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Genetics

Meiotic recombination between yeast artificial chromosomes yields a single clone containing the entire *BCL2* protooncogene

(DNA cloning/polymerase chain reaction/lymphoma/human genome)

GARY A. SILVERMAN*†, ERIC D. GREEN‡, ROBERT L. YOUNG§, JENNIFER I. JOCKEL¶, PETER H. DOMER‖, AND STANLEY J. KORSMEYER§¶‖

Departments of *Pediatrics, ‡Genetics, ¶Medicine, and §Molecular Microbiology, and the ‖Howard Hughes Medical Institute, Box 8045, Washington University School of Medicine, St. Louis, MO 63110

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ABSTRACT **The common translocation found in human follicular lymphoma, t(14;18)(q32;q21), results in deregulation of the *BCL2* protooncogene. The isolation of the intact gene would provide an essential substrate to analyze the molecular basis of this malignancy. Pulsed-field gel electrophoresis suggested that this three-exon gene was several hundred kilobases (kb) long. Therefore, a library of yeast artificial chromosome (YAC) clones was screened to isolate the intact *BCL2* gene. Two clones, yA85B6 (200 kb) and yB206A6 (700 kb), were isolated by using polymerase chain reaction (PCR) assays specific for exon I/II and exon III, respectively. However, neither YAC contained the entire *BCL2* locus. Since the two YACs were found to overlap by 60 kb, we sought to take advantage of the high recombination frequency in yeast and induce physical recombination between the two clones. Cells containing each YAC were mated and induced to undergo meiotic division and sporulation. Analysis of the resulting tetrads revealed a spore containing a single recombinant YAC of 800 kb. PCR assays and Southern blotting demonstrated that this recombined YAC contained the entire ≈230-kb *BCL2* gene. Furthermore, probe order was conserved and there was no evidence of overt rearrangements or deletions. These results indicate the feasibility of reconstructing large genomic segments with overlapping YAC clones to study genes spanning hundreds of kilobases.**

Over 80% of the cases of follicular B-cell lymphoma are associated with a specific interchromosomal translocation, t(14;18)(q32;q21) (1). This translocation places the *BCL2* protooncogene from 18q21 into the immunoglobulin heavy-chain locus (*IGH*) located on 14q32 (2–4). The newly created *BCL2–IGH* fusion gene generates heterogeneously sized chimeric transcripts composed of 5′ *BCL2* exons and varied 3′ *IGH* untranslated regions (5). The 2.5-hr half-life of the *BCL2–IGH* fusion mRNA is not different from that of the normal *BCL2* mRNA. Preliminary studies suggested that the rate of newly initiated transcription from the translocated allele is increased (5). The precise mechanism(s) responsible for the full deregulation of the *BCL2–IGH* fusion gene is uncertain, but the consequence is an inappropriately high level of *BCL2–IGH* mRNA and BCL2 protein relative to the mature B-cell stage of these tumors (5–7).

BCL2 is a unique oncoprotein that extends the survival of ordinarily senescent cells without affecting cellular proliferation (8–12). In turn, the prolonged survival allows for the accumulation of additional transforming events. Support for this theory is provided by gene-transfer studies which show that *BCL2* improves the cloning efficiency of lymphoblastoid cells in soft agar (10), prevents cell death after interleukin deprivation in some interleukin-dependent cell lines (8, 11), and potentiates the transforming potential of a *MYC* construct overexpressed in lymphoblastoid (10) and bone marrow cells (8). Further, mice containing a deregulated *BCL2* transgene demonstrate an expanded B-cell compartment and enhanced B-cell survival (9, 12).

The normal human *BCL2* gene has three exons. The first intron is only 220 base pairs (bp) long, but pulsed-field gel electrophoresis (PFGE) suggested the second intron was ≈370 kilobases (kb) long (5). The t(14;18) frequently introduces the *IGH* joining, enhancer, and constant elements into either of two sites located in the 3′ untranslated region of *BCL2* (2–4). These have been designated the *BCL2* major breakpoint (MBR) and minor cluster (MCR) regions (5, 13). This implies that the effects of the *IGH* locus introduced into either the MBR or MCR are conveyed across the large second intron to the 5′ *BCL2* promoters (5). Therefore, examination of the mechanisms of transcriptional regulation and deregulation would benefit from a cloned, intact DNA segment containing the entire *BCL2* gene within its genomic context. However, the size of *BCL2* precludes its isolation in a single phage or cosmid clone. Yeast artificial chromosomes (YACs) are vectors that permit the cloning of genomic fragments as large as 1000 kb (14, 15). We have isolated the entire *BCL2* gene in a single clone by the physical recombination between two smaller overlapping YACs.

MATERIALS AND METHODS

YAC Library Construction and Screening. The YAC library was constructed in the Center for Genetics in Medicine at Washington University. This library was derived from an *Eco*RI partial digest of human genomic DNA which was ligated into the pYAC4 vector as described (15). A screening protocol based on the polymerase chain reaction (PCR) was used to isolate clones from the library (16).

DNA Analysis by PFGE. Restriction digests of high molecular weight yeast DNA from the YAC clones or human genomic DNA from the lymphoblastoid cell line CGM-1 (derived from the same donor used to construct the library; ref. 15) were performed in low-melting-point agarose plugs (17, 18). Restriction fragments or intact yeast chromosomes were separated in a 1% agarose gel with a contour-clamped homogeneous electric field (CHEF) apparatus (19). Electrophoresis conditions included a 6-V/cm (160 V) field strength, 0.5× TBE buffer (20) at 12°C, and a 24-hr electrophoresis time. Switch intervals are indicated in the figure legends. DNA fragments were transferred to reinforced nitrocellulose

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Abbreviations: CHEF, contour-clamped homogeneous electric field; MBR, major breakpoint region; MCR, minor cluster region; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; YAC, yeast artificial chromosome.

†To whom reprint requests should be addressed.

by routine blotting techniques (20). Filters were hybridized with ^{32}P-labeled probes and washed under stringencies previously described (21). Autoradiograms were developed 1–7 days. Filters were stripped of a probe by 0.1% SDS at 80°C. Fragment sizes were determined by comparison to concatemers of λ phage DNA and the electrophoretic karyotype of *Saccharomyces cerevisiae* strain AB1380 (22).

DNA Analysis by Conventional Electrophoresis. Either 0.1 μg of total yeast DNA or 15 μg of total human genomic DNA was digested with *Bam*HI, *Eco*RI, or *Hin*dIII. The difference in amount of yeast to human DNA reflects the lower (1/200) complexity of the yeast genome. Fragments were separated in 0.9% agarose gels in 2× TAE buffer (21).

PCR. Assays were performed in 5 μl reaction volumes containing purified yeast DNA (0.01 μg) or yeast cells (half of a colony ≈2 mm in diameter) (23), 5′ and 3′ primers (5 μM each), dNTPs (200 μM each), 0.5 unit of *Thermus aquaticus* DNA polymerase, 50 mM KCl, 100 mM Tris (pH 8.3), and 1.5 mM $MgCl_2$ (23, 24). Thirty-five cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and elongation (72°C, 1 min) were performed. Amplified products were separated by electrophoresis through a 1% agarose gel.

Probes. The genomic *BCL2* probes corresponding to exon II (0.6 kb, *Bam*HI–*Eco*RI), exon III (1.5 kb, *Hin*dIII–*Eco*RI), and the MBR (2.8 kb, *Eco*RI–*Hin*dIII) have been described (5). The A85R, A85L, B206R, and B206L probes were derived from the right and left ends of YAC clones yA85B6 and yB206A6. These fragments were isolated by a modified, inverse PCR technique (21). The DNA sequences used to synthesize PCR oligonucleotide primer pairs from these (25) and the *BCL2* exon probes (5) have been described. The In-15 probe is a unique 2.1-kb *Sac* I fragment isolated from *BCL2* intron II. The MCR probe was amplified from genomic DNA and corresponds to nucleotides 351–895 (13). The left-arm and right-arm YAC vector probes are the 2.7-kb and 1.7-kb *Pvu* II–*Bam*HI fragments of pBR322, respectively (14). ^{32}P-labeled probes were generated by PCR (26) or by random priming (27).

Yeast Strains and Manipulations. AB1380 (*MATa*, *ura3*, *trp1*, *ade2-1*, *can1-100*, *lys2-1*, *his5*) was the transforming strain used for construction of the YAC library (14). The pYAC4 vector contained the complementing *URA3* and *TRP1* genes. The yA85B6 YAC was transferred to a *MATα* strain by simple outcrossing with AB1610 [*MATα*, *ura3*, *trp5-2*, *leu1*, *ade2-1*, *lys2-1*]. Spores were obtained by microdissection of asci (28, 29). A PCR assay capable of discriminating between mating types was used to screen colonies derived from single spores for the presence of a *MATα*, yA85B6-containing clone (23).

RESULTS

YAC Clones Containing Portions of *BCL2*. Oligonucleotide primer pairs specific for the exons flanking the large *BCL2* intron were used in a PCR assay to screen a human genomic YAC library (50,000 clones). Two clones, yA85B6 (200 kb) and yB206A6 (700 kb), were isolated. Clone yA85B6 hybridized with the *BCL2* exon II probe but not with the exon III probe (Fig. 1 *a* and *c*). Conversely, yB206A6 hybridized with the exon III probe (Fig. 1*c*) but not with the exon II probe (data not shown). To confirm that these clones contained *BCL2* and not cross-hybridizing sequences, restriction endonuclease analysis was performed. The sizes of the restriction fragments identified by the exon II and exon III probes within

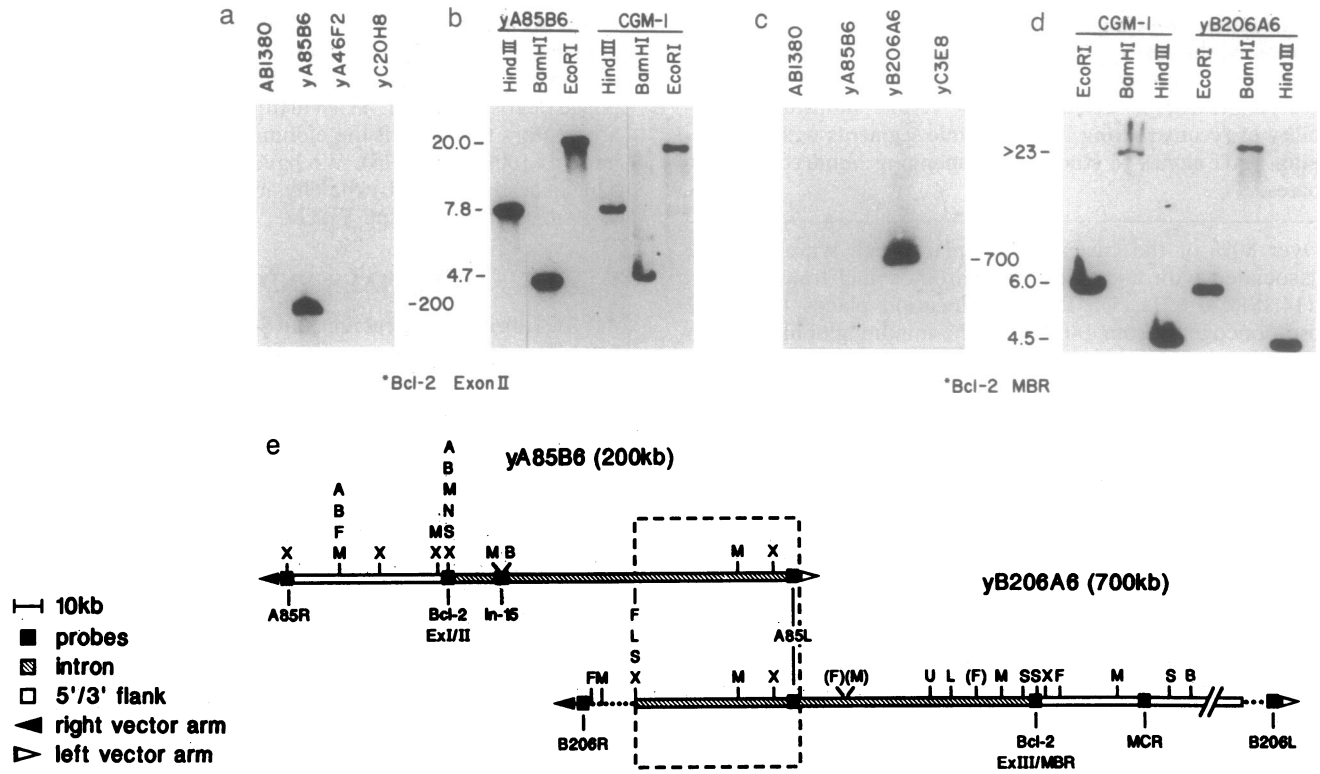

FIG. 1. Identification and characterization of the *BCL2*-containing YAC clones. (*a–d*) Autoradiograms of size-fractionated chromosomes (*a* and *c*) and restriction digests (*b* and *d*) of yA85B6 and yB206A6. The ^{32}P-labeled probes were *BCL2* exon II (*a* and *b*) and MBR (*c* and *d*). Yeast chromosomes were separated by CHEF electrophoresis using a constant 72-sec switch interval. Clones yA46F2, yC20H8, and yC3E8 are YACs containing human DNA inserts. AB1380 is the host strain. CGM-1 is a lymphoblastoid cell line derived from the same donor used to construct the library. Sizes of hybridizing fragments are indicated in kilobases. (*e*) Rare-cutting restriction maps of yA85B6 and yB206A6. Boxed area indicates the region of overlap between the clones. Dotted lines represent the fragments in yB206A6 that were derived from chromosome 7 (B206R) or 1 (B206L) (25). A, *Nae* I; B, *Bss*HII; F, *Sfi* I; L, *Sal* I; M, *Sma* I; N, *Not* I; S, *Sac* II; U, *Mlu* I; X, *Xho* I.

yA85B6 and yB206A6 were identical to those of human genomic DNA (Fig. 1 *b* and *d*).

These data suggested that yA85B6 and yB206A6 contained the 5′ and 3′ portions of *BCL2*, respectively. To determine whether the entire *BCL2* gene was contained collectively within these two YACs, rare-cutting restriction endonucleases and PFGE were used to construct physical maps of the clones. Probes specific for the vector arms and fragments (A85R, A85L, B206R, B206L) corresponding to the right (R) and left (L) ends of the cloned inserts facilitated the mapping process. Analysis of yA85B6 revealed that *BCL2* exon I/II was located ≈65 kb from A85R and ≈135 kb from A85L (Fig. 1*d*). A85L was identified within yB206A6. This suggested that a left portion of yA85B6 overlapped with a right portion of yB206A6. However, the B206R probe did not hybridize to yA85B6. This discrepancy was resolved by finding that the B206R probe mapped to chromosome 7 and not to chromosome 18 (25). Thus, the rightmost segment of yB206A6 was derived from a noncontiguous chromosomal sequence. This type of cloning artifact has been detected in other clones isolated from this library (21, 25, 29). Nonetheless, yA85B6 and yB206A6 shared a region of overlap spanning ≈60 kb. Further analysis of yB206A6 revealed that *BCL2* exon III was located ≈90 kb 3′ of A85L. This suggested that the entire *BCL2* gene was contained within the two YAC clones and that intron II was 225 kb (135 kb plus 90 kb) long.

Construction of a Single *BCL2*-Containing YAC by Meiotic Recombination. The isolation of two clones that overlapped by ≈60 kb provided an opportunity to construct a single YAC containing the entire *BCL2* gene. This could be accomplished by introducing both YACs into the same diploid yeast strain, inducing meiosis, and examining any resulting YAC-containing spores for evidence of meiotic recombination. In yeast, meiotic recombination occurs between homologous sequences at a rate of ≈0.4 centimorgan per kilobase (30, 31). To avoid the generation of mitotically unstable dicentric or acentric YACs, the overlapping clones should be in the same 5′ → 3′ vector orientation (Fig. 1*e*).

Clone yA85B6 (*MATα*) was mated with yB206A6 (*MAT***a**). The resulting diploid strain was induced to undergo meiosis and sporulation. Asci containing four spores (tetrads) were dissected onto complete medium and subsequently plated on complete medium minus uracil. This selects for the presence of the YAC vector (*URA3*) in a Ura$^-$ host. The YAC-containing spores were analyzed by PCR assays specific for the multiple loci present on the parental YACs (Figs. 1*e* and 2*a*). The desired tetrad should yield four spores, each containing a different YAC (tetratype): (*i*) the yA85B6 parental (200 kb), (*ii*) the yB206A6 parental (700 kb), (*iii*) an 800-kb recombinant YAC containing the entire *BCL2* gene, and (*iv*) a 90-kb reciprocal recombinant YAC.

A total of 15 tetrads were dissected. All four spores germinated from only four of the asci. Two of these tetrads were analyzed in detail. The first revealed a complex pattern of rearranged or deleted YACs not consistent with the desired tetratype (data not shown). PCR analysis of spores from the second tetrad (Fig. 2*b*) showed that spores I and II contained a PCR "fingerprint" identical to that observed for the parentals yA85B6 and yB206A6, respectively. Detection of all the correctly sized markers except B206R suggested

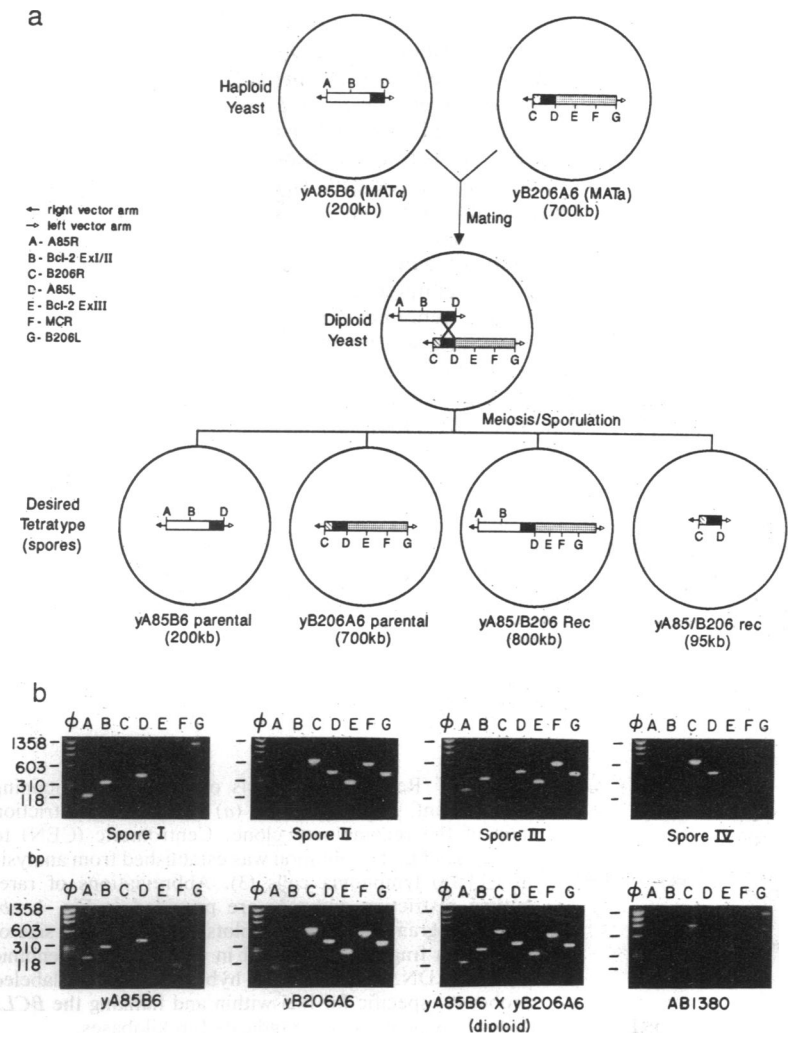

FIG. 2. Analysis of spores derived from the mating between yA85B6 and yB206A6. (*a*) Schematic representation of the mating, crossover, and desired tetratype that would yield a spore containing a single YAC with the entire *BCL2* gene. Ex, exon. (*b*) Ethidium bromide-stained 1% agarose gels of PCR-amplified products of DNA isolated from the indicated spores and primers that amplify the regions depicted in *a*. Sizes of the amplified products for A–G, in base pairs, are 149 (A85R), 275 (*BCL2* ExI/II), 660 (B206R), 420 (A85L), 225 (*BCL2* ExIII), 550 (MCR), and 410 (B206L). (*Upper*) Analysis of four spores derived from a single tetrad. (*Lower*) As controls, amplified products derived from parental strains. ϕ, *Hae* III-digested ϕX174 phage DNA (size standards).

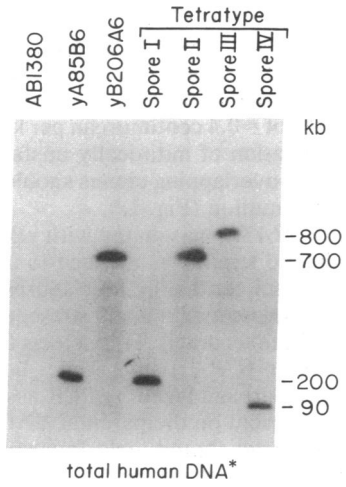

FIG. 3. Sizing of the YACs contained within spores derived from the yA85B6 × yB206A6 mating. Yeast chromosomes were separated by CHEF electrophoresis (45-sec/90-sec linearly ramped switch interval), blotted, and hybridized to ^{32}P-labeled total human DNA. AB1380 is the nontransformed host. yA85B6 and yB206A6 are the parental clones and the tetratype is the same as described in Fig. 2.

that spore III contained a recombinant YAC encompassing all of *BCL2*. Detection of only the B206R and A85L markers suggested that spore IV contained the reciprocal recombinant YAC.

Although the PCR assays suggested that a tetratype with the desired recombinant (spore III) had been isolated, the presence of contaminating diploids or two haploid spores with nonrecombined parental YACs was not excluded. In a PCR assay that can discriminate mating types (23), the detection of a single allele confirmed that the spores were truly haploid (data not shown). Analysis of high molecular weight DNA by PFGE confirmed that each spore contained a single YAC (Fig. 3). Further, the sizes of the YACs in spores I (200 kb), II (700 kb), III (800 kb), and IV (90 kb) were identical to those predicted for the tetratype containing the yA85B6 parental, the yB206A6 parental, a *BCL2*-containing recombinant (yA85/B206 Rec), and a reciprocal recombinant (yA85/B206 rec), respectively (Figs. 2*a* and 3).

Characterization of the Recombinant YAC Containing *BCL2*. The previous experiments suggested that the recombinant YAC in spore III (yA85/B206 Rec) contained the entire *BCL2* gene. To determine whether the gene was intact or whether the recombination event resulted in gross rearrangements or deletions, restriction analysis was performed. First, a rare-cutting restriction map was constructed using PFGE and multiple probes (Fig. 4*a*). The map of yA85/B206 Rec was in accordance with that predicted by recombination within the 60-kb region shared by yA85B6 and yB206A6. Specifically, the 5′ → 3′ order of the markers and the lengths of the restriction fragments were preserved. For example, the three contiguous *Sal* I fragments [which correspond to the segments containing exon I/II (≈145 kb), A85L (≈75 kb), and exon III (≈110 kb), respectively] present within the parentals (Fig. 1*b*) were detected in digests of yA85/B206 Rec (Fig. 4*a*). Furthermore, routine Southern blotting revealed that the sizes of the *Bam*HI or *Eco*RI restriction fragments detected within yA85/B206 Rec by the *BCL2* exon I/II, In-15, A85L, *BCL2* exon III, and MCR probes were identical to those of genomic DNA (Fig. 4*b*). Since the sizes of the amplified products detected in the PCR assays of yA85/B206 Rec were identical to those of the parentals (Fig. 2*b*), these data suggest that no overt rearrangements or deletions were present within yA85/B206 Rec.

DISCUSSION

The discovery of genes associated with follicular lymphoma (5), Duchenne muscular dystrophy (32), cystic fibrosis (33), colorectal carcinoma (34), and chronic myelogenous leukemia (35) that span hundreds of kilobases has fostered a new series of questions regarding the molecular mechanisms that control their expression. Are some genes, due to their large size, governed by regulatory events different from those envisioned to control smaller genes? For example, does the altered transcription of the *BCL2–IGH* fusion gene observed in lymphoma cells result from placement of the *IGH* enhancer 225 kb away from the 5′ *BCL2* promoter elements or result from a modification of higher-order chromatin structure (36)? Although gene-transfer studies using minigene constructs containing truncated gene segments and putative regulatory elements provide some insight, they cannot be used to evaluate events that require extensive genomic sequence. The study of gene regulation within the genomic context would be facilitated greatly if methods were available to isolate large genomic sequences containing intact genes.

In this study we have taken a major step in the effort to understand *BCL2* deregulation by constructing a single DNA segment that contains the entire gene plus extensive flanking sequence. An initial screen of a YAC library yielded two

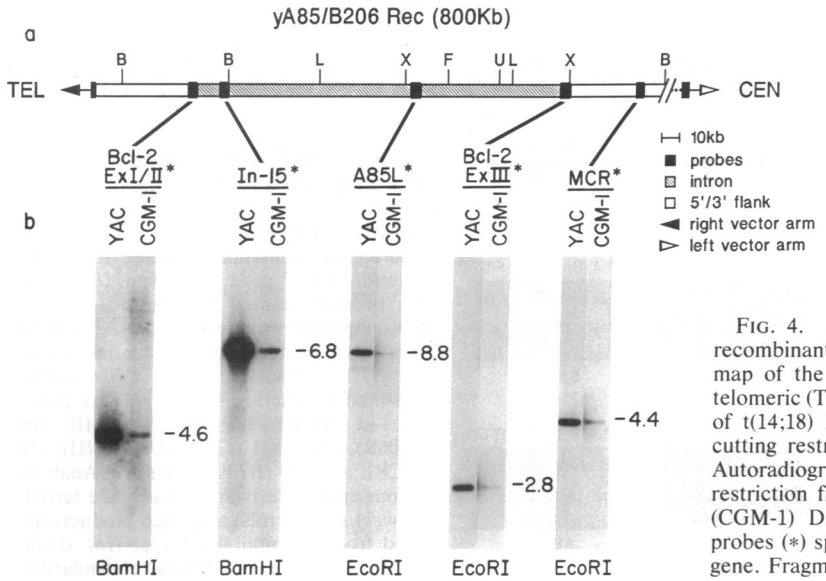

FIG. 4. Restriction analysis of the *BCL2*-containing recombinant, yA85/B206 Rec. (*a*) Rare-cutting restriction map of the recombinant clone. Centromeric (CEN) to telomeric (TEL) orientation was established from analysis of t(14;18) lymphoma cells (3). Abbreviations of rare-cutting restriction enzymes are provided in Fig. 1. (*b*) Autoradiograms of Southern blots comparing the size of restriction fragments detected in the YAC vs. genomic (CGM-1) DNA. Blots were hybridized to ^{32}P-labeled probes (*) specific for loci within and flanking the *BCL2* gene. Fragment sizes are indicated in kilobases.

overlapping clones, neither of which contained the entire gene. However, we employed an approach using meiotic recombination to generate a single 800-kb YAC containing the entire gene. In this recombinant YAC, probe order was preserved and no obvious rearrangements or deletions were detected, even within the region of recombination. Recently, an analogous strategy was used to derive a single YAC containing the entire cystic fibrosis gene (29). This suggests that YAC cloning and meiotic recombination will prove to be useful adjuncts in the isolation and manipulation of large genes. Furthermore, recent studies detecting the stable integration and expression of YACs within mammalian cells suggest that functional analysis of these large clones is feasible (37, 38).

Isolation of the intact *BCL2* gene in a single YAC has provided new information regarding its genomic organization. Previous analysis of human genomic DNA by PFGE suggested that intron II was ≈370 kb long (5). This was based on the detection of a 370-kb *Sac* II fragment that hybridized to both the exon II and the exon III probe. Analysis of genomic λ phage clones revealed *Sac* II sites just 5′ of exon II and within the region recognized by the exon III probe (5). This suggested that the 370-kb *Sac* II fragment was demarcated by these sites. However, analysis of the YAC clones revealed an intron of 225 kb and additional *Sac* II sites located ≈5 kb 5′ and ≈50 kb 3′ of exon III. This paradox in the intron's size can be explained by interference with *Sac* II digestion by DNA methylation in human genomic DNA. The CpG motif present in the recognition sequence of many rare-cutting restriction endonucleases (e.g., *Sac* II) is methylated at the 5 position of cytosine in human but not in *S. cerevisiae* genomic DNA (39). Using probes flanking the 5′ *Sac* II site, we confirmed that this site was digested in both human and yeast genomic DNA (G.A.S., unpublished observations). However, in *Sac* II digests of yeast DNA, the exon II and exon III probes did not hybridize to a common fragment. The exon III probe detects a unique 5-kb fragment and an ≈50-kb piece that also hybridizes to the distal MCR probe. Since the exon III probe does not detect these fragments in *Sac* II digests of human genomic DNA, the *Sac* II sites 5′ and within exon III may be methylated. This hypothesis was supported by analysis using the MCR probe. Although this probe is located 3′ of these *Sac* II sites, it hybridized to a human genomic *Sac* II fragment of the same size as that recognized by both the exon II and the exon III probe (G.A.S., unpublished observations). Thus, the *Sac* II site demarcating the 3′ limit of the human genomic 370-kb fragment is not in exon III and must be distal to the MCR locus. This accounts for the overestimation of the intron's size by PFGE analysis of genomic DNA.

HTF islands (genomic regions rich in unmethylated CpG motifs that are frequently identified by the clustering of rare-cutting restriction sites) are usually associated with the 5′ regions of "housekeeping" genes (40). The detection of a CpG island in the 5′ region of *BCL2*, although not unique, is unusual for a tissue-specific gene. In addition, new CpG islands have been detected ≈40 kb 5′ and at variable locations 3′ of *BCL2*. Although we have yet to determine whether these regions are associated with unidentified genes associated with *BCL2* regulation, the generation of a single YAC encompassing *BCL2* should provide the substrate necessary to analyze the relevance of these syntenic elements.

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