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Formation of a Hybrid *bcl*-2/Immunoglobulin Transcript as a Result of t(14;18) Chromosomal Translocation

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A. Introduction

Specific chromosomal changes are consistently associated with several morphologically or histologically distinct forms of cancer [1]. In a few hematolymphoid malignancies, chromosomal translocations are known to occur near or within the cytologic loci for cellular homologues of retroviral-encoded oncogenes [2]. For example, both the c-*abl* and c-*myc* proto-oncogenes are targets for interchromosomal translocations in chronic myelogous leukemia and Burkitt's lymphoma, respectively [3, 4]. Recent molecular studies have elucidated the structural consequences that chromosomal translocations have upon these cellular proto-oncogenes [5–7]. However, most of the recurring chromosomal translocations which have been described, particularly in hematolymphoid malignancies, do not result in structural alterations of cellular homologues for known retroviral oncogenes. Presumably these cytogenetic abnormalities affect cellular genes which have not been transduced by retroviruses and therefore remain largely undefined. One approach toward isolation and characterization of these candidate human proto-oncogenes is to clone from appropriate tumors chromosomal translocation breakpoints. The breakpoint DNAs can then be used to identify closely linked transcriptional units for further study.

A particularly common translocation is the reciprocal t(14;18) translocation that occurs in at least 85%–90% of human follicular lymphomas [8]. We and others have shown that the points of crossover on chromosome 14 for these translocations always occur within or directly adjacent to an Ig heavy chain joining region [9–11]. The molecular features of t(14;18) crossovers suggested that these interchromosomal translocations result from errors in V-D-J joining during B-cell differentiation. Clustering of t(14;18) chromosomal breakpoints also occurs on chromosome 18, adjacent to or within a previously undefined transcriptional unit. The term *bcl*-2 was proposed for this potentially new human proto-oncogene [12], which may be involved in the pathogenesis of human follicular lymphomas. We describe here the structural characterization of the *bcl*-2 gene and its mRNA product. Our data show that the proposed *bcl*-2 protein shares significant homology with a hypothetical Epstein-Barr virus (EBV) protein. We also show that most t(14;18) translocations disrupt the *bcl*-2 gene, creating a hybrid *bcl*-2/IgH transcriptional unit. The results indicate that the molecular mechanism of most t(14;18) translocations is a regulatory alteration of the expression of the *bcl*-2 gene.

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B. Structure of the *bcl*-2 Gene, RNA, and Predicted Protein

A variety of hematolymphoid cell lines were screened for expression of *bcl*-2 mRNA, us-

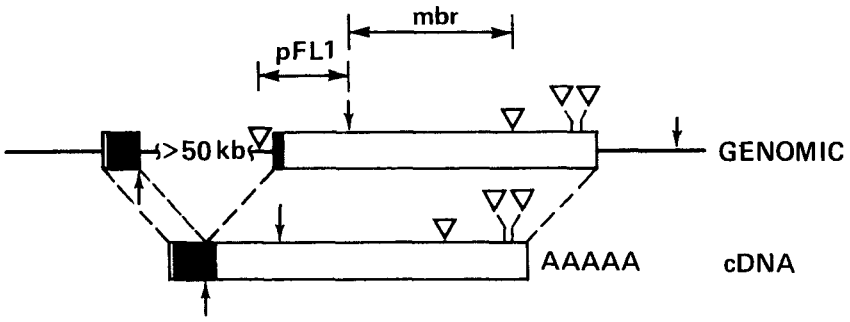

Fig. 1. Physical maps of the *bcl*-2 gene and its mRNA transcript. The physical structure determined for the configuration of the *bcl*-2 gene is shown along with a map of the *bcl*-2 mRNA predicted by the nucleotide sequence [14]. The gene consists of two exons separated by more than 50 kb of intervening sequence as determined by restriction enzyme mapping studies and nucleotide sequence determinations. The genomic locations of the pFL-1 hybridization probe and the t(14;18) major breakpoint cluster region (*mbr*) are denoted with *brackets*. Restriction enzyme cutting sites are as follows: *Bam*H1(↑), *Eco*R1(↓), and *Hin*dIII(∇)

ing a 1.5 kb fragment of chromosome 18 DNA (pFL-1) derived from a cloned t(14;18) breakpoint [9]. The results indicated that the normal transcription product of the *bcl*-2 gene, at least in B-lineage cell lines, was a 6.0 kb mRNA and that the B-cell precursor cell line SUP-B2 derived from a common acute lymphoblastic leukemia [13] expressed reasonably high levels of this mRNA. A lambda gt10 cDNA library was constructed using poly(A)$^+$ RNA from SUP-B2, and hybridizing phages were identified and purified using the pFL-1 chromosome 18 DNA probe. Analysis of phage DNA inserts showed that overlapping cDNA had been obtained comprising a total of 6 kb (as shown in Fig. 1). Nucleotide sequence determinations showed that the largest open reading frame initiated at the first ATG codon at the 5′ end of the cDNA [14]. The reading frame initiated by this codon terminated 717 nucleotides downstream at the first in-phase stop codon, followed thereafter by multiple stop codons in all three reading frames. The sequence predicted the unusual structure for the *bcl*-2 mRNA shown in Fig. 1, which consisted of a short open reading frame at the 5′ end followed by a long 3′ untranslated region.

The nucleotide sequence predicted a 239 amino acid polypeptide as the putative product of the *bcl*-2 gene, whose sequence is shown in Fig. 2. When this sequence was compared with those currently contained in the NBRF protein database, significant homology was observed with the Epstein-Barr virus (EBV) protein BHRF1, a hypothetical protein corresponding to an open reading frame in the EBV genome [15]. A computer, generated alignment of the two sequences is shown in Fig. 2. There is 25% identity between the two sequences over a 149 amino acid overlap, which increases substantially if conservative amino acid replacements are considered to be identical. The findings indicate that the predicted *bcl*-2 polypeptide is evolutionarily related to the hypothetical BHRF1 protein.

The *bcl*-2 cDNAs were used to determine the genomic configuration of *bcl*-2 DNA. The results indicated that a major portion of the *bcl*-2 cDNA was contained within a single large exon, which overlapped with the pFL-1 hybridization probe, as shown in Fig. 1. Nucleotide sequence determinations confirmed that all but the 5′ 614 nucleotides of the cDNA were contained within this single large exon. The 5′ 614 nucleotides of the *bcl*-2 cDNA are encoded greater than 50 kb away, as determined by restriction mapping studies. This distance may be considerably larger since DNA was not isolated spanning the entire linkage. The structure of the genomic DNA showed that the major t(14;18) breakpoint cluster region (*mbr*) containing most t(14;18) translocations was located within the gene, in the middle of the large exon containing the 3′ untranslated region of the *bcl*-2 mRNA. The nucleotide sequences of several previously described

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  1'    MAHAGRTGYDNREIVMKYIHYKLSQRGYEWDAGDVGAAPPGAAPAPGIFSSQPGHTPHTA
  1"                                                    MAYSTREILLALCI

 61'    ASRDPVARTSPLQTPAAPGAAAGPALSPVPPVVHLTLRQAGDDFSRRYRRDFAEMSRQLR
                                  *** .** * .  ... * . .*.* ...
 15"    RDSRVHGNGTLHPVLELAARETPLRLSPEDTVV-LRYHVLLEEIIERNSETFTETWNRFI

121'    LTPFTARGRFATVVEELF-RDGVNWGRIVAFFEFGGVMCVESVNREMSP-LVDNIALWMT
          .  .   *..*  *.* *.. . ** .*....    * .   .. .*  * .....
 74"    THTEHVDLDFNSVFLEIFHRGDPSLGRALAWMAWCMHACRTLCCNQSTPYYVVDLSVRGM

179'    EYLNRHLHTWIQDNGGWDAFVELYGPSMRPLFDFSWLSLKTLLSLALVGACITLGAYLGHK
          .  *..**.....*...*   *. *.   *** . . *.*.*.  *  *
134"    LEASEGLDGWIHQQGGWSTLIEDNIPGSRR---FSWTLFLAGLTLSLLVICSYLFISRGRH
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Fig. 2. Alignment of the predicted *bcl*-2 and BHRF1 amino acid sequences. The predicted amino acid sequences for the *bcl*-2 protein and the hypothetical EBV protein BHRF1 are shown following alignment for maximum homology determined by the program DFASTP [19]. *Asterisks* denote identical amino acids and *dots* indicate conservative replacements shared by the two sequences. The single letter amino acid designations are according to Dayhoff [20]. *Numbers* denote residue positions for *bcl*-2 (*prime*) and BHRF1 (*double prime*)

t(14;18) breakpoints were compared with the nucleotide sequences determined for the *bcl*-2 genomic DNA and cDNA; this comparison showed that seven t(14;18) crossovers occurred within 500 nucleotides of each other in the 3′ untranslated segment of the *bcl*-2 gene. These data indicate that the majority of t(14;18) translocations divide the *bcl*-2 gene within the middle of a *bcl*-2 exon.

C. Structure of the Translocated *bcl*-2 Gene and mRNA

We determined the genomic configuration of the translocated *bcl*-2 gene in the SU-DHL-4 cell line [16], which contains a t(14;18) translocation, by means of genomic Southern blot hybridizations. The results indicated that the *bcl*-2 gene was split by t(14;18) and the 3′ half of the gene was lost from the translocated allele. The results showed a head-to-tail juxtaposition of the truncated *bcl*-2 gene with an Ig heavy chain gene, as shown in Fig. 3. For the *bcl*-2 gene, the point of crossover was within the exon containing the 3′ untranslated portion; on chromosome 14 the point of crossover was at joining region J4. The results indicated that in the SU-DHL-4 cell line, following t(14;18) translocation, a hybrid *bcl*-2/IgH transcriptional unit was created.

To investigate the structure of *bcl*-2 mRNA in cells containing the t(14;18) translocation, Northern blot hybridization analyses were carried out on poly(A)$^+$ RNA isolated from the SU-DHL-4 cell line. When a 5′ *bcl*-2 cDNA fragment was used as a probe, two abnormally sized RNAs of 5.8 and 3.8 kb were detected in SU-DHL-4 cells (Fig. 4). However, when the same RNA preparation was hybridized with a 3′ cDNA probe, no transcripts were detected in SU-DHL-4, confirming that the translocation in this cell line prematurely truncates the *bcl*-2 mRNA. Since karyotype analyses and Southern blot hybridizations of this cell line indicated that a normal chromosome 18 was present, the lack of a detectable 6 kb mRNA with the 3′ probe also indicated that the non-translocated *bcl*-2 allele in SU-DHL-4 was silent. Both the 5.8 and 3.8 kb abnormally sized *bcl*-2 transcripts in this cell line also hybridized to a gamma heavy chain probe, suggesting that two hybrid *bcl*-2/Ig transcripts were produced by t(14;18) translocation.

To confirm that the abnormal transcripts actually encoded hybrid *bcl*-2/Ig mRNAs, a cDNA library was constructed from SU-DHL-4 mRNA, and overlapping cDNAs representative of the 5.8 kb transcript were

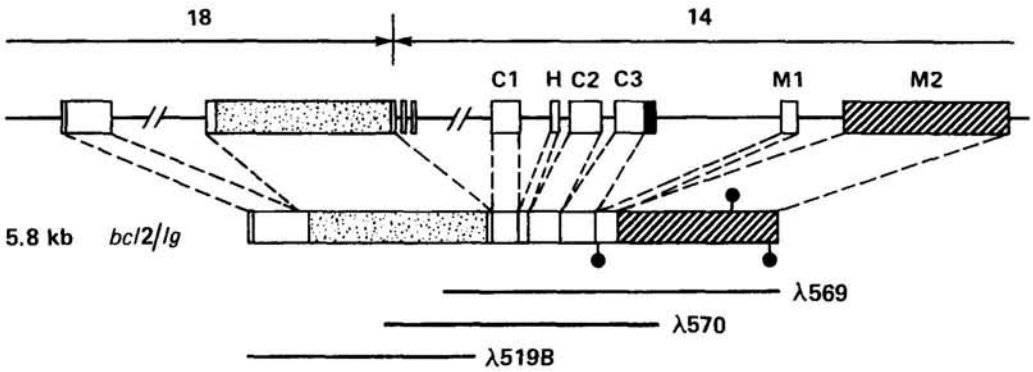

Fig. 3. Schematic diagram of the translocated *bcl*-2 gene and its 5.8 kb hybrid transcription product synthesized in the SU-DHL-4 cell line. A schematic physical map of the fused *bcl*-2 and immunoglobulin Cγ heavy chain genes in SU-DHL-4 based on Southern blotting experiments is shown. The structure of the 5.8 kb hybrid *bcl*-2/IgH mRNA transcribed from the fused genes is shown immediately below the configuration of the genes with necessary processing events indicated. Three overlapping cDNA clones isolated from SU-DHL-4 which together contain the entire 5.8 kb hybrid transcript are shown below the schematic. Open reading frames and 5′ untranslated regions for both *bcl*-2 and Cγ are represented by *open boxes*. The *bcl*-2 3′ untranslated region is shown as a *stippled box*. The *Ig* $C\gamma^m$ 3′ untranslated region is represented as a *crosshatched box* and that for *Ig* $C\gamma^s$ as a *solid box*. Restriction enzyme cutting sites: *Sma*I (♦ and *Sac*II (♦)

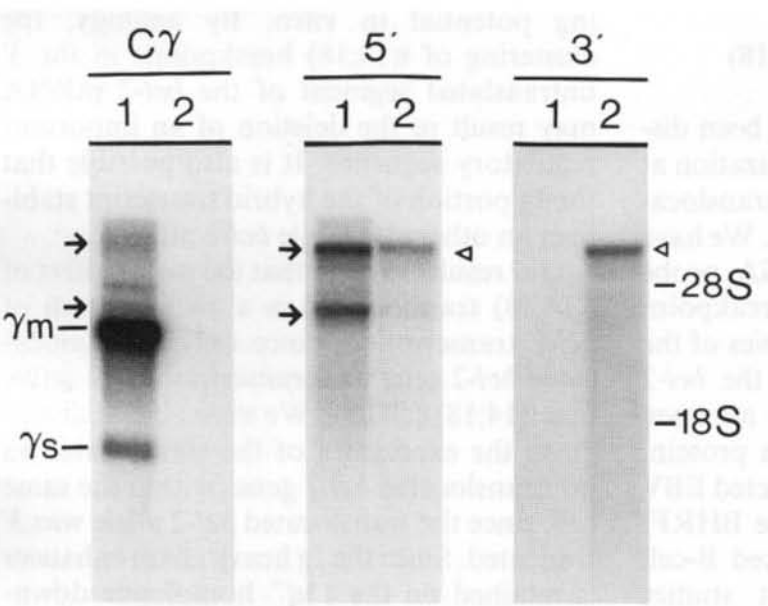

Fig. 4. Autoradiograms of Northern blots analyzing *bcl*-2- and immunoglobulin-homologous transcripts in the B-lineage cell lines SU-DHL-4 and SUP-B2. Three micrograms of poly(A^+) RNA from cell lines SU-DHL-4 (*lanes 1*) and SUP-B2 (*lanes 2*) were fractionated through formaldehyde/0.8% agarose gel. Following transfer to nylon membranes, the immobilized, paired RNAs were hybridized separately with a 5′ *bcl*-2 cDNA fragment, a 3′ *bcl*-2 cDNA fragment, and a human γ4 Ig constant region probe. The 6.0 kb *bcl*-2 mRNA detected in SUP-B2 is denoted with a *triangle;* the two abnormal 5.8 and 3.8 kb *bcl*-2 mRNAs detected in SU-DHL-4 are denoted with *arrows*, as are the Cγ-homologous mRNAs of identical size observed in this cell line

purified and characterized. Nucleotide sequence analyses showed that this abnormal RNA consisted of the 5′ approximate half of the *bcl*-2 mRNA fused to a decapitated Ig heavy chain mRNA, as shown in Fig. 3. A translation termination codon in the *bcl*-2 3′ untranslated segment adjacent to the point of fusion is in phase with the gamma heavy chain translational reading frame, thus preventing production of a chimeric Ig protein. The sequence and restriction mapping results for the hybrid *bcl*-2/Ig transcript from

this cell line indicated that transcription proceeds from the *bcl*-2 gene on the chromosome 18 portion of the translocated chromosome, across the t(14;18) breakpoint, and into the Ig heavy chain gene on chromosome 14, to be terminated somewhere downstream of the gamma membrane RNA exons, at an immunoglobulin gene termination site. The *bcl*-2 and Ig heavy chain mRNA introns are excised from the precursor in a manner similar to that which occurs during processing of normal *bcl*-2 and heavy chain mRNAs, respectively. However, the point of t(14;18) crossover, where sequences of the *bcl*-2 3′ untranslated segment join an Ig J_H segment, is not spliced out of the hybrid mRNA. The 3.8-kb hybrid transcript most likely results from differential processing of the immunoglobulin portion at the 3′ end of the hybrid *bcl*-2/Ig precursor.

D. Molecular Mechanisms of t(14;18)

The *bcl*-2 transcriptional unit has been discovered solely by virtue of its localization at the site of frequent chromosomal translocations in human B-cell malignancies. We have now used a chromosome 18 DNA probe flanking the major t(14;18) breakpoint cluster region to isolate cDNA copies of the normal transcription product of the *bcl*-2 gene. The *bcl*-2 mRNA contains an open reading frame for a 26 kilodalton protein, which is distantly related to a predicted EBV protein. It is not clear whether the BHRF1 protein participates in EBV-induced B-cell immortalization; however, recent studies have identified a BHRF1 protein in EBV-infected cells and suggest it is a highly abundant immediate early viral protein (Pearson and Kieff, personal communication). Purely structural analyses of the *bcl*-2 sequence do not suggest a specific subcellular role for the *bcl*-2 protein. Since there is no apparent transmembrane hydrophobic segment, it is unlikely to span the nuclear or plasma membrane. It also does not share homology with the conserved domains of all known protein kinases. It is likely that, since the *bcl*-2 gene is the frequent target of chromosomal translocations in developing B cells, and its protein product shares significant homology with a probable regulatory viral protein, *bcl*-2 likely plays some role in controlling the proliferation of early B cells.

Our results show that most t(14;18) translocations result in the formation of a hybrid *bcl*-2/Ig transcriptional unit, which produces hybrid transcripts lacking the 3′ half of the normal *bcl*-2 mRNA. Since these hybrid mRNAs continue to encode a normal *bcl*-2 protein, the results suggest that the major effect of t(14;18) is a regulatory alteration of *bcl*-2 expression. Part of this regulatory alteration may be posttranscriptional, since the hybrid transcripts lack the 3′ half of the normal *bcl*-2 mRNA. There is precedent for activation of cellular proto-oncogene expression due to alterations of the 3′ untranslated portions of the mRNA, as described by Meijlink et al. for the c-*fos* gene [17]. Deletion of a 67 nucleotide A-T rich region from the 3′ untranslated segment of the c-*fos* mRNA is sufficient to activate its transforming potential in vitro. By analogy, the clustering of t(14;18) breakpoints in the 3′ untranslated segment of the *bcl*-2 mRNA may result in the deletion of an important regulatory sequence. It is also possible that the Ig portion of the hybrid transcript stabilizes an otherwise labile *bcl*-2 mRNA.

Our results suggest that the major effect of t(14;18) translocation is a *cis* alteration of *bcl*-2 transcription, since only the translocated *bcl*-2 gene was transcriptionally active in a t(14;18) cell line. We were able to distinguish the expression of the translocated vs nontranslocated *bcl*-2 genes within the same cell, since the translocated *bcl*-2 allele was 3′ truncated. Since the Ig heavy chain enhancer is retained on the $14q^+$ homologue downstream of the *bcl*-2 portion within the hybrid transcriptional unit following t(14;18), it may play a role in *cis* alteration of *bcl*-2 gene expression. However, our results indicate that the 5′ end of the *bcl*-2 gene is at least 50 kb away from the point of t(14;18) crossover, requiring that the enhancer exert its effect on the *bcl*-2 promoter over a considerable distance. It is also possible that other dominant cis-acting long-range properties of the Ig heavy chain locus affect *bcl*-2 gene expression similar to the deregulation of c-*myc* gene expression in Burkitt's lymphoma.

The mechanism for alteration of *bcl*-2 expression presented here applies for most t(14;18) translocations since two-thirds of all

crossovers occur within the major breakpoint cluster region (*mbr*). However, approximately one-third of t(14;18) breakpoints cluster at another site on chromosome 18 which has not been linked within 20–30 kb of the *bcl*-2 transcriptional unit [18]. It is possible therefore that there are additional mechanisms for activation of *bcl*-2 or, alternatively, there is a second gene at 18q32 whose expression can be altered by t(14;18).

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