition to the nuclease-sensitive site we have identified a 45-Kd protein that binds to the G-C rich oligo c. This 20-bp sequence contains the human minisatellite consensus \(^{14,24,39,40}\) (Table 1) that is homologous to the procaryotic activator of recombination (CHI). This element stimulates recombination via a protein-specific pathway (RecBC enzyme) in *E. coli*.\(^{41}\) We find it therefore intriguing that the
45-Kd protein binds to analogous sequences in the BCL-2 breakpoint regions as well as to sequences on chromosome 14 (Table I). We have defined an 11 bp motif from the mbr that contains the best matches with the mcr as well as the IgH D and J regions. The protein-DNA binding is sequence specific as shown by the competition experiments, but will tolerate some base pair mismatches. This may facilitate heteroduplex formation and make it likely that secondary structure is important. However, a simple poly(dG.dC) oligonucleotide as well as an oligo mutated within the two GGGAGG-motifs will not compete for binding. It is interesting to note that Collick and Jeffrie9 have identified a murine protein of approximately 40 Kd (Msbp-1) that binds to minisatellite repeats with similar specificity. We also note that the sequence contains a 9 bp match with a human alu direct repeat.10

The binding of a protein to sequences that can stimulate homologous, site specific recombination in conjunction with a nuclease is very indicative of the involvement of a human RecA-RecBC like recombination/repair system. Thus, our findings may also be important for other chromosomal translocations. Interestingly, we found corresponding sites at the t(9;22) breakpoint partners21 as well as at a t(2;8).19 Most significantly, the binding sequence is also present at a t(11;14) breakpoint.22 This translocation occurs in lymphoma and joins the BCL-1 locus to the IgH gene by a similar mechanism as the t(14;18) (Table 1).

While the binding protein is highly expressed in early B cells and binds to specific sites, the tissue distribution as well as the specificity of the nuclease is still unclear. The different nucleolytic pattern of REH and SU-DHL-6 may simply be due to an increased presence of nucleases in immature cells. On the other hand its activity may be modulated by bp45, which could target the nuclease to the breakpoint sequences.

Based on our observations we propose the following model for the t(14;18) (Fig 7): (1) The translocation is initiated by base pairing between the CHI-like sequences in one of the BCL-2 breakpoint regions and their homologous counterparts in the IgH D or J regions according to the strand invasion model. Bp45 could possibly act as a clamp through its capacity to bind to sequences on both chromosomes. Alternatively it could bind to heteroduplexes, or, like RecA, it could be involved in the formation or resolution of triple helices43 or tetraplexes44 generated in the process of homologous recombination. (2) The fact that both breakpoint sequences are in close contact with each other should facilitate the cross-over, which involves DNA cleavage by the endonuclease on chromosome 18 and V(D)J recombinase on chromosome 14. Bp45 could also play a role as a tissue specific accessory molecule for the nuclease. The break on chromosome 14 is most likely mediated by V(D)J recombinase and represents the attempted D to J rearrangement. (3) The chromosomes are religated after DNA repair and N-segment addition45 (4). This model can explain the selection of defined breakpoint regions for the translocation by a concerted action of sequence homology, DNA binding proteins, nucleases, and recombinatorial enzymes (polymerases?). It can also deal with the problem that of the several nuclease cleavage sites in Fig 1 only one is selected, because it contains the homologous DNA binding motif.

It remains to be established whether homologous recombination precedes the V(D)J recombinase-mediated recombination or if they are both part of a simultaneous event. Nevertheless, the localization of the protein binding sites as well as the expression of bp45 at the appropriate stage of B-cell development suggest that this protein DNA-interaction is involved in the translocation. Further purification of bp45 and the nuclease from early B cells should enable us to test the model in functional experiments.

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Mechanism of the chromosomal translocation t(14;18) in lymphoma: detection of a 45-Kd breakpoint binding protein

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