Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10137–10141, October 1995
Genetics

The *BCL2* major breakpoint region is a sequence- and cell-cycle-specific binding site of the Ku antigen

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Communicated by Stanley M. Gartler, University of Washington, Seattle, WA, July 26, 1995

ABSTRACT **The majority of translocations involving *BCL2* are very narrowly targeted to three breakpoint clusters evenly spaced over a 100-bp region of the gene's terminal exon. We have recently shown that the immediate upstream boundary of this major breakpoint region (mbr) is a specific recognition site for single-strand DNA (ssDNA) binding proteins on the sense and antisense strands. The downstream flank of the mbr is a helicase binding site. In this report we demonstrate that the helicase and ssDNA binding proteins show reciprocal changes in binding activity over the cell cycle. The helicase is maximally active in G_1 and early S phases; the ssDNA binding proteins are maximally active in late S and G_2/M phases. An inhibitor of helicase binding appears in late S and G_2/M. Finally, at least one component of the helicase binding complex is the Ku antigen. Thus, a protein with helicase activity implicated in repair of double-strand breaks, variable(diversity)joining recombination, and, potentially, cell-cycle regulation is targeted to the *BCL2* mbr.**

The t(14,18) translocation of human follicular lymphoma fuses the 250-kb *BCL2* gene with joining segments of the immunoglobulin heavy chain locus (1), resulting in constitutive *BCL2* expression (2) and interruption of programmed cell death (3–5). Seventy per cent of these translocations occur within a 100-bp-long segment of the untranslated portion of exon 3, designated the major breakpoint region (mbr; refs. 1, 6, and 7). At the immediate 5′ border of the mbr are two tandem octamer sequences representing 8-for-8 and 7-for-8 matches, respectively, of the human minisatellite consensus GC(A/T)GG(A/T)GG (8), which resembles the prokaryotic activator of recombination, χ (9). Approximately 15 translocations are clustered just downstream of the 8/8 χ; two other clusters are spaced 50 bp apart further downstream of cluster 1 (see Fig. 1; ref. 10). Translocation abruptly terminates after cluster 3.

The highly organized spacing of *BCL2* translocation breakpoints within the mbr suggests that the translocation process may be directed to, and constrained within, this region by DNA–protein interactions defining an unusual chromatin structure/function. Employing mobility shift assays (MSAs) with double- and single-strand target DNAs representing various portions of the mbr, we have recently demonstrated that the region just downstream of the termination of translocation (cluster 3) corresponds to an 85-bp-long binding site. DNA sequence specificity of binding has been established by standard competition assays as well as by deletion analysis. The complex that formed on this site has a number of other unusual properties. (*i*) It is very large, migrating at the top of 4% polyacrylamide gels. (*ii*) Though the complex is produced with extracts from every human and murine cell line tested, it is extremely abundant in lymphocyte extracts. (*iii*) In extracts from human leukocyte cell lines expressing *BCL2*, such as Jurkat and HL60, a variant pattern is observed. The large complex is greatly reduced or absent; in its place a diffuse, slightly smaller, complex occurs. (*iv*) The variant complex contains fully single-stranded target DNA (even though the target is initially double-stranded) and can be competitively inhibited specifically by sense and antisense mbr single-strand DNA (ssDNA). (*v*) The formation of the variant, but not the standard, complex requires energy. These characteristics imply that, in some cell lines, the mbr is the target of a site-specific helicase activity. The reduction of variant complex formation in favor of a ladder of smaller complexes in the presence of target excess further suggests a distributive, rather than processive, mechanism of action.

The χ sequences at the 5′ boundary of the mbr have also proven to be sites interacting with specific nuclear factors. In this case, binding occurs to single-, rather than double-strand, target DNA. Mutation analysis has revealed that the sense strand binding factor requires both χ octamers in the recognition sequence. The antisense factor(s) produces an array of complexes that require either one of the two χ octamer sequences for specific binding.

Thus, the region of the *BCL2* gene into which translocations are targeted is demarcated by recognition sites for helicase and ssDNA binding proteins. Since proteins of this description have been implicated in transcription, replication, recombination, and repair, we entertained the hypothesis that the mbr specifies a region important for the control of *BCL2* expression and/or the replication of the gene. We now report that mbr complex formation is highly orchestrated in the cell cycle and that at least one protein component of the complexes is the Ku antigen. The implications of these findings for *BCL2* translocation and function will be discussed.

MATERIALS AND METHODS

Preparation of Nuclear Extracts. Crude nuclear extracts were prepared from the cell lines Jurkat (human $CD8^+$ T-cell leukemia), PD31 (Abelson leukemia virus-induced murine pre-B-cell leukemia), and HL60 (human promyelocytic leukemia) by the method of Dignam *et al.* (11) with modifications. Only 10^8 Jurkat cells from elutriation pools were used for extraction; also, dialysis was performed with only 50 volumes of Dignam buffer D. Extracts of induced HL60 cells were prepared after pretreatment of cells with 10 ng of 12-*O*-tetradecanoylphorbol 13-acetate per ml (TPA; Sigma) for 24, 72, or 120 hr. Following extraction, total protein was determined by Bradford assay (Bio-Rad), and extracts were stored frozen in small aliquots at −70°C.

Preparation of Target DNA. Double-strand targets representing various *BCL2* mbr germ-line fragments were amplified and labeled by the polymerase chain reaction (PCR). The oligonucleotides used for amplification of the target fragments

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Abbreviations: mbr, major breakpoint region; MSA, mobility shift assay; PCNA, proliferating cell nuclear antigen; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; ssDNA, single-strand DNA; dsDNA, double-strand DNA.

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are listed below, and a schematic description of targets is provided in Fig. 1. The template sequence was a 279-bp germ-line fragment subcloned into pBSM13+ (10). The labeled fragments were then isolated by electrophoresis through 8% polyacrylamide/TBE (TBE: 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) gels, followed by elution in buffer (500 mM ammonium acetate/0.1% SDS/1 mM EDTA) at 37°C for 16 hr. DNA was extracted with phenol/chloroform/isoamyl alcohol (24:24:1) and then precipitated in 2.5 volumes of 95% ethanol. ssDNA targets containing the sense and antisense χ mbr sequences were end-labeled with [γ-^{32}P]ATP (DuPont/NEN) and T4 polynucleotide kinase (New England Biolabs).

Oligonucleotides. The double-strand *BCL2* mbr target fragments were amplified by PCR using the following oligonucleotides as primers: 5′-CCTTTAGAGAGTTGCTTTACGTG-3′ (RTW2; bases 2989–3012, GenBank accession no. M14745) and 5′-TCCATATTCATCACTTTGACAA-3′ (RTW3; bases 3266–3245). The single-strand *BCL2* mbr targets were 45-nt oligonucleotides; the sense strand was 5′-CTCCCAGAGC-**CCTCCTGCCCTCCTTC**CGGGGGGGCTTTCTCATGG-3′ and the antisense strand was 5′-GACAGCCATGAGAAAG-CCCCCCCG**GAAGGAGGGCAGGAGG**GCTCT-3′. These were bases 3032–3076 and 3081–3037, respectively, of the GenBank sequence. The χ octamer doublet is shown in boldface in each sequence.

MSAs. Thirty-microliter DNA–protein binding reactions were carried out in 10 mM Tris, pH 7.5/40 mM NaCl/1 mM EDTA/1 mM 2-mercaptoethanol/4% glycerol/3–6 μg of poly(dI-dC)·poly(dI-dC), 10,000 cpm of labeled target DNA (specific activity $>10^8/\mu$g), and 10 μg of crude nuclear extract (12). The samples were incubated for 30 min at room temperature; the reaction products were then separated by electrophoresis through prewarmed 4% polyacrylamide/Tris acetate gels (Fig. 2) or 4% polyacrylamide/Tris borate gels (Figs. 3–6). The gels were then blotted onto Whatman 3-mm paper, dried at 80°C for 1 hr under vacuum, and exposed to Kodak XAR film. In supershift assays, the anti-Ku monoclonal antibodies were 162 (IgG2a anti-p70/p80), 111 (IgG1 anti-p80), and N3H10 (IgG2b anti-p70) (refs. 13 and 14; a kind gift of W. Reeves), and the antibody to proliferating cell nuclear antigen (anti-PCNA antibody) was an IgG2a purchased from Santa Cruz Biotechnology. Antibodies were preincubated with Jurkat extract for 1 hr at 4°C prior to the addition of target DNA.

Counterflow Centrifugal Elutriation. An asynchronous population of Jurkat cells was separated via counterflow centrifugal elutriation using a JE-6B elutriation rotor and a J2-21 ME elutriation centrifuge system (Beckman). Logarithmic phase cells were concentrated by centrifugation at 1000 × *g* and resuspended at 4×10^7 cells per ml in complete culture medium. Cells (4×10^8) were loaded with a peristaltic pump into the 4°C chamber with an initial flow rate of 3.0 ml/min at 2250 rpm. The system was then equilibrated at 3.0 ml/min for 10 min. Nineteen elutriation fractions were obtained by increasing the flow rate 2.0 ml/min per fraction, with the first fraction collected at the rate of 4.5 ml/min. Fractions at each flow rate were collected for 4 min in 50-ml conical tubes on ice. For each fraction, an aliquot of cells was used to determine DNA content by fluorescence-activated cell sorter (FACS) analysis using a FACScan analyzer (Becton Dickinson Immunocytometry Systems). Prior to analysis, the cells were prepared as described by Taylor (15). Jurkat cells in appropriate fractions were then pooled as follows: P1 contained G_1 cells in fractions obtained from flow rates of 16.5 ml/min, 18.5 ml/min, and 20.5 ml/min; P2 contained early S cells from 24.5 ml/min, 26.5 ml/min, and 28.5 ml/min; P3 contained middle to late S cells from 30.5 ml/min, 32.5 ml/min, and 34.5 ml/min; and P4 contained G_2 plus M cells from 38.5 ml/min and 40.5 ml/min.

RESULTS

Variation of mbr Complex Formation with TPA Treatment of HL60 Cells. To determine if complex formation involving the mbr was altered with changes in *BCL2* expression and the state of cellular differentiation, we examined the promyelocytic leukemia cell line HL60. When HL60 is exposed to the phorbol ester TPA, *BCL2* transcription ceases (16), and cells are induced to differentiate into nonproliferating monocytes (17). MSAs were performed with nuclear extracts from HL60 cells treated for 24, 72, or 120 hr with TPA. The extracts from uninduced cells (Fig. 2) yielded the "variant" binding pattern of two complexes described in the Introduction; these were similar to Jurkat (Fig. 2). For comparison, the single large complex obtained with most lymphocyte extracts is shown in the lane labeled PD31. However, the two complexes were only

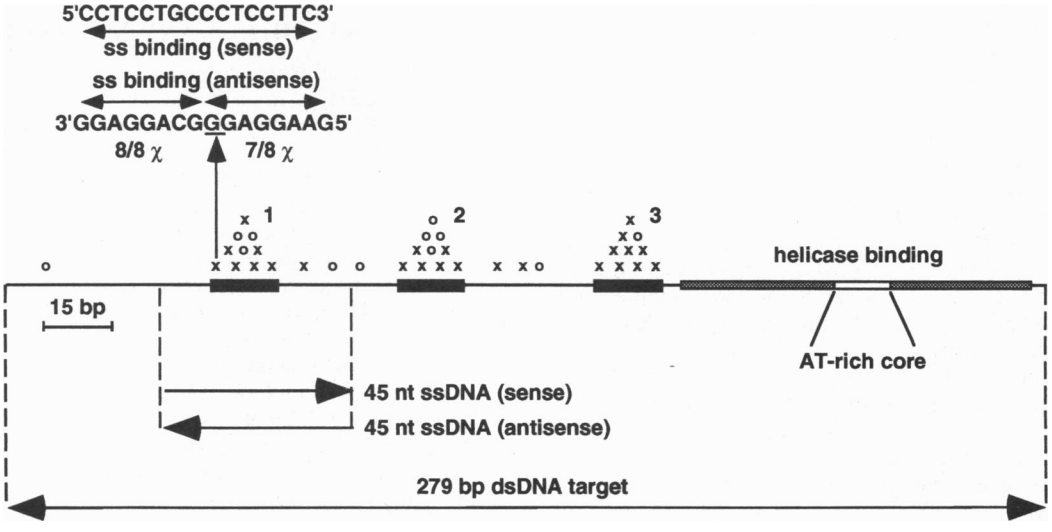

FIG. 1. Organization of MSA targets, protein binding sites, and translocation clusters in the *BCL2* major breakpoint region. The 279-bp segment containing the mbr is depicted. Three translocation clusters, each ≈15 bp wide, are schematically indicated by filled rectangles. The Xs and Os represent approximate sites of individual translocation breakpoints summarized in ref. 10. The tandem χ doublet octamer sequence is shown above cluster 1. The underlined "G" represents the site of the first translocation breakpoint in cluster 1. The extent of the 85-bp helicase binding site is shown downstream of cluster 3. Target DNAs used in MSAs are shown below the mbr. dsDNA, double-strand DNA.

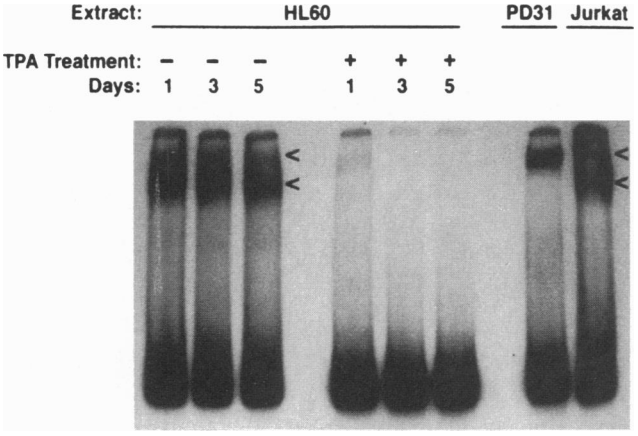

FIG. 2. mbr complex formation following TPA treatment of HL60 cells. Nuclear extracts were prepared from HL60 cells treated with TPA for 24, 72, and 120 hr as well as from the untreated parent culture. MSAs were then performed with the 279-bp mbr target. Complexes formed with PD31 and Jurkat are shown for comparison. Arrowheads denote upper and lower complexes.

faintly visible in cells treated with TPA for 24 hr and were absent with longer treatment (Fig. 2). This effect paralleled the diminution of *BCL2* expression previously reported. During this same interval, the sense strand χ binding activity was somewhat diminished; the antisense χ complexes increased dramatically (not shown). Therefore, both types of mbr-specific nuclear factors showed pronounced changes in binding activity in response to changes in the state of cellular differentiation.

Cell-Cycle Control of mbr Binding Factor Expression. Given the dependence of complex formation on *BCL2* expression and, potentially, cell division, we wished to determine if mbr binding activity demonstrated variation with specific stages of the cell cycle. Preliminary studies using cells synchronized with hydroxyurea indicated G_1/early S compartmentalization of helicase binding activity but gave conflicting results with the single-strand χ binding proteins. To obviate possible drug effects and clarify interactions with ssDNA binding proteins, we subjected populations of unsynchronized Jurkat cells to counterflow centrifugal elutriation and pooled cell fractions with DNA content characteristic of G_1 (P1), early S (P2), middle to late S (P3), and G_2/M (P4) stages of the cell cycle (Fig. 3*B*). Nuclear extracts were prepared from each of these pools and used in MSAs with 279-bp mbr target to assay helicase binding and sense and antisense 45-nt targets to detect ssDNA χ binding activity (see Fig. 1 for target descriptions). The results are depicted in Fig. 3*A*.

Helicase binding activity was sharply limited to G_1 and early S; PhosphorImager quantitation of complexes in Fig. 3*A* showed a 95% reduction of binding in late S and G_2/M. The ssDNA binding proteins showed a reciprocal pattern of expression. Complex formation with both sense and antisense χ targets was maximal in late S and G_2/M; activity was reduced by >90% in early S and by 70% (sense strand) or 85% (antisense strand) in G_1.

Specific Inhibition of mbr Helicase Complex Formation in Late S Phase. The disappearance of helicase binding in late S and G_2/M could have resulted from the absence of factors or the presence of an inhibitor. To differentiate between these two broad alternatives, positive extracts (P1 and P2) and negative extracts (P3 and P4) were mixed 1:1 in all combinations of two prior to conducting MSAs with the 279-bp mbr target. Complementary factors could not be detected in P3 and P4, as evidenced by the continued lack of complex formation when these extracts were mixed (Fig. 4, lane 5). However, inhibition of helicase binding occurred in P1 and P2 when these

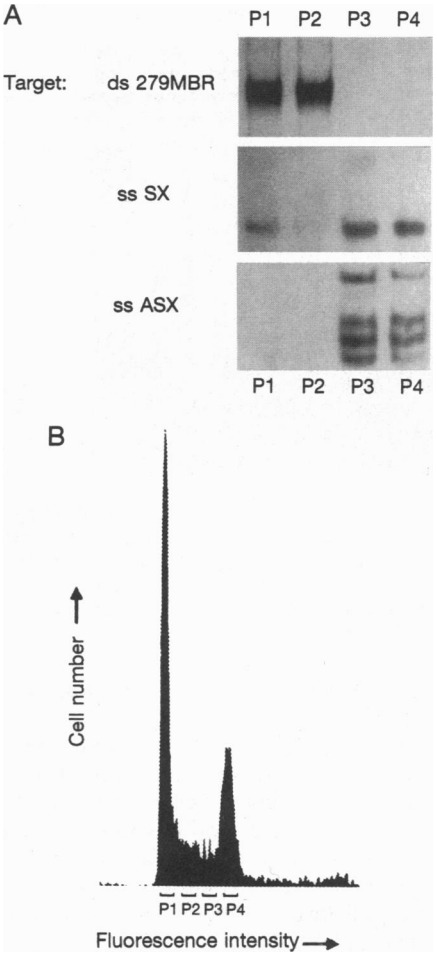

FIG. 3. Cell-cycle variation of mbr complex formation. (*A*) Unsynchronized Jurkat cells were subjected to counterflow centrifugal elutriation and pooled (see text). Nuclear extracts were then prepared and used in MSAs with double-strand 279-bp mbr target (ds 279MBR, *Top*), 45-nt single-strand sense target (ss SX, *Middle*), and 45-nt single-strand antisense target (ss ASX, *Bottom*). (*B*) FACS profile of cellular DNA content of culture used in *A*. P1, G_1 DNA content; P2, early S DNA content; P3, middle to late S DNA content; and P4, G_2/M DNA content. The indicated gaps between pools are accurate.

positive extracts were mixed with either P3 or P4 prior to MSA. This inhibition was specific to helicase complex formation since normal levels of sense and antisense ssDNA binding activity were still present in such mixtures (Fig. 4 *Right*).

Involvement of Ku Antigen in mbr Helicase Complex Formation. The targeting of helicase activity within the mbr, which demonstrated sequential loading onto substrate DNA and which potentially interacted with the variable(diversity)joining recombinase (given the nature of *BCL2* translocation), suggested the involvement of Ku antigen. We therefore undertook supershift assays with Jurkat extracts to characterize further the proteins present in mbr binding complexes. Murine monoclonal antibody to PCNA served as a control; it showed no effect on complex formation (Fig. 5, lane 2), except for mild inhibition in occasional experiments (Fig. 6). In contrast, murine antibodies (13, 14) directed against either the p70/p80 heterodimer of the Ku antigen (antibody 162), the p70 subunit (N3H10), or the p80 subunit (antibody 111) all reproducibly increased helicase complex formation by 4- to 8-fold. In addition, the anti-p70 antibody supershifted the lower complex quantitatively. Incubation of labeled target with antibody alone produced no complex formation (not shown). Maximal effects (increased binding and supershifting) were observed by

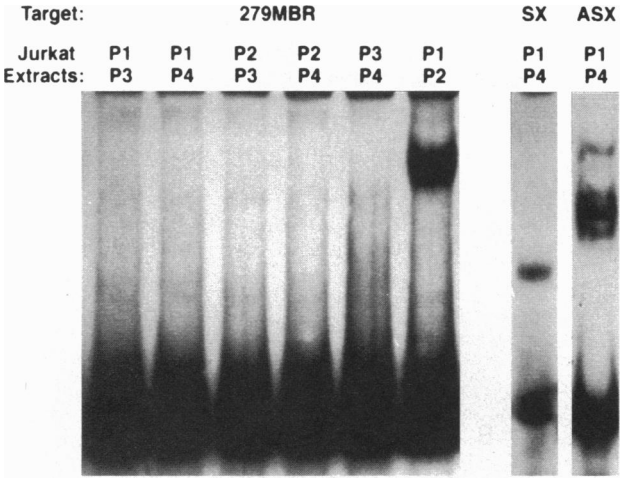

FIG. 4. Inhibition of helicase complex formation by late S and G_2/M extracts. The nuclear extracts used in the experiments of Fig. 3 were mixed 1:1 in all possible combinations of two. MSAs were then performed with the 279-bp mbr dsDNA target. For comparison, a P1/P4 mixture was also incubated with either sense or antisense ssDNA target (*Right*).

incubating antibody with nuclear extracts prior to addition of labeled target DNA.

The solitary appearance of p70 in Jurkat complexes was somewhat puzzling. Purified Ku does not dissociate upon binding DNA, although some experimental evidence (14) has suggested that p70 alone may bind DNA in chromatin. Therefore, we examined nuclear extracts of another cell line, HL60, demonstrating Jurkat-like complex formation. In these experiments (Fig. 6), all three antibodies demonstrated clear-cut supershifts. This indicated that the HL60 complex possessed both subunits. The differences between HL60 and Jurkat were not related to the extent of electrophoresis into 4% gels; HL60 complexes still showed clear-cut supershifts run under conditions identical to those of Fig. 5 (not shown). Interestingly,

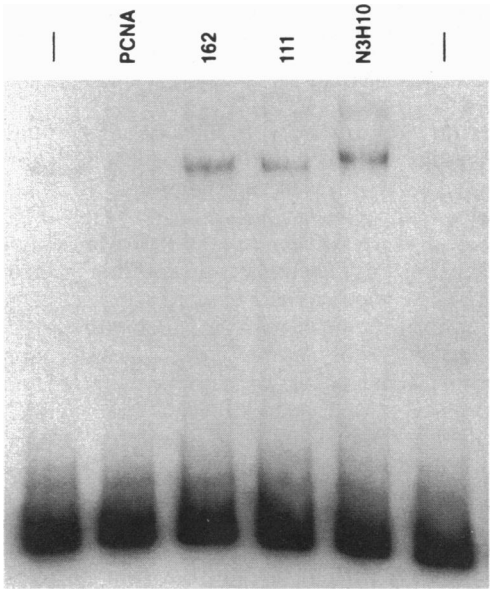

FIG. 5. Supershift MSAs with the 279-bp mbr dsDNA target and Jurkat nuclear extracts. Jurkat extract was preincubated for 1 hr at 4°C with murine monoclonal antibodies against PCNA, the Ku p70/p80 heterodimer (162), the Ku p80 subunit (111), or the Ku p70 subunit (N3H10). Control MSAs without antibody are in the first and last lanes.

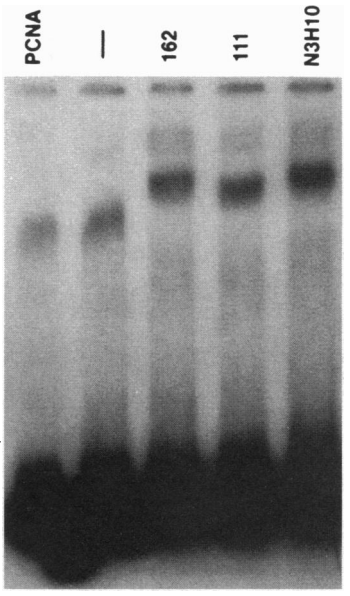

FIG. 6. Supershift MSAs with the 279-bp mbr dsDNA target and HL60 nuclear extracts. HL60 extract was preincubated for 1 hr at 4°C with murine monoclonal antibodies against PCNA, the Ku p70/p80 heterodimer (162), the Ku p80 subunit (111), or the Ku p70 subunit (N3H10). Control MSA without antibody is in the second lane.

anti-Ku antibodies increased HL60 complex formation by only 2- to 4-fold.

DISCUSSION

The occurrence of translocation breakpoints at very specific sites within the mbr implies that double-strand breaks are generated in this *BCL2* gene segment. Therefore, the identification within mbr–protein complexes of a nuclear factor recently associated with repair and recombination of double-strand breaks provides highly suggestive information on the mechanism of *BCL2* translocation. The Ku antigen, consisting of 70-kDa (p70) and 86-kDa (p80, p86) subunits, was originally identified as the nuclear protein target of autoantibodies present in patients with systemic lupus erythematosus and other autoimmune disorders (13, 18, 19). The heterodimer and p70 bind DNA (20). Ku also demonstrates helicase activity (21), entering from double-strand ends without any apparent sequence specificity (22).

Recently, Ku has been identified as the DNA binding component of a large (≈350 kDa) DNA-dependent protein kinase (DNA–PK; ref. 23) with substrates including replication factor A (24), p53 (25, 26), RNA polymerase II (27), and Ku itself (25). Thus, the multisubunit array is implicated in the control of transcription and, potentially, cell-cycle progression. Furthermore, the Ku–DNA–PK complex has been definitively identified as a key component in repair of double-strand breaks (28–32) and proper end ligation during immunoglobulin and T-cell receptor gene recombination (30–32). These latter attributes and our identification of Ku within the mbr helicase complex together provide strong evidence in favor of our prior hypothesis (10) that *BCL2* translocation is mediated by protein factors common to repair and variable(diversity)-joining recombination. Whether the DNA–PK also appears in a mbr-specific complex—perhaps the slower of the two species appearing at the top of MSA gels—will require further analysis.

Binding of purified Ku protein to substrate is independent of DNA sequence. Yet we have conclusively demonstrated site specificity of mbr complex formation involving the p70 Ku subunit (unpublished data), implying that a different nuclear

factor must be required to target Ku to the downstream flank of translocation cluster 3. This result may parallel the recent observation that Ku could apparently be targeted to the promoter of the human transferrin receptor gene by a cognate transcription factor (33). Another singular aspect of the interaction of the mbr with Ku is the enhancement of complex formation by antibody to either subunit and to the heterodimer. We speculate that some Ku antigen may be released from sequestration by inhibitor through antibody binding or that complex formation is stabilized by antibody binding. Our present interpretation of the Jurkat–HL60 disparity is that both Ku subunits are implicated in complex formation and target denaturation but that additional factors (perhaps merely different levels of Ku activity or relative levels of each subunit) are required for progressive dissociation (Jurkat) or maintenance (HL60) of the heterodimer. We leave open the possibility that the larger complex contains Ku but does not supershift because of its size.

The inhibitor we defined in late S and the putative Ku inhibitor we proposed to be released by antibody binding may be identical. However, the most attractive candidates for the late-S inhibitors of Ku complex formation—because of their suggestive appearance when mbr helicase activity disappears and because their binding might inhibit the loading of Ku onto its substrate—are the sense and antisense χ binding proteins. We attempted to remove inhibitor activity from P3 and P4 prior to mixing with P1 by adding a large excess of single-strand χ competitor DNA, alone and in various sequential combinations of sense and antisense, prior to incubation with labeled dsDNA target. However, these experiments were not successful. Therefore, the identification of both inhibitory activities awaits further purification of binding proteins and characterization of the unwinding reaction.

The limitation of Ku interaction with the mbr to G_1 and early S, the appearance of a specific inhibitor of this interaction in late S and G_2/M, and, above all, the myriad and quite disparate functions attributed to Ku suggest that mbr complex formation may have physiologic significance beyond the simple recognition of fortuitously occurring double-strand breaks. In fact, the cell-cycle restriction of Ku binding indicates that complex formation just precedes, and may be coincident with, the replication of the *BCL2* gene. Also, the region is conserved in the murine *bcl2* gene, and murine "mbr" binds the same complexes as the human segment (unpublished results). These considerations, together with the fact that the Ku binding region is removed by the translocation event, implicate the mbr as a site regulating *BCL2* expression and replication. Future studies, in establishing this functional role, may also shed light on another intriguing issue: whether double-strand breaks are an integral feature of a mbr-based regulatory process or, at the very least, a prominent by-product of it.

This work is dedicated to the memory of our late colleague, Dr. Janine André-Schwartz. We are grateful to Drs. Peter Bullock, David Potter, and Naomi Rosenberg for helpful discussions; to Drs. Rosenberg and Brian Schaffhausen for comments on the manuscript; and to Dr. Westley Reeves for anti-Ku antibody preparations. This work was supported by a grant from the National Cancer Institute (CA51985).

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