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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

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The t(14;18) chromosomal translocation is the most common translocation in human cancer, and it occurs in all follicular lymphomas. The 150-bp *bcl-2* major breakpoint region (Mbr) on chromosome 18 is a fragile site, because it adopts a non-B DNA conformation that can be cleaved by the RAG complex. The non-B DNA structure and the chromosomal translocation can be recapitulated on intracellular human minichromosomes where immunoglobulin 12- and 23-signals are positioned downstream of the *bcl-2* Mbr. Here we show that either of the two coding ends in these V(D)J recombination reactions can recombine with either of the two broken ends of the *bcl-2* Mbr but that neither signal end can recombine with the Mbr. Moreover, we show that the rejoining is fully dependent on DNA ligase IV, indicating that the rejoining phase relies on the nonhomologous DNA end-joining pathway. These results permit us to formulate a complete model for the order and types of cleavage and rejoining events in the t(14;18) translocation.

Antigen receptor loci recombine V, D, and J segments to create exons that encode the variable domains of immunoglobulins and T-cell receptors. The recombination is catalyzed by the RAG complex, consisting of RAG1, RAG2, and HMG1. The RAG complex binds to signal sequences adjacent to the V, D, and J segments. These signal sequences consist of a palindromic heptamer and an A/T-rich nonamer, separated by either a 12- or a 23-bp spacer. One recombination event consists of the action of the RAG complex on a pair of signals, one with a 12-bp spacer (designated a 12-signal) and the other with a 23-bp spacer (designated a 23-signal). After double-strand cleavage, there are four DNA ends: a 12-signal end, a 23-signal end, and two coding ends (designated the 12- and 23-coding ends to indicate to which signal each coding end was formerly attached) (7, 13). The two signal ends are joined together, and the two coding ends are joined together, all using the nonhomologous DNA end-joining pathway (NHEJ) (23, 25).

NHEJ begins with the binding of Ku to the DNA ends. Ku recruits a nuclease complex consisting of Artemis and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). The Artemis–DNA-PKcs complex opens the DNA hairpins that are formed at the coding ends during the RAG cleavage process (26). This complex also trims 5′ and 3′ overhangs during the end processing of NHEJ. The XRCC4-DNA ligase IV complex ligates the two signal ends together, typically without nucleotide loss, to form a signal joint (15, 35). The two coding ends are joined together with nucleotide loss (due to nuclease action) and with template-independent nucleotide addition, due to terminal deoxynucleotidyl transferase (TdT) (25), all contributing to junctional diversification.

The t(14;18) translocation is the single most common translocation in human cancer, occurring in all follicular lymphomas and in a subset of other lymphomas (2, 3, 5, 19, 29, 34, 36). The break at chromosome 18 occurs within the *bcl-2* gene, primarily within a 150-bp region called the major breakpoint region (Mbr) (19, 29, 36). The events at chromosome 14 begin with cuts at the D and J segments. However, instead of the D end being joined to the J end, the D end is joined to the downstream end of the *bcl-2* Mbr break, and the J end is joined to the upstream end of the *bcl-2* Mbr break (Fig. 1) (20, 21, 29).

It has been unclear why the *bcl-2* Mbr is so precisely focused to this 150-bp region and what proteins cause the break. Recently, we showed that the *bcl-2* Mbr adopts a non-B DNA structure and that the RAG complex cleaves within this non-B DNA region. We showed that many aspects of the t(14;18) translocation can be recapitulated on a human minichromosome (30). The non-B DNA conformation of the Mbr is stable and can be recapitulated on bacterial plasmids and even on shorter PCR fragments (28, 30).

Despite this progress, many questions remain. Once the breaks at the D and J segments occur, can any of the four ends (two signal and two coding ends) recombine with the two ends of the Mbr? Can either of the two coding ends (D or J) recombine with either end of the Mbr, or are there constraints intrinsic to the manner of breakage? Are all six DNA ends (two signal, two coding, and two Mbr ends) rejoined simultaneously, or are some ends rejoined before others (Fig. 1)? Finally, what is the pathway of rejoining? Here, we address each of these questions.

MATERIALS AND METHODS

Oligonucleotides. Oligomers are from QIAGEN/Operon (Richmond, CA) or from the USC Norris Cancer Center Microchemical Core Facility. The following

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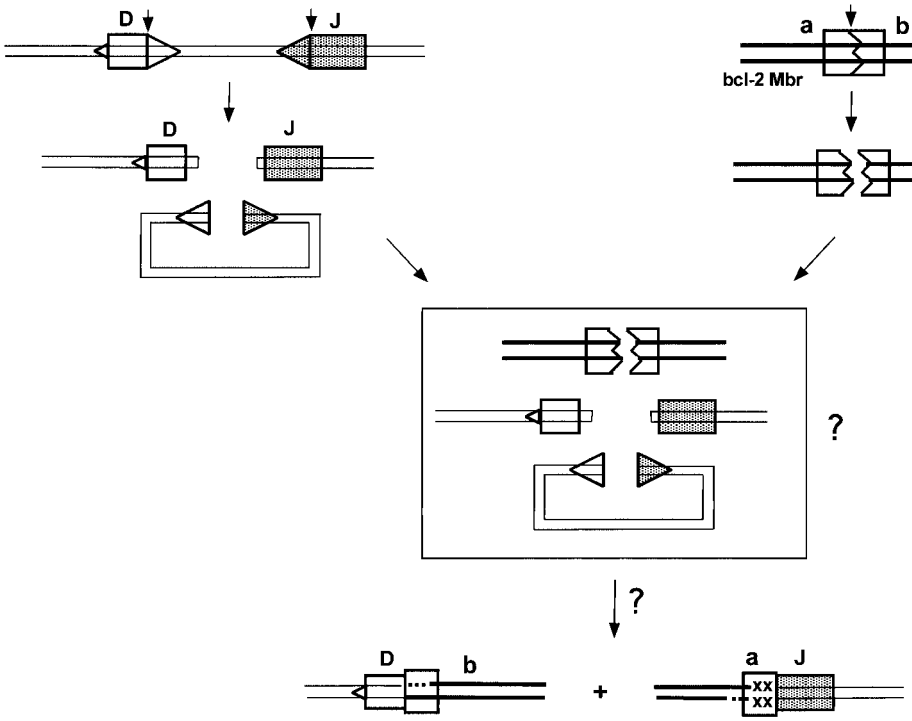


FIG. 1. Six DNA ends are generated during the t(14;18) translocation involving the Ig heavy-chain locus and *bcl-2*. The D and J segments at the Ig heavy-chain locus on human chromosome 14 are shown in this diagram, and the DNA for this locus is indicated by double lines. The downward vertical arrows mark where the V(D)J recombination process creates DSBs. The open and stippled triangles at the Ig heavy-chain locus represent the heptamer/nonamer recombination signal sequences. Open triangle, 12-signal; stippled triangle, 23-signal. The DNA between the two signals is normally joined to form a signal joint, and the D and J segments are joined together to form a coding joint. In the t(14;18) translocation, the D and J segments fail to join together. In addition to this error in the process, a break occurs at the 3′ untranslated region exon of the *bcl-2* gene (ends indicated by a and b). Therefore, during the t(14;18) translocation, six different ends are generated (two Mbr ends, two signal ends, and two coding ends). The order of joining between the ends is unclear, as reflected by the question marks. The final products of the t(14;18) translocation are shown as the derivative chromosome 18 (left) and the derivative chromosome 14 (right). The J subexon joins with the 3′ end (end a) of the *bcl-2* gene, and this joining leads to the upregulation of the *bcl-2* gene and follicular lymphoma. The derivative chromosome 18 is generated by joining of the D subexon and the 5′ end (end b) of the *bcl-2* gene. The pathway of all of the joining is unclear.

oligomers were used in this study: SCR21, 5′-AGTGCCACCTGACGTCTAAG-3′; GHG1, 5′-GAACGGTCTGGTTATAGGTAC-3′; SCR97, 5′-CGCGTCCCATTCGCCATTC-3′; SCR98, 5′-CAATACGCAAACCGCCTCTCC-3′; XW8, 5′-AGCGTCGACCCTTTAGAGAGTTGCTTTACG-3′; XW9, 5′-GCTGTCGACCTCGAGTAGCAGCACAGGATTGGATAT-3′; KY28, 5′-GATCAGCTGATAGCTACCACAGTGCTACAGACTGGAACAAAAACCCTGCT-3′; KY29, 5′-TAGCAGGGTTTTTGTTCCAGTCTGTAGCACTGTGGTAGCTATCAGCTGAT-3′; KY36, 5′-GATCAGCTGACAGTAGCACAGTGGTAGTACTCCACTCTCTGGCTGTACAAAAACCCTGCT-3′; KY37, 5′-TAGCAGGGTTTTTGTACAGCCAGAGAGTGGAGTACTACCACTGTGCTACTGTCAGCTGAT-3′; SCR116, 5′-GACCTGCCGAGTGGTTCAGCAGG-3′; SCR173, 5′-CCTGTTGACAATTAATCATC-3′; SCR199, 5′-GTTTTTGTTCCAGTCTGTAGCAC-3′; SCR200, 5′-CAGACAGTGGAGTACTACCAC-3′; SCR201, 5′-CTCGCCAAGCTGATCCCCGG-3′; and SCR202, 5′-CCTAGTTAACTATACGCAGC-3′.

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

Plasmid construction. The plasmid constructs were made by modifying the simian virus 40-based plasmid, pGG51, pGG49, or pGG52 (11). A SalI-digested, 300-bp PCR fragment of the *bcl-2* Mbr was cloned into a SalI-digested pGG51 (pXW5). In pXW6, the *bcl-2* Mbr is in the opposite orientation (30). Plasmid pSCR1 was constructed by cloning the 300-bp SalI fragment into the SalI site of pBSS3, a derivative of a Bluescript plasmid in which the *lacZ* promoter has been deleted (30).

A 167-bp fragment containing 12- and 23-signals was cloned into pXW5 after replacing the existing 23-signal fragment to construct pSCR45 (30). A weak prokaryotic transcriptional terminator is present between the 12- and 23-signals of pSCR45. In order to remove this weak terminator, a 354-bp lambda fragment was cloned into the ClaI site of pGG51, pGG49, and pGG52. A 497-bp SpeI-SmaI fragment, containing the two signals and the 354-bp lambda fragment, was then cloned into pXW5 after replacing the 23-signal by BamHI digestion. The new constructs generated are pSCR71, pSCR72, pSCR73, pSC74, pSCR75, and pSCR76 (Fig. 2A and B). The plasmids pSCR77, pSCR79, pSCR81, pSCR85, and pSCR87 are the same as pSCR71, pSCR72, pSCR73, pSCR75, and pSCR76, respectively, except that the *bcl-2* Mbr is in the reverse orientation.

Human V(D)J recombination assay. The human lymphoid cell line Reh was grown logarithmically, transfected with the appropriate plasmid substrates by using the electroporation/DEAE-dextran method described previously (8), and cultured for 48 h at 37°C. In some experiments, Nalm6 cells or N114P2 cells (which are genetically null for ligase IV and are derived from the Nalm6 cell line) were also used for transfection (16). The various steps involved in the human V(D)J recombinase assay were described previously (9, 11, 12). In brief, episomal DNA was harvested from mammalian cells after 48 h of incubation at 37°C by a rapid alkaline harvest method. The DNA was recovered and resuspended in 50 μl of Tris-EDTA (pH 8.0). The recovered DNA was then transformed into *Escherichia coli*, with or without DpnI digestion (to quantitate eukaryotic replication), for further analysis. The recombinants (Fig. 2A) recovered on chloramphenicol-ampicillin doubly resistant plates were further examined by extracting the episomal DNA and by DNA sequencing. Sequencing reactions were carried out using a SequiTherm Excel II sequencing kit (Epigenetics) and an MWG thermal cycler, model Primus 96 Plus (MWG Biotech, High Point, NC). Automated sequencing was carried out using a Li-Cor DNA analyzer, model 4200 (Li-Cor, Lincoln, NE). The recombinant plasmids conferred resistance to ampicillin (designated A) alone and to ampicillin and chloramphenicol (designated

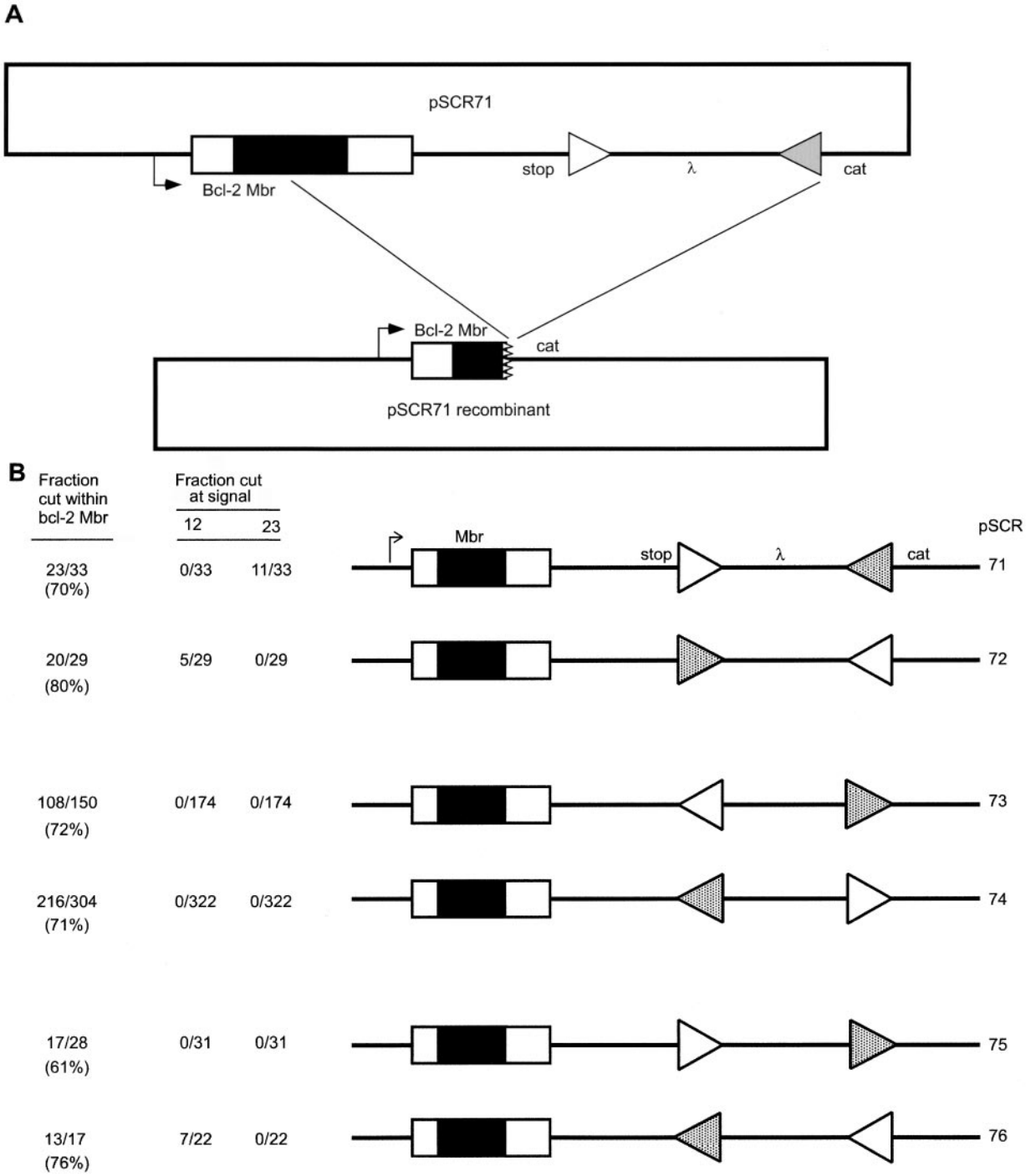


FIG. 2. In vivo distribution of breakpoints on extrachromosomal substrates, which cluster within the *bcl-2* Mbr and at the recombination signal sequences. (A) Depiction of a recombination substrate (pSCR71) and its recombination product (pSCR71 recombinant) derived after the joining between the *bcl-2* Mbr and a 23-signal. (B) The episomes (pSCR71 through pSCR76) were transfected into mammalian cells (Reh cells), recovered 48 h later, and analyzed in bacteria, as described in Materials and Methods. The fraction of the recombinants cleaved within the *bcl-2* Mbr and at the 12- or 23-signal is also shown. Designation of a cleavage event "at the 12- or 23-signal" means that the recombination occurred within 13 bp of the coding end proximal to the adjacent C nucleotide of the heptamer. In normal V(D)J recombination, over 98% of the recombination points within the coding end are within 13 bp of the heptamer (11). In both panels, the Mbr regions are represented by black within the boxes. The white regions within the boxes correspond to the DNA that is outside of the Mbr but which is the flanking DNA that is naturally adjacent to the Mbr in the human chromosome. The 12- and 23-signals are indicated by the open and shaded triangles, respectively. The transcriptional promoters are indicated by arrows. A 354-bp fragment of DNA from bacteriophage lambda serves as a spacer between the 12- and 23-signals and is indicated by λ. "Stop" indicates the region of a prokaryotic transcription terminator, and "cat" represents the chloramphenicol gene.

TABLE 1. Comparison of breakpoint frequencies in recombinant plasmid molecules

Plasmid[a]	DA[b]	DAC[c] with:		% Recombination[d]		No. of molecules (% recombination) with Mbr joined to the 12- or 23-signal[e]
		Breakpoint within *bcl-2* Mbr	Cut at the 12- or 23-signal	Mbr	12- or 23-signal	
pSCR71	238,000	23	11 (23-signal)	0.00966	0.00462	10 (0.00420)
pSCR72	294,800	20	5 (12-signal)	0.00678	0.00170	3 (0.00102)
pSCR73	2,150,500	108	0 (23-signal)	0.00502	<0.000047	0 (<0.000047)
pSCR74	3,884,600	216	0 (12-signal)	0.00556	<0.000026	0 (<0.000026)
pSCR75	270,400	17	0 (23-signal)	0.00629	<0.00037	0 (<0.00037)
pSCR76	314,000	13	5 (12-signal)	0.00414	0.00159	5 (0.00159)

[a] The episomes (pSCR71 through pSCR76) were transfected into mammalian cells (Reh cells), recovered 48 h later, and analyzed in bacteria as described in Materials and Methods.
[b] Substrate molecules that replicated in the Reh cells.
[c] Bacterial transformants that are ampicillin-chloramphenicol (doubly) resistant, also referred to as recombinants. The DAC column is subdivided according to the left and right boundaries of recombination. The left boundary is the subset of double-resistant recombinants that have a breakpoint within the *bcl-2* Mbr. The right boundary is the subset of recombinants that utilize the 12-signal or 23-signal sequence for recombination.
[d] The % recombination of the Mbr is calculated by the equation (DAC of the Mbr/DA) × 100 and is the recombination frequency for all events that utilize the Mbr. The % recombination of the 12- or 23-signal is calculated by the equation (DAC of the 12- or 23-signal/DA) × 100 and is the recombination frequency for all events that utilize either the 12- or the 23-signal.
[e] The recombination frequencies of molecules with one break at the *bcl-2* Mbr and a second break at a 12- or 23-signal are shown.

AC). The ratio of AC colonies to A colonies reflects the fraction of recovered substrates that underwent V(D)J recombination. A more meaningful ratio can be obtained by focusing on those ampicillin-resistant molecules that have undergone replication in the mammalian cells (24). The ratio of AC to A among replicated molecules is determined by the equation $R = (\mathrm{DAC/DA}) \times 100$, where DAC is the number of transformants that arose because of transformation with replicated recombinant molecules, DA is the number of transformants that arose from any replicated plasmid, and R represents the recombination frequency of the substrate. We controlled for replication by digesting the recovered substrates with DpnI before bacterial transformation. DpnI cleaves the plasmid molecules that did not lose their prokaryotic Dam methylation pattern by replication in eukaryotic cells. Only molecules that replicated in eukaryotic cells (DA) remain undigested by DpnI and can transform the bacteria. The replication frequency of a given substrate is calculated by use of the equation (DA/A) × 100. Each eukaryotic transfection was typically analyzed with 10 to 20 *E. coli* transformations to determine R [(DAC/DA) × 100]. The average number of multiple eukaryotic transfections of the same substrate in Reh cells was calculated by summing the total DAC counts (after restriction analysis and sequencing verification of representative transformants) and dividing that total by the total number of transformants from replicated plasmids (DA). The resulting number is expressed as a percentage and is the weighted average [$R = (\mathrm{DAC/DA}) \times 100$].

RESULTS

In vivo tests for 12- or 23-coding-end recombination with the *bcl-2* Mbr. Recombination between the *bcl-2* Mbr and signal or coding ends can be detected by using a human V(D)J recombination assay. Previously, we reported that recombination between the *bcl-2* Mbr and the 23-coding end is dependent on the RAG1 and RAG2 proteins (30). In the present study, we examined other possible end-joining combinations and requirements of the t(14;18) translocation by using a series of minichromosomes that carry the *bcl-2* Mbr and 12/23-signal pairs in various orientations (Fig. 2B). The Mbr and signal sequences are separated by a prokaryotic transcriptional terminator. If recombination occurs between either of the signal ends and the Mbr, then the transcription terminator will be deleted, resulting in chloramphenicol resistance (Fig. 2A).

The episome pSCR71 or pSCR72 was transfected into the mammalian pre-B-cell line Reh (known to have high levels of RAG expression) and harvested after 48 h. The replication efficiency was calculated by transformation into *E. coli* as described above (see Materials and Methods). The recombinants were obtained by plating on chloramphenicol-ampicillin-containing plates, and each recombinant was further analyzed by DNA sequencing.

In 70% (23/33) of the recombinants of pSCR71, the left breakpoint in the recombination zone was within the *bcl-2* Mbr (Fig. 2B). There were no cases where the 12-coding end of this substrate joined with the Mbr. However, the recombination frequency of molecules with a break at the Mbr and a second break at the 23-signal coding end was 0.0042% (Table 1). We also found nucleotide additions at the junctions of the recombinants and some joining events that utilize microhomology.

In actual patient t(14;18) translocations, the 23-coding end is joined to the *bcl-2* Mbr. In the case of the pSCR72 construct, we positioned the 12/23-signals in such a way that we could test for recombination of the other coding end, the 12-coding end, with the *bcl-2* Mbr. With this construct, ~80% of the breakpoints on the left side of the recombination zone were within the Mbr (Fig. 2B), with a recombination frequency of 0.00678% (Table 1). Interestingly, on the right side of the recombination zone, we found that the 12-coding end also can join with the Mbr (frequency of 0.00102%) (Table 1). We found nucleotide additions and microhomology usage at the recombinant breakpoint junctions here also. Parenthetically, three recombinants were found where the 12- to 23-signal rearrangement occurred, but no Mbr recombination was found; in these cases, the chloramphenicol resistance observed was due to point mutations within the transcription terminator. As above, here also we do not find any signal end joining to the *bcl-2* Mbr (Fig. 2B).

In a different construct, pSCR76, we paired the *bcl-2* Mbr sequence with a pair of 12/23-signals oriented for inversional recombination with respect to one another (Fig. 2B). After transfection into Reh cells and analysis of the recombinants, we found, as above, that the Mbr is more fragile (76% of recombinants within the Mbr) than the surrounding sequences, with a recombination frequency of 0.00414%. Similar to pSCR72, the 12-coding end joined with the Mbr, with a recombination frequency of 0.00159% (Table 1). Though the coding end of the 12-signal was able to join with the Mbr, we did not see any joining of the 23-coding end to the Mbr. The

generation of such recombinants would require three independent double-stranded DNA ligation events to generate a completely intact recombinant plasmid—one at the retained signal joint and two more at the two Mbr-to-coding-end junctions. In summary, the studies described above show that either of the two coding ends is capable of joining to the *bcl-2* Mbr.

In vivo test for 12- or 23-signal end recombination with the *bcl-2* Mbr. From the studies described above, we have seen that signal ends are not able to join with the Mbr. Because two or more independent ligation events would be required for the joining of a signal end to the Mbr, the efficiency of such joining might be expected to be lower than that of the single junction events. Therefore, we analyzed another set of episomal substrates, pSCR73 and pSCR74, for which a single ligation between a signal end and the Mbr would be sufficient to generate the recombinant (Fig. 2B).

Results showed that, for both pSCR73 and pSCR74, most of the breakpoints at the *bcl-2* side of the recombination zone are located within the Mbr (72% and 71%, respectively) and have comparable recombination frequencies (0.00502% for pSCR73 and 0.00556% for pSCR74) (Fig. 2B and Table 1). We further found that neither the 23-signal end of pSCR73 nor the 12-signal end of pSCR74 can join with the Mbr (Fig. 2B). The recombination frequency of the 23-signal end joining to the Mbr in pSCR73 was undetectable and less than 0.000047%, which is >89-fold lower than that of pSCR71 (Table 1). Similarly, the recombination frequency of the 12-signal end joining to the Mbr in pSCR74 was less than 0.000026%, which is >162-fold lower than that of pSCR71 (Table 1). Therefore, signal ends appear not to be able to join to the Mbr. In these recombinants, a random break accounts for the recovery on the double-selection plates because this break is not located adjacent to either recombination signal sequence (RSS). [The cause of the random break is unknown, and these may arise during replication. Such breaks are unlikely to initiate from the signals, because signal ends do not consistently suffer nucleolytic resection in V(D)J recombination within wild-type cells (11).] It is also important to point out that in both pSCR73 and pCSR74, the recombination signal close to the Mbr is a coding end. These coding ends are not used for recombination to the Mbr. This again may be due to the possible requirement of two independent ligations to yield such a recombinant. Another possibility is that after RAG cleavage at 12- and 23-signals, the fragment containing the lambda region may be lost, so that the 12- or 23-coding ends of pSCR73 and pSCR74 may not be available at all for rejoining.

We also tested another substrate, pSCR75, where the Mbr could recombine with 12- and 23-signals oriented for inversional recombination relative to each other. In pSCR75, the orientation of the signal sequences is the reverse of that in pSCR76 (Fig. 2B). We found that, in this case also, 17 out of 28 recombination events occurred at the Mbr, with a recombination frequency of 0.00629% (Table 1). None of the recombination events utilized the 12- or the 23-signal end. The recombination frequency was less than 0.00037%, which is >12-fold lower than that of pSCR71, where recombination occurs between the Mbr and the 23-coding end (Table 1).

Since we were not able to detect any recombinants with a signal end joined to the Mbr by a transformation assay, we used a PCR assay to further screen for such recombinants. We digested the recovered DNA with a restriction enzyme that cleaves only in the zone between the Mbr and the 12/23-signals to eliminate unrecombined DNA molecules. The PCR was then carried out using the enzyme-digested DNA. Results showed that there is no amplification of the digested transfection products (data not shown), further indicating that the signal ends are not joined to the *bcl-2* Mbr.

These results indicate that, unlike coding ends, signal ends are unable to join with the *bcl-2* Mbr, implying that they are either already joined with one another to form a signal joint or that they are bound by RAG proteins in a manner that is distinct from the coding ends (1). Our results also show that the break at the *bcl-2* Mbr occurs independently of the break at the 12/23-signal pair because the recombination rate and the targeting to the Mbr are independent of whether there is only a 12- or a 23-signal, or both, on the plasmid (30). The overall recombination frequency for the Mbr is only slightly higher for pSCR71 and pSCR72 than for pSCR73 to -76, consistent with the fact that targeting to both the Mbr and a coding end account for only about one-third of recombinants (30).

We also found that similar joining events occur even when the *bcl-2* Mbr is placed in reverse orientation relative to the 12/23-signals (pSCR77, -79, -81, -85, and -87) (data not shown). These results indicate that either of the resulting two DNA ends of the broken *bcl-2* Mbr is capable of joining with either coding end.

Sequences of junctions reveal T nucleotides. It has previously been noted that the *bcl-2* Mbr translocations have a distinct, highly significant junctional feature called T nucleotides (where T stands for "templated"), in which the additions are a direct or inverted repeat of a portion (1 to 10 nucleotides [nt]) of one of the participating DNA ends with various numbers of mismatches (19, 30). The template for the T nucleotides can be either from the intact sequences or from the deleted portions and can be from either the top or the bottom strands of any of the four involved DNA ends. These T nucleotides are sufficiently common that they represent a signature feature of the in vivo t(14;18) translocations seen in patients.

In our study, we found nucleotide additions at the junctions of recombinants. Some of these insertions appear to have resulted from N nucleotide addition by TdT. Interestingly, many of the junctions also had T nucleotides (Fig. 3). In our study, we designated additions as T nucleotides when 3 or 4 nt of perfect homology were present. In cases where the junctional additions were 5 nt or more, a minimum of 80% homology was required. The frequent presence of this signature feature in our recombination substrates indicates that the t(14;18) translocation mechanism is recapitulated at the sequence level of the break at the *bcl-2* Mbr.

In vivo test of the ligase IV dependence of the 23-coding-end recombination with the *bcl-2* Mbr. In the minichromosomal substrate studies of mammalian cells described above, we noted that many of the recombinants utilized microhomology during the joining process, suggesting that NHEJ is the mechanism of rejoining the breaks. It is well established that NHEJ is involved in V(D)J recombination (7, 13, 25). Studies of patients show that during t(14;18) translocation, the break at chromosome 14 occurs during V(D)J recombination, and the above-described and earlier intracellular minichromosomal studies show that the breaks at the *bcl-2* Mbr are generated by

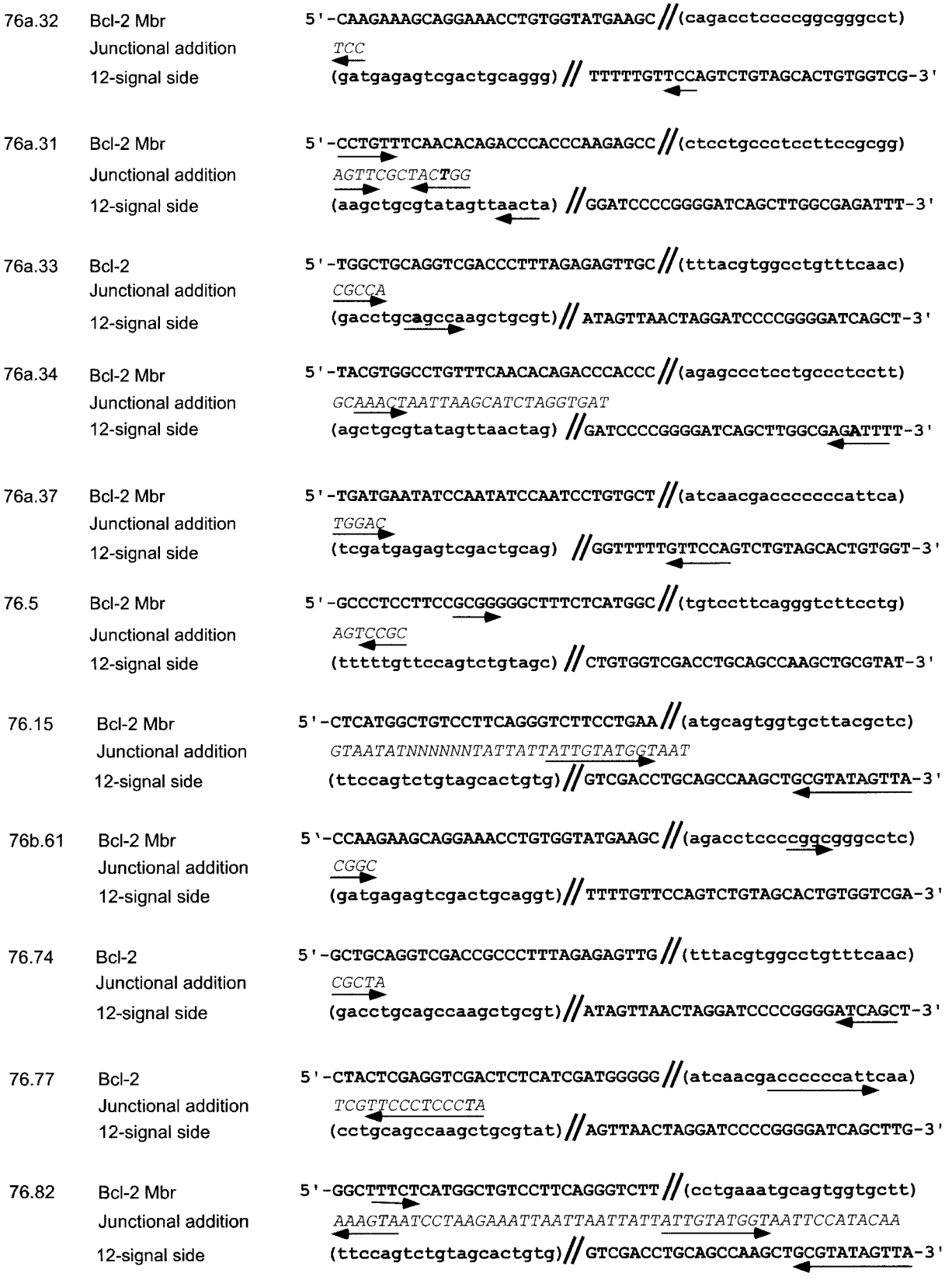
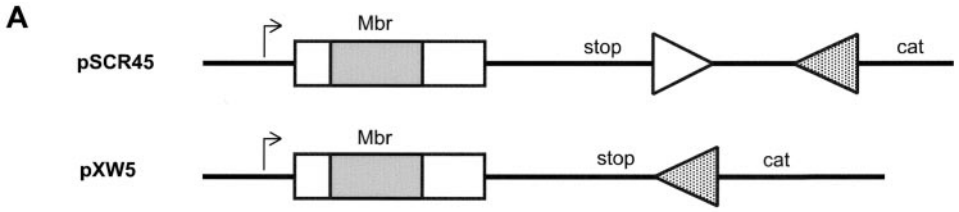


FIG. 3. T nucleotides are present at the junctions of the *bcl-2* recombinants. The top strand of each substrate sequence is shown. The bold capital letters represent nucleotides that are retained on the substrate. The double slashes indicate the breakpoints. The italicized capital letter sequences are the nucleotide additions at the junction. T nucleotides are underlined, and the orientations are indicated by arrows. Mismatches within the T nucleotides are represented in bold within the arrow-designated regions. The DNA (20 nt) deleted and immediately adjacent to the breakpoints from each end are shown within parentheses and in small letters. The number at the left of each sequence indicates the name of that molecular clone.



B

Plasmid name	Cells	DA	Mbr		signal end	
			inside	outside	at or within	outside
pSCR45	Nalm6	632,900	17/24 (71%)	7/24 (29%)	13/24 (54%)	11/24 (46%)
pSCR45	N114P2	643,400	1/2 (50.0%)	1/2 (50.0%)	0/2 (0.0%)	2/2 (100%)
pXW5	Nalm6	278,900	9/13 (69.2%)	4/13 (30.8%)	3/13 (23.1%)	10/13 (76.1%)
pXW5	N114P2	294,500	0/0	0/0	0/0	0/0

FIG. 4. Ligase IV is required for recombination between the *bcl-2* Mbr and the signal ends. Episomes pSCR45 and pXW5 were transfected into ligase IV knockout cells (N114P2) and the corresponding wild-type cells (Nalm6). (A) DNA was recovered 48 h later and analyzed. The Mbr region is represented by a gray box. The white boxed regions adjacent to the Mbr are the flanking regions that are naturally adjacent to the Mbr in the human chromosome. The 12-signal is indicated by an open triangle, and the 23-signal is indicated by a stippled triangle. The transcriptional promoter is indicated by a short arrow above and parallel to the plasmid backbone. "stop" indicates the region of a transcription terminator, and "cat" represents the chloramphenicol gene. (B) DA, number of substrate molecules that replicated in the Reh cells. The recombinants that are ampicillin-chloramphenicol (doubly) resistant are classified according to the left and right boundaries of recombination. The left boundaries of recombinants are grouped according to whether the break is within the Mbr or outside of it. The right boundaries of recombinants are grouped by whether or not they utilize the 23-signal.

the RAG complex (30). In order to test whether NHEJ is involved in t(14;18) translocation, we used human pre-B cells that are genetically knocked out at the ligase IV gene (16).

We transfected two episomal substrates, pSCR45 and pXW5, into the ligase IV knockout cells, N114P2 (16), and the wild-type pre-B parental line, Nalm6. The DNA was harvested at 48 h (Fig. 4). Recombination products were recovered and analyzed by sequencing as described above. For pSCR45, we found that in 71% of the cases, the left boundary of recombination occurred within the Mbr, with a recombination frequency of 0.0027% in the wild-type, pre-B cells (Nalm6) (Fig. 4 and Table 2). In about 54% of the events, the right recombination boundary break occurred at the 23-signal. The frequency of molecules where a 23-coding end joined with the Mbr was 0.002%. We also found T nucleotides at the junctions of these recombinants.

Interestingly, when we transfected pSCR45 into the ligase IV knockout cells, we found a dramatic reduction in the number of recombinants. We found only two recombinants, and of those, only one had a breakpoint at the Mbr. The recombination frequency was reduced to 0.00016%, which is about 17-fold lower than that of the wild-type cells (Table 2). More importantly, there were no recombinants with a break at the Mbr and a second break at either the 12- or the 23-signal (recombination frequency of <0.00016%) (Fig. 4 and Table 2). These results indicate that ligase IV is likely to be the ligase responsible for all or at least 95% of the rejoining events.

We found an even more dramatic difference when we transfected pXW5, the one-signal substrate, into Nalm6 and N114P2 cells. About 69% of the breaks occurred at the *bcl-2* Mbr when pXW5 was harvested from Nalm6, with a recombi-

TABLE 2. Comparison of recombination frequencies of recombinant plasmid molecules from Nalm6 and N114P2 cells

Plasmid[a]	Location of recombination event	Recombination frequency (%)		
		Nalm6 cells	N114P2 cells	Fold difference
pSCR45	MBR	0.0027	0.00016	17
	23-signal	0.0021	<0.00016	<13
pXW5	MBR	0.0032	<0.00034	<9

[a] Episomes pSCR45 and pXW5 were transfected into mammalian cells (either Nalm6 cells or N114P2 cells, which are ligase IV knockout derivatives of Nalm6), recovered 48 h later, and analyzed in bacteria as described in Materials and Methods. The recombination efficiency of pSCR45 is defined according to the left and right boundaries of recombination. The left boundary is the recombination frequency of the Mbr, and the right boundary is the recombination frequency of the 23-signal.

nation frequency of 0.0032%. We found no recombinant when we transfected the same episome into N114P2 cells, yielding a recombination frequency of <0.00034% (Fig. 4B and Table 2). These results further confirm the requirement of ligase IV. The involvement of DNA ligase IV suggests that NHEJ is the pathway to rejoin the breaks during the *bcl-2* translocation. In the absence of ligase IV, other ligases are unable to compensate for it at the same level of efficiency.

DISCUSSION

Model for the events during the t(14;18) translocation. There have been many unanswered questions about the t(14;18) order of events and the nature of those events (21). Six DNA ends (two coding ends, two signal ends, and two Mbr ends) must be accounted for in this process, whereas the normal V(D)J recombination reactions require accounting for only four (29). Can any one of the six ends join to any other of the six? Is the specific RAG complex that cleaves the 12- and 23-signals the same one as that which cleaves the *bcl-2* Mbr?

Our results showed that only coding ends, not signal ends, can join with the *bcl-2* Mbr. The recombinants that we observed in the case of signal end substrates (pSCR73 and pSCR74) did not involve the 12- or 23-signals. In these recombinants, the break on the left side of the recombination zone was within the Mbr, but the break on the right side of the recombination zone was not at the signal. Usually these right-side breaks are between the two signals and far from either signal. Occasionally, the breaks are far to the right side of the rightmost signal boundary. Such breaks are highly unlikely to initiate from the signals, as signal ends usually do not suffer nucleolytic resection during the V(D)J recombination within wild-type cells (11).

The fact that the signal ends do not participate in the joining to either Mbr end is most easily explained either by the fact that they have already been joined to form a signal joint or by the fact that they have been bound by RAGs (1, 18). The fact that any of the four remaining ends (two coding ends and two Mbr ends) can join to any other of the four raises the possibility that there could be a four-ended intermediate just prior to the ligation of these four ends (22). Substrates, such as those used here, permit all combinations of ends to be assessed. However, in the chromosome, cell viability would be at risk if an immunoglobulin (Ig) heavy-chain 12-coding end joined to the Mbr, because this would yield one dicentric and one acentric chromosome. Hence, the substrates used here permit inferences that chromosomal observations cannot.

The reliance on DNA ligase IV indicates that NHEJ is responsible for the rejoining of these four ends. The sequence features of the chromosomal and minichromosomal translocation junctions, including P nucleotides due to hairpin opening by the Artemis/DNA-PKcs complex and template-independent addition due to TdT, also indicate that NHEJ is the joining pathway (26, 33).

Based on these findings, a detailed model for the events at the t(14;18) translocation can now be formulated (Fig. 5). In this model, one RAG complex cuts both strands independently at the *bcl-2* Mbr. We know that the RAG complex can cut both strands (top and bottom strands) of the Mbr independently in vitro (30, 30a). We have also seen RAG cleavage on short double-stranded DNA containing bubbles or loops, indicating that the RAG complex generally cleaves at single- or double-strand transitions (30a). This cleavage does not involve hairpin formation at the break sites, in contrast to action at synapsed RSS sites. In addition, we have seen RAG-induced double-strand break (DSB) formation on 248- to 339-bp DNA fragments containing the *bcl-2* Mbr structure, and these fragments do not have any recombination signals. Hence, no synapsis of the Mbr with any RSS can occur.

A second RAG complex simultaneously cuts the 12- and 23-signals in the standard synapsis that is thought to form during V(D)J recombination (7). We have observed 12- and 23-signal recombination events on substrates that also carry the Mbr, and their recombination is indistinguishable from that of substrates that do not carry the Mbr. In such a standard V(D)J recombination reaction, there is a period of time when there is a four-ended intermediate containing two signal ends and two coding ends. Such a four-ended intermediate is functionally known to exist because any coding end can join with any signal end to form not only the standard signal and coding joints but also hybrid joints and open-and-shut joints (22). There is also biochemical evidence to suggest the existence of such intermediates (1, 18). The fact that we did not find signal end recombination with the Mbr suggests that the two signal ends are ligated to form a signal joint or are synapsed by the RAG complex such that they cannot be used for ligation with the Mbr ends. This also rules out any direct attack by the RAG–12-signal end–23-signal end complex on the Mbr to create the DSB there, consistent with our observation that Mbr breaks are not stimulated by 12- or 23-signals located nearby on cellular substrates.

Since we found that both the *bcl-2* Mbr and the RSS are independently cleaved by RAGs, the question arises whether there is a place in the nucleus where RAG-mediated cleavage occurs. This appears to be unlikely, based on observations in human and yeast cells. For example, the human MLL gene on chromosome 11 can break and join with 38 different partners, leading to the development of infant acute lymphoblastic leukemia (31, 32). If the MLL gene has 38 partners, then it seems unlikely that the MLL gene and all 38 partners are positioned adjacently in the human nucleus. Therefore, in the case of the MLL gene, the search for and rejoining of ends appear to involve a large fraction of the genome and, hence, the nucleus. We suspect that the same considerations apply to the t(14;18) translocation. In addition, during yeast homologous recombination events that result in intrachromosomal deletion or reciprocal translocation, the broken ends of mitotic chromosomes are free to search the entire genome for appropriate partners; thus, mitotic chromosomes are not functionally confined to isolated domains of the nucleus (17).

One might wonder to what extent the RAG proteins remain bound to the coding ends and/or to the Mbr ends following cleavage. Signal ends are known to be held relatively tightly in a postcleavage complex. In contrast, the binding of coding ends in such postcleavage complexes may not be as tight (1, 18). In the small proportion of D-J recombination events that result in translocations, we suspect that these are cases where the D and J coding ends are released prematurely. There is also no reason to believe that the binding of the RAG complex to the Mbr is tight, following the nicking events on the top or bottom

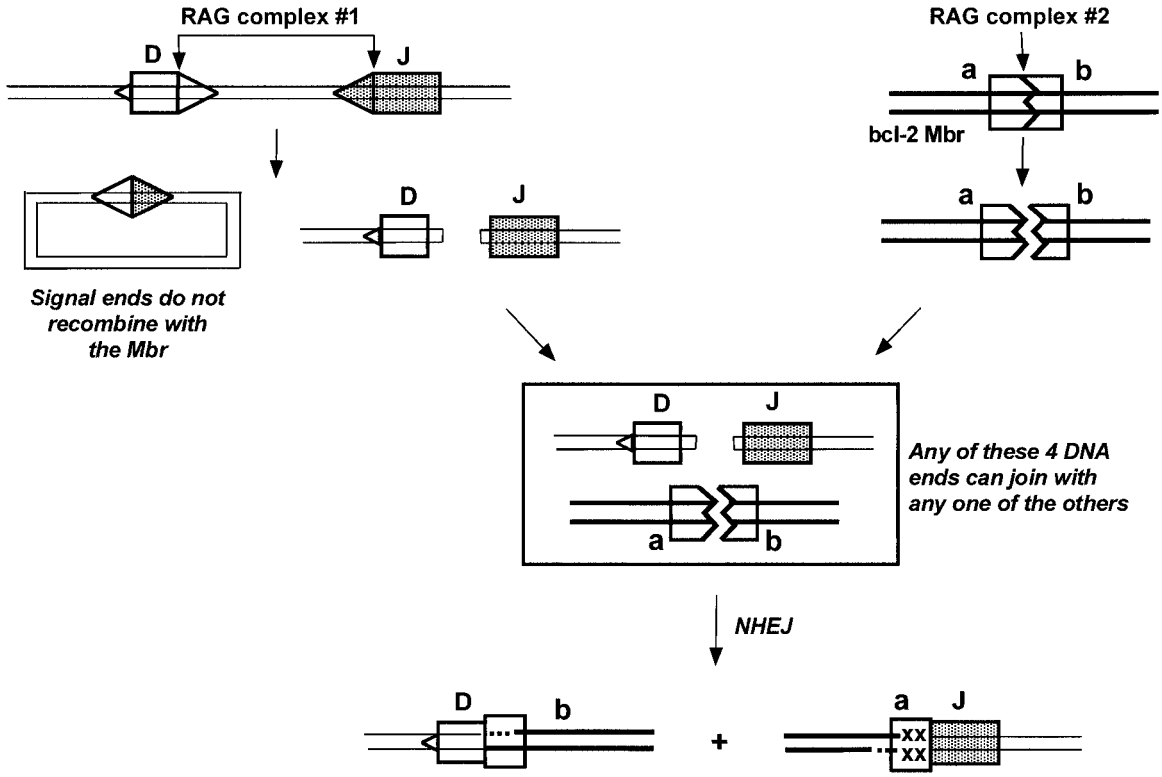


FIG. 5. Biochemical model for the mechanism of the t(14;18) chromosomal translocation. The aspects of the mechanism depicted by the two question marks in Fig. 1 are significantly clarified by this study. Our previous work indicated that the RAG cleavage at the Mbr (denoted by "RAG complex #2" and downward arrows) is independent of the RAG cleavage at the paired 12/23-signal pair (denoted by "RAG complex #1"), and the work here has confirmed and extended this point (30). Because the signal ends do not recombine with the Mbr, they may already be joined; alternatively, they may still be bound by the RAG complex that cleaved them (1). In either case, they are unavailable to recombine with the Mbr. This reduces the translocation intermediate step to a four-DNA-end problem rather than a six-DNA-end problem. NHEJ is the pathway for rejoining the ends, based on the new findings from the present study, which are shown in italics.

strand of the Mbr (30a). Hence, we do not necessarily believe that a RAG complex at the D-J coding ends and another at the Mbr are involved in the synapsis of these two sites. Rather, we believe that the ends may come together in a RAG-independent manner, probably by NHEJ. If the D, J, and two Mbr ends are the only ones in the nucleus, their rejoining by NHEJ in any possible configuration may not be so surprising in light of data on simultaneous breaks in the genomes of yeast (17).

One might also consider why the t(14;18) translocation does not involve the V and D rather than the D and J segments. Distance issues are one possibility. Because we do not know the full limits over which the Ig heavy-chain enhancer and locus control regions act, it is difficult to rule this possibility in or out (27). Nicking efficiency by the RAGs at the V-D pairs versus the D-J pairs is a second factor. Nicking at the V segment and the upstream side of the D segment is not as efficient as it is at the downstream side of the D segment and at the J segment (10, 37–39). Part of this is due to lower signal sequence strength, but part is due to coding-end sequence effects (14). This factor could contribute as much as 2 orders of magnitude preference to the use of D-J pairs instead of V-D pairs.

In addition to the P nucleotides (due to hairpin opening) and the N nucleotides (due to TdT), we also found T nucleotides at the junctions of the *bcl-2* recombinants. Such T nucleotides are a signature feature of t(14;18) translocation in follicular lymphoma patients (19, 29). The reproduction of such T nucleotides in the episomal recombinants indicates that these recombinants recapitulate the fine structural features of this translocation process.

The final phase of the translocation process is the joining of the two coding ends with the two Mbr ends. We have shown that this is dependent on the XRCC4-DNA ligase IV complex and hence is a normal NHEJ process at both joints (see below).

Ligase IV dependence of chromosomal translocations. NHEJ has been suspected to be important for many translocations, and the studies here support that. This issue has been complicated by the fact that in NHEJ null mice, translocations (none that correspond to the *bcl-2* translocation) can still be detected, and it has been unclear what enzymes replace the NHEJ components under these circumstances (6). Alternative methods for end joining may be used when NHEJ is absent (29). In the absence of ligase IV or XRCC4, the ligation of some translocation must be done by either DNA ligase I or III. Ligase I can ligate when the two nicks of a DSB are sufficiently far apart. Ligase III can ligate DSBs when they are compatible (4). Our studies here do not shed light on this. Rather, we only know that when ligase IV is absent, the efficiency of these

alternative pathways is not adequate to quantitatively restore the level of the *bcl-2* translocation. Hence, the efficiencies of these alternative enzymatic methods of joining are not as high as that of NHEJ for this particular translocation process.

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