

ORIGINAL ARTICLE

## DNA Topoisomerase II in Therapy-Related Acute Promyelocytic Leukemia

Anita R. Mistry, Ph.D., Carolyn A. Felix, M.D., Ryan J. Whitmarsh, B.A., Annabel Mason, B.Sc., Andreas Reiter, M.D., Bruno Cassinat, Pharm.D., Anne Parry, Ph.D., Christoph Walz, Joseph L. Wiemels, Ph.D., Mark R. Segal, Ph.D., Lionel Adès, M.D., Ian A. Blair, Ph.D., Neil Osheroff, Ph.D., Andrew J. Peniket, B.A., Marina Lafage-Pochitaloff, Ph.D., Nicholas C.P. Cross, Ph.D., Christine Chomienne, Ph.D., Ellen Solomon, Ph.D., Pierre Fenaux, Ph.D., and David Grimwade, Ph.D.

### ABSTRACT

#### BACKGROUND

Chromosomal translocations leading to chimeric oncoproteins are important in leukemogenesis, but how they form is unclear. We studied acute promyelocytic leukemia (APL) with the t(15;17) translocation that developed after treatment of breast or laryngeal cancer with chemotherapeutic agents that poison topoisomerase II.

#### METHODS

We used long-range polymerase chain reaction and sequence analysis to characterize t(15;17) genomic breakpoints in therapy-related APL. To determine whether topoisomerase II was directly involved in mediating breaks of double-stranded DNA at the observed translocation breakpoints, we used a functional in vitro assay to examine topoisomerase II-mediated cleavage in the normal homologues of the *PML* and *RARA* breakpoints.

#### RESULTS

Translocation breakpoints in APL that developed after exposure to mitoxantrone, a topoisomerase II poison, were tightly clustered in an 8-bp region within *PML* intron 6. In functional assays, this “hot spot” and the corresponding *RARA* breakpoints were common sites of mitoxantrone-induced cleavage by topoisomerase II. Etoposide and doxorubicin also induced cleavage by topoisomerase II at the translocation breakpoints in APL arising after exposure to these agents. Short, homologous sequences in *PML* and *RARA* suggested the occurrence of DNA repair by means of the nonhomologous end-joining pathway.

#### CONCLUSIONS

Drug-induced cleavage of DNA by topoisomerase II mediates the formation of chromosomal translocation breakpoints in mitoxantrone-related APL and in APL that occurs after therapy with other topoisomerase II poisons.

From the Department of Medical and Molecular Genetics, Guy's, King's, and St. Thomas' School of Medicine, London (A.R.M., A.M., E.S., D.G.); the Division of Oncology, Children's Hospital of Philadelphia, Philadelphia (C.A.F., R.J.W.); the Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia (C.A.F.); Fakultät für Klinische Medizin Mannheim der Universität Heidelberg, Mannheim, Germany (A.R., C.W.); Unité de Biologie Cellulaire, Service de Médecine Nucléaire, Hôpital St. Louis, Paris (B.C., A.P., C.C.); the Department of Epidemiology and Biostatistics, University of California, San Francisco, San Francisco (J.L.W., M.R.S.); Hôpital Avicenne-Paris 13 Université, Bobigny, France (L.A., P.F.); the Center for Cancer Pharmacology, University of Pennsylvania, Philadelphia (I.A.B.); the Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville (N.O.); the Department of Haematology, John Radcliffe Hospital, Oxford, United Kingdom (A.J.P.); Institut Paoli-Calmettes, INSERM UMR 599, and Université de la Méditerranée, Marseille, France (M.L.-P.); Wessex Regional Genetics Laboratory, Salisbury, United Kingdom (N.C.P.C.); and the Department of Haematology, University College London Hospitals, London (D.G.). Address reprint requests to Dr. Grimwade at the Cancer Genetics Laboratory, Department of Medical and Molecular Genetics, 8th Fl., Guy's Tower, Guy's Hospital, London SE1 9RT, United Kingdom, or at david.grimwade@genetics.kcl.ac.uk.

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**A**CUTE MYELOID LEUKEMIA (AML) IS commonly associated with reciprocal balanced chromosomal translocations that underlie the formation of chimeric proteins that have key roles in the development of leukemia.<sup>1,2</sup> The most frequent translocation, t(15;17)(q22;q21), occurs in 10 to 15 percent of cases of AML<sup>1</sup> and is the hallmark of acute promyelocytic leukemia (APL). This translocation creates the *PML-RARA* and *RARA-PML* fusion genes.<sup>3</sup> The resultant *PML-RAR $\alpha$*  fusion protein determines not only the phenotype of APL but also the response of APL to all-*trans*-retinoic acid and arsenic trioxide treatment.<sup>3,4</sup>

The transforming function of leukemia-associated fusion proteins has been widely studied, but little is known about the mechanisms that cause the underlying translocations. Insights can be gained from investigations of therapy-related leukemias, all of which have counterparts in primary leukemias. Exposure to drugs that poison topoisomerase II — the anthracyclines daunorubicin, doxorubicin, and epirubicin; the anthracenedione mitoxantrone; and epipodophyllotoxins such as etoposide — predisposes patients to secondary leukemias with balanced chromosomal rearrangements, including *MLL* translocations involving band 11q23, t(8;21), inv(16), t(15;17), t(9;22), and *NUP98* translocations involving band 11p15.<sup>5-7</sup> The association of such chromosomal rearrangements with exposure to drugs that affect topoisomerase II suggests a role for topoisomerase II-mediated cleavage of DNA in forming translocations, but how this occurs remains to be established.

Topoisomerase II relaxes supercoiled DNA by cleaving and religating both strands of the double helix through the formation of a transient covalent cleavage intermediate.<sup>8</sup> Chemotherapeutic drugs termed “topoisomerase II poisons” convert topoisomerase II into a DNA-damaging enzyme. They disrupt the cleavage-religation equilibrium and thereby increase the concentration of topoisomerase II-mediated cleavage complexes.<sup>8</sup> Although the enzyme does not have a known DNA recognition sequence that it is most likely to target, genomic sequencing studies have suggested possible binding sites for the enzyme at translocation breakpoints in primary and treatment-related leukemias with *MLL*, *AML1-ETO*, *PML-RARA*, and *NUP98* rearrangements.<sup>9-15</sup>

In early studies, less than 5 percent of APL cases were a consequence of chemotherapy,<sup>16,17</sup> but more recently, the European APL group reported that ther-

apy-related APL accounted for 22 percent of all cases.<sup>17</sup> The rising incidence of therapy-related APL parallels the increased use of topoisomerase II poisons, particularly in the treatment of breast cancer. APL with t(15;17) is one of the most frequent secondary cancers that arise after the treatment of breast cancer<sup>17-20</sup>; mitoxantrone has been implicated in almost half these cases.<sup>17,19,20</sup> In the present study, we examined genomic breakpoint regions in patients with APL after exposure to topoisomerase II poisons, particularly mitoxantrone, and used functional assays to gain further insight into mechanisms underlying the formation of the t(15;17) chromosomal translocation.

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## METHODS

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### PATIENTS AND SAMPLES

Genomic breakpoint locations in *PML* and *RARA* genes were studied in six patients with therapy-related APL, which arose after mitoxantrone treatment for breast carcinoma (in five) or multiple sclerosis (in one). To determine whether breakpoint clustering in *PML* intron 6 detected in mitoxantrone-related APL was statistically significant, a comparison was made with breakpoint locations in 7 patients with secondary APL arising after other types of exposure, mostly radiotherapy, and in 35 patients with primary APL. Chromosomal breakpoint mechanisms were subsequently investigated with the use of functional topoisomerase II cleavage assays in four of the patients with mitoxantrone-related cases (Patients 1 through 4) and an additional patient (Patient 5) with secondary APL that developed after exposure to doxorubicin and etoposide (Table 1). All patients gave written informed consent, in accordance with the Declaration of Helsinki.

### CHARACTERIZATION OF GENOMIC BREAKPOINTS

Three breakpoint regions have been identified within the *PML* locus in APL: intron 3 (bcr3), exon 6 (bcr2), and intron 6 (bcr1); virtually all breakpoints in *RARA* occur in intron 2.<sup>21</sup> The breakpoint pattern in *PML* was determined by nested reverse-transcriptase polymerase chain reaction (RT-PCR)<sup>22</sup>; appropriate primers were used to amplify the sequences of genomic breakpoint junctions by long-range or “bubble” PCR, and the PCR products were sequenced.<sup>21</sup> Breakpoint junction sequences obtained in this way were confirmed by a patient-specific breakpoint PCR with the use of a fresh aliquot of genomic DNA as template.

**Table 1. Characteristics of Five Patients with APL Arising after Exposure to Topoisomerase II Poisons.**

Patient No.	Primary Cancer	Treatment of Primary Tumor*	Interval between Primary Cancer and APL <i>mo</i>	Karyotype	PML Breakpoint			RARA Breakpoint		
					der(15)†	der(17)†	Proximal Drug-Induced Cleavage Site	der(15)†	der(17)†	Proximal Drug-Induced Cleavage Site
1	Breast carcinoma	Surgery; radiotherapy, 60 Gy; mitoxantrone, 120 mg; VCR, 12 mg; CPM, 3.6 g; 5-FU, 3.6 g	36	46,XX, del(13)(q13q21), t(15;17)(q22;q21)	1482–1486	1488–1489	1484	2694–2698	2698–2699	2695
2	Breast carcinoma	Surgery; radiotherapy, 45 Gy; mitoxantrone, 120 mg; CPM, 5.1 g; 5-FU, 5.1 g	21	46,XX, t(15;17)(q22;q21)	1483–1485	1484–1486	1484	634–636	633–635	635
3	Breast carcinoma	Surgery; radiotherapy, 48 Gy; mitoxantrone, 15 mg; tamoxifen	20	46,XX, -6, t(15;17)(q22;q21), +mar	1488	1488–1489	1484	2434	2433–2434	2431
4	Breast carcinoma	Radiotherapy, 58 Gy; mitoxantrone, 80 mg; CPM, 3.4 g	15	46,XX, t(15;17)(q22;q21)	1485–1487	1486–1489	1484	11570–11572	11571–11574	11569
5	Laryngeal carcinoma	Radiotherapy, 64 Gy; doxorubicin, 304 mg; CPM, 4.56 g; VP16, 760 mg	36	46,XY, t(15;17)(q22;q21)	1239	1240	1239	16090	16088	16089

\* VCR denotes vincristine, CPM cyclophosphamide, 5-FU fluorouracil, and VP16 etoposide.

† DNA fragments of the normal homologues of PML (GenBank accession numbers S51489 [bcr3] for Patient 5 and S57791 [bcr1] for Patients 1 through 4) and RARA (GenBank accession numbers AF088889 for Patient 2 and AJ297538 for Patients 1, 3, 4, and 5) that encompassed the relevant translocation breakpoints were examined.

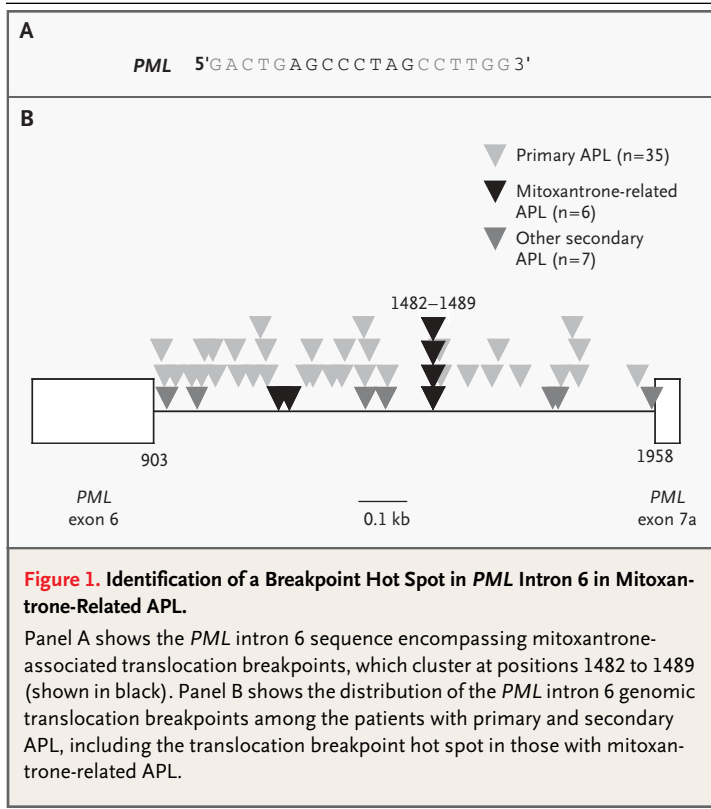
**IN VITRO TOPOISOMERASE II CLEAVAGE ASSAYS**

We examined DNA fragments of the normal homologues of PML (GenBank accession numbers S51489 and S57791) and RARA (GenBank accession numbers AF088889 and AJ297538) that encompassed the relevant translocation breakpoints using an in vitro topoisomerase II cleavage assay.<sup>23</sup> Substrate DNA was incubated with human topoisomerase II $\alpha$  in the presence of ATP and exposed to drugs that target topoisomerase II. Final concentrations of etoposide, etoposide catechol, etoposide quinone, and mitoxantrone were 20  $\mu$ M<sup>24</sup>; we selected a final concentration of doxorubicin of 25 nM on the basis of a titration using concentrations between 1 nM and 200 nM (data not shown). Cleavage complexes were irreversibly trapped on the addition of sodium dodecyl sulfate, and cleavage products were resolved in a gel containing 8 percent

polyacrylamide and 7.0 M urea alongside a DNA-sequencing ladder. This procedure allowed us to map cleavage sites precisely at the sequence level and to analyze the positions of the cleavage sites with respect to translocation breakpoint sites. Cleavage products were visualized by means of autoradiography and quantified with the use of a Phosphorimager and IMAGEQUANT software (Molecular Dynamics).

**STATISTICAL ANALYSIS**

The significance of the putative mitoxantrone cluster in cases of therapy-related APL or primary plus therapy-related APL was assessed with the use of scan statistics,<sup>25</sup> which have been used widely for the assessment of spatial and temporal clustering of events.<sup>26</sup> Generally, they are based on the maximal number of events occurring in a prescribed re-



gion or interval. This statistic is then referenced against a uniform (null) distribution (over the entire region or period) reflecting the absence of clustering. In the case of translocation breakpoint clustering, the interval is the occurrence of a breakpoint, the interval is the number of base pairs spanning the putative cluster, and the reference interval is the relevant intron length.<sup>25</sup> Because the distribution of the scan statistic is exceedingly complex, a number of approximations have been developed. Here, we used the accurate, end-point-corrected, large-deviation approximation to the one-dimensional scan statistic.<sup>27</sup>

## RESULTS

### IDENTIFICATION OF A TRANSLOCATION BREAKPOINT HOT SPOT IN MITOXANTRONE-RELATED APL

Genomic breakpoint junction sequences on the derivative (der) chromosomes 15 and 17 were characterized in APL that arose after mitoxantrone-based treatment for breast cancer in five patients and mitoxantrone treatment for multiple sclerosis in one

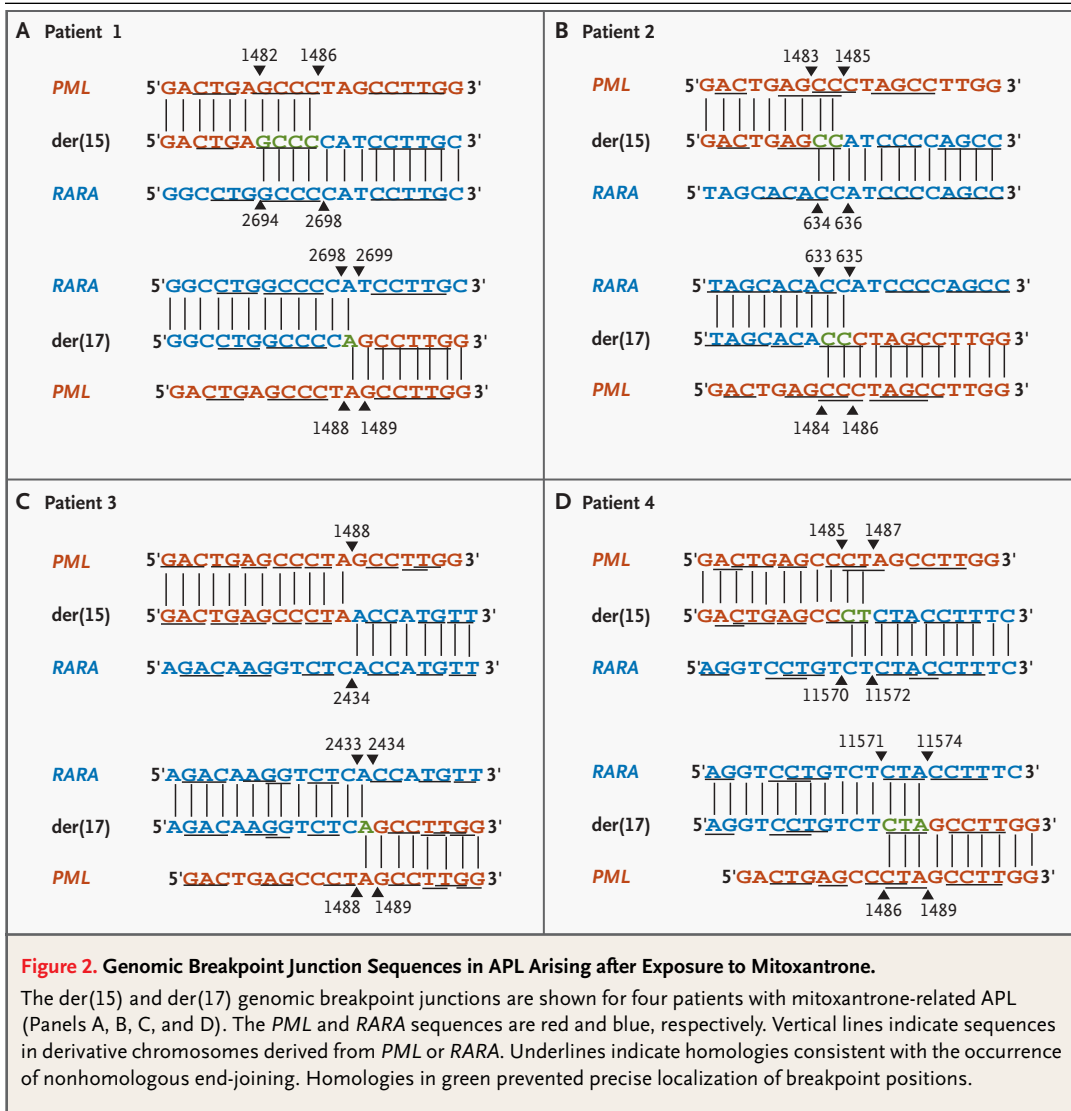
patient. Remarkably, the der(15) and der(17) PML breakpoints in four of these patients (Patients 1, 2, 3, and 4) (Table 1) were tightly clustered in an 8-bp region (positions 1482 to 1489; GenBank accession number S57791) in PML intron 6 (Fig. 1 and 2), a result consistent with the presence of a hot spot of DNA damage. Scan statistics indicated that the clustered breakpoints within an intron longer than 1 kb were unlikely to have arisen by chance ( $P < 0.001$  for the comparison with 7 cases of APL related to other therapy,  $P < 0.05$  for the comparison with the 7 other therapy-related cases plus 35 cases of primary APL, and  $P < 0.05$  for the comparison with the 35 cases of primary APL alone).

In contrast to the clustering of the PML breakpoints in cases of mitoxantrone-related APL, the RARA breakpoints were dispersed (Table 1 and Fig. 2). Study of the der(15) and der(17) sequences in the four patients with mitoxantrone-related cases associated with the hot spot indicated that the breakpoint junctions were formed without the gain or loss of any bases relative to the native PML and RARA sequences (Fig. 2). The short sequence homologies between PML and RARA (underlined in Fig. 2) are characteristic of DNA repair by the non-homologous end-joining pathway (NHEJ),<sup>28</sup> which requires minimal overlapping sequences between nonhomologous chromosomes to repair breaks in double-stranded DNA.

### SITE OF FUNCTIONAL TOPOISOMERASE II-MEDIATED CLEAVAGE AT THE PML INTRON 6 TRANSLOCATION BREAKPOINT HOT SPOT

We evaluated topoisomerase II-mediated cleavage of the normal homologue of the PML translocation breakpoint hot spot in vitro using a 268-bp double-stranded DNA substrate encompassing the 8-bp hot spot in the presence of mitoxantrone, etoposide or its catechol, or quinone metabolites and in the absence of these agents.<sup>24</sup> Few cleavage sites were observed in the absence of drug (Fig. 3A). Bands of various sizes and intensities showed where cleavage sites were enhanced by the different agents (Fig. 3A).

The 8-bp translocation breakpoint hot spot at positions 1482 to 1489 corresponded to a topoisomerase II-mediated cleavage site at position 1484, where the position indicates the base immediately 5' to the cleavage (-1 position). Cleavage at position 1484 was detected in the absence of drug, but it was markedly enhanced by etoposide, both etoposide metabolites, and mitoxantrone. Position 1484 was a preferred site of cleavage by topoisom-



erase II in the presence of mitoxantrone, as evidenced by the intensity of the cleavage band (Fig. 3A). Mitoxantrone-induced cleavage was enhanced by a factor of 8.9 and 2.5, respectively, relative to cleavage at this site without drug or in the presence of etoposide. Cleavage at many sites decreased substantially or was eliminated after heating (Fig. 3A). By contrast, the mitoxantrone-induced cleavage at position 1484 remained detectable after heating (Fig. 3A), indicating stability of the cleavage complexes. These results show that the PML intron 6 translocation breakpoint hot spot in mitoxantrone-related APL is a preferred and stable mitoxantrone-induced site of cleavage by topoisomerase II.

**RARA TRANSLOCATION BREAKPOINTS IN MITOXANTRONE-RELATED APL**

We performed in vitro topoisomerase II cleavage assays on double-stranded DNA substrates spanning the normal homologues of the RARA translocation breakpoints in Patients 1, 2, 3, and 4 to determine whether topoisomerase II also mediated the breakage at the RARA locus (Fig. 3B and Table 1). In Patient 1 (Fig. 3B), topoisomerase II-mediated cleavage was observed at position 2695 of the RARA intron 2 proximal to the der(15) and der(17) RARA translocation breakpoints and was heat-stable (Fig. 3B). The RARA breakpoints on both derivative chromosomes in specimens from Patients 2, 3, and 4 were also at, or proximal to, sites of func-

tional mitoxantrone-induced cleavage by topoisomerase II (Table 1). Assays on all substrates were repeated, and the repeated assays confirmed these results.

#### SITES OF MITOXANTRONE-INDUCED CLEAVAGE BY TOPOISOMERASE II AND t(15;17) BREAKPOINT JUNCTIONS

We used the functional sites of topoisomerase II-mediated cleavage of DNA at the translocation breakpoints to generate a model for the formation of the t(15;17), incorporating known repair mechanisms of breaks in double-stranded DNA. Figure 4 shows how recombination of mitoxantrone-enhanced cleavage sites at *PML* position 1484 and *RARA* position 2695 would form the der(15) and der(17) genomic breakpoint junctions identified in the APL in Patient 1. The sites of topoisomerase II-mediated cleavage of each DNA strand are four bases apart, thereby creating 5' overhangs,<sup>8</sup> as shown in Figure 4. In the model, repair of the overhangs in *PML* and *RARA* entails exonucleolytic digestion, pairing of complementary bases, and joining of the DNA free ends by means of the NHEJ pathway, with template-directed polymerization to fill in any gaps. Models were also generated showing that mitoxantrone-induced cleavage by topoisomerase II formed the der(15) and der(17) breakpoint junctions in the leukemias in Patients 2, 3, and 4 (data not shown).

#### PML AND RARA TRANSLOCATION BREAKPOINTS IN ETOPOSIDE- AND DOXORUBICIN-RELATED APL

A fifth patient (Patient 5) received a diagnosis of APL after being treated with etoposide and doxorubicin for laryngeal cancer (Table 1). Study of the breakpoint junction sequences indicated that the translocation occurred with the loss of a single G nucleotide from *RARA* (position 16089) (Fig. 1A in the Supplementary Appendix, available with the full text of this article at [www.nejm.org](http://www.nejm.org)). Short sequence homologies were observed in *PML* and *RARA* characteristic of DNA repair by the NHEJ pathway. In the in vitro assay, etoposide, its catechol and quinone metabolites, and doxorubicin induced topoisomerase II-mediated cleavage at the *PML* and *RARA* translocation breakpoints. Cleavage was shown to be heat-stable with each of these drugs at the *PML* breakpoint (Fig. 1B in the Supplementary Appendix) and with etoposide quinone at the *RARA* breakpoint (Fig. 1C in the Supplementary Appendix). A model (Fig. 1D in the Supplementary Ap-

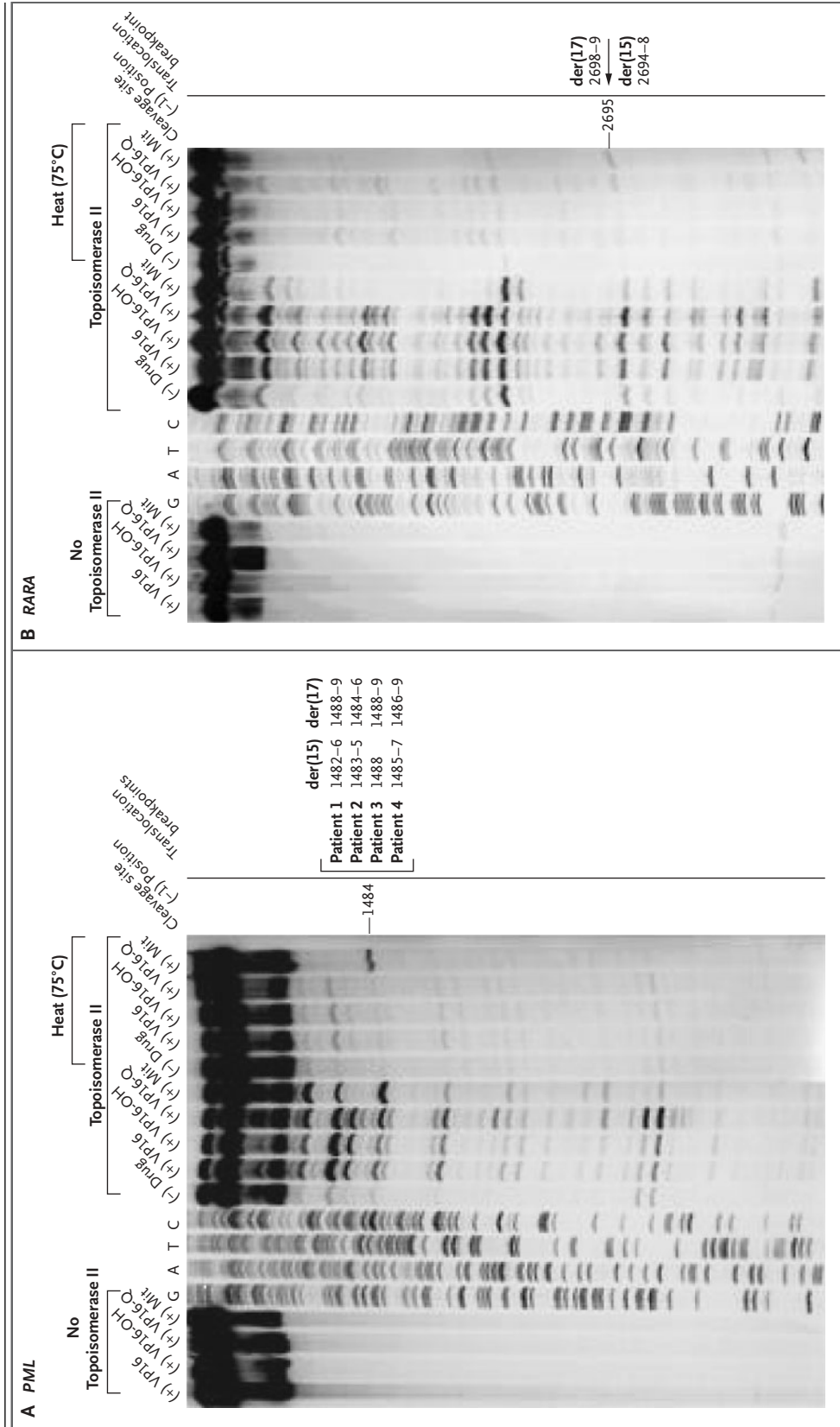
pendix) indicated how recombination of cleavage sites at *PML* position 1239 and *RARA* position 16089 would form the der(15) and der(17) genomic breakpoint junctions in this case of APL.

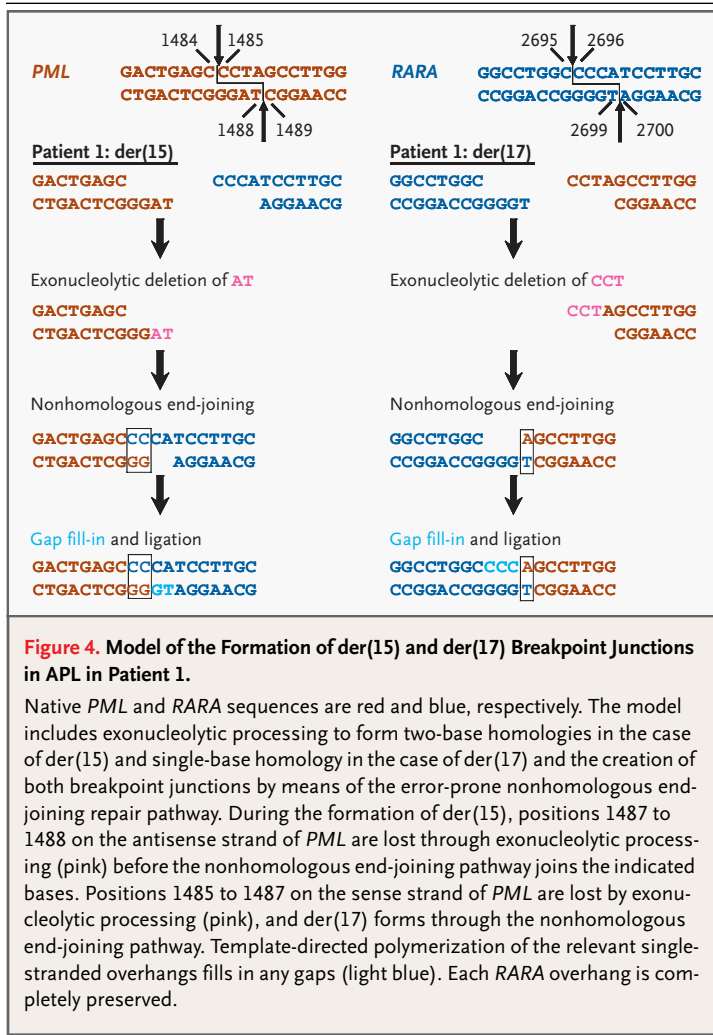
#### DISCUSSION

Leukemia characterized by balanced translocations, including t(15;17), can be a complication of treatment with anticancer drugs that poison topoisomerase II.<sup>29,30</sup> The mechanism by which these drugs predispose patients to leukemia remains in dispute.

#### Figure 3 (facing page). Functional Topoisomerase II Cleavage Sites at Mitoxantrone-Associated Translocation Breakpoints.

Chromosomal breakpoint regions were examined by means of a functional in vitro assay, which identifies topoisomerase II-dependent cleavage of DNA induced by various chemotherapeutic agents and their metabolites. Cleavage products were fractionated according to size and compared with a DNA-sequencing ladder to allow precise mapping of sites of DNA cleavage. Panel A shows topoisomerase II-mediated cleavage of DNA substrate spanning positions 1284 to 1551 of *PML* intron 6 (GenBank accession number S57791) encompassing the 8-bp translocation breakpoint hot spot (positions 1482 to 1489). The cleavage products in 25 ng (30,000 cpm) of DNA labeled only at the 5' end were examined after 10 minutes' incubation at 37°C with 147 nM human topoisomerase II $\alpha$ , 1 mM ATP, and the following drugs at final concentrations of 20  $\mu$ M: etoposide (VP16), etoposide catechol (VP16-OH), etoposide quinone (VP16-Q), and mitoxantrone (Mit). Cleavage complexes were irreversibly trapped on the addition of sodium dodecyl sulfate (SDS), and purified cleavage products were resolved in a gel containing 8 percent polyacrylamide and 7.0 M urea, alongside DNA sequencing reactions primed at the same 5' end. Although very few cleavage sites were visible in the absence of drug (indicated by the minus signs), cleavage sites were enhanced by exposure to the various topoisomerase II-targeted agents (indicated by the plus signs). Specified reactions were incubated for an additional 10 minutes at 75°C before the addition of SDS in order to examine the stability of the cleavage complexes formed. The nucleotide 1484 is on the 5' side of the cleavage site (-1 position), which corresponds to the der(15) and der(17) translocation breakpoints in four patients with mitoxantrone-related APL. Panel B shows DNA topoisomerase II-mediated cleavage of the normal homologue of the der(15) and der(17) *RARA* translocation breakpoints in APL in Patient 1. The substrate spanning positions 2603 to 2871 of *RARA* intron 2 (GenBank accession number AJ297538) contained the translocation breakpoints. The nucleotide 2695 indicates the (-1) position of the cleavage site corresponding to the der(15) and der(17) translocation breakpoints.





with the absence of a dose–response effect in the development of leukemia that follows epipodophylotoxin treatment<sup>37</sup> and the detection of a leukemia-associated *MLL* translocation after a low total dose of doxorubicin.<sup>38</sup>

The mitoxantrone-related *PML* translocation breakpoint hot spot corresponded with a preferred site of topoisomerase II–mediated cleavage that was not religated after heating. In vitro, mitoxantrone stimulated cleavage at the translocation breakpoint hot spot that was nine times that observed in the absence of the drug. The *RARA* translocation breakpoints in each of the four patients with mitoxantrone-related APL investigated by functional assays were also found to correspond to mitoxantrone-induced sites of cleavage of DNA by topoisomerase II. Models were devised in which recombination of broken DNA at sites of topoisomerase II–mediated cleavage formed the der(15) and der(17) breakpoint junctions. These studies indicate that mitoxantrone induces cleavage of *PML* and *RARA* by topoisomerase II and that this cleavage resulted in the observed translocation breakpoint junctions in mitoxantrone-related APL.

To determine whether topoisomerase II–mediated cleavage is relevant to other drugs in therapy-related APL, we evaluated a patient in whom APL developed after exposure to etoposide and doxorubicin. Etoposide and its metabolites and doxorubicin induced topoisomerase II to cleave DNA at the *PML* and *RARA* translocation breakpoints. The cleavage sites could recombine to form the der(15) and der(17) breakpoint junctions observed in this patient. These results suggest that topoisomerase II–mediated cleavage is a general mechanism causing DNA damage in APL that develops after treatment with various agents that target topoisomerase II.

Recent reports of treatment-related APL indicate that epirubicin and mitoxantrone are the most common antecedent drugs and that a substantial proportion of the patients had breast cancer.<sup>17–19</sup> Although etoposide is implicated in some cases of treatment-related APL,<sup>17–19,36</sup> this drug is more often associated with *MLL* translocations that disrupt band 11q23.<sup>30,39</sup> These observations suggest that different chemotherapeutic agents predispose patients to different translocations. A key question is how such specificity is conferred. Our in vitro assays show that mitoxantrone and etoposide or its metabolites stimulate topoisomerase II to cleave different sites in *PML* and *RARA*, implying the existence of different genomic hot spots for topoisom-

Some evidence supports a direct role for topoisomerase II in causing the DNA damage that leads to chromosomal rearrangements.<sup>6,23,24,31</sup> An indirect mechanism involving the induction of apoptosis-inducing nucleases has also been proposed.<sup>32–35</sup>

Few genomic breakpoint junctions have been characterized in therapy-related APL.<sup>36</sup> Our study of the der(15) and der(17) genomic breakpoint junctions in APL arising after mitoxantrone treatment revealed clustering of breakpoints in the *PML* gene within an 8-bp region in intron 6, a result consistent with the existence of a translocation breakpoint hot spot. The *PML*-*RARA* rearrangements occurred without the gain or loss of any bases relative to the native genes, indicating that the translocation breakpoints in intron 6 were the sites of DNA damage.<sup>24</sup> Interestingly, one patient (Patient 3) received only 15 mg of mitoxantrone. This finding is consistent

erase II-mediated cleavage in the presence of the different drugs. It is likely that such hot spots occur throughout the genome but that only translocations that confer a proliferative or survival advantage in an appropriate hematopoietic progenitor lead to leukemia. The identification of this translocation mechanism has important implications for the chemotherapy of cancer.

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