

A Precise Interchromosomal Reciprocal Exchange between Hot Spots for Cleavable Complex Formation by Topoisomerase II in Amsacrine-treated Chinese Hamster Ovary Cells¹

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Abstract

Among the *aprt* mutations induced in confluence-arrested Chinese hamster ovary D422 cells by the topoisomerase II poison amsacrine, there was a reciprocal exchange between the *aprt* gene and an unrelated sequence, accompanied by a chromosomal translocation at the *aprt* locus. The breakpoints in both parental sequences were hot spots for amsacrine-stimulated DNA cleavage *in vitro*, and the novel junctions formed were precisely as expected for a mechanism involving reciprocal exchange of topoisomerase II subunits followed by resealing of the breaks and correction of mismatches in the cohesive ends. The results are consistent with a role for direct subunit exchange in the production of chromosomal translocations by topoisomerase poisons, although more complex models involving double-strand breakage and repair could produce reciprocal exchanges of similar specificity.

Introduction

Inhibitors of DNA topoisomerase II that stabilize the cleavable complex and prevent religation are potent inducers of large deletions, gene rearrangements, and sister chromatid exchanges (for reviews, see Refs. 1 and 2). Characteristic chromosomal translocations are also frequently found in second malignancies associated with chemotherapy that includes a topoisomerase inhibitor (for a review, see Ref. 3). Because all of these effects necessarily involve DNA breakage and rejoining, they could in theory result from an exchange of topoisomerase subunits between two cleavable complexes in different segments of DNA (4). Such a model is supported by the finding that purified topoisomerase II can catalyze an illegitimate recombination *in vitro* (5, 6). However, despite the potent recombinogenic effects of topoisomerase poisons in intact cells, there is little direct evidence that these effects involve topoisomerase subunit exchange. Indeed, the few topoisomerase-mediated deletions, insertions, and rearrangements that have been analyzed at the molecular level have in general not conformed to the rather stringent specificities imposed by subunit exchange models (7, 8). Here, however, we report a reciprocal translocation in which the breakpoints and novel junctions are consistent with a precise reciprocal exchange of topoisomerase subunits between cleavable complexes in two unrelated sequences in endogenous chromosomal DNA.

Materials and Methods

Mutants were generated by treatment of confluence-arrested CHO³ cells for 2 days with *m*-AMSA (4'-(9-acridinylamino)methanesulfon-*m*-anisidide), using a protocol described previously (9). Rearrangement mutations were detected and mapped as described previously (10).

Rearrangement breakpoints were amplified by nested ligation-mediated PCR using pUC19 as an anchor (11). Mutant genomic DNA was cleaved with *Tsp509I* (for the upstream breakpoint) or *MboI* (for the downstream breakpoint), and the fragments (0.5 μ g) were ligated to dephosphorylated *EcoRI*- or *BamHI*-cut pUC19 DNA (2 μ g). Ligated DNA (0.6 μ g) was subjected to 25 cycles of PCR (1 min at 94°C, 30 s at 55°C, and 1 min at 72°C) as described previously (7), using 0.5 μ g each of an outer nested *aprt* primer (corresponding to bp 2034–2053 or 2522–2504) and the outer nested pUC19 primer (TGT-GCTGCAAGGCGATTAAG). A 5- μ l aliquot of this reaction mixture was then used as the template for a second PCR, but with inner nested *aprt* (bp 2106–2125 or 2475–2458) and pUC19 (TTTCCCAGTCACGACGTTGT) primers. A third inner nested *aprt* primer (bp 2112–2131 or 2392–2373) was used to sequence the resulting product across the breakpoint using an Epicentre cycle sequencing kit. *aprt* is numbered according to Ref. 12.

For *in vitro* cleavage studies, cloned 5' end-labeled fragments containing each of the *aprt* exons were prepared as described previously (10). The labeled non-*aprt* parental fragments were prepared by PCR using labeled primers (7). Labeled fragments were treated with 4 units of human topoisomerase II α (p170 form; Topogen) at 37°C for 10 min in the presence of 1 mM ATP and 0–20 μ M *m*-AMSA. Cleavable complexes were trapped with SDS plus proteinase K, and the DNA was phenol-extracted, precipitated, and analyzed on 7% polyacrylamide gels (7). Cleavage was quantitated by phosphorimager analysis (10). Experiments with other fragments have indicated that the cleavage specificity of the human enzyme is indistinguishable from that of the rodent enzyme (purified from murine L1210 cells and provided by Y. Pommier, National Cancer Institute, Bethesda, MD).⁴

For fluorescence *in situ* hybridization, metaphase spreads from the cell lines were prepared and GTG-banded by standard procedures (13). The λ DM2 and λ B21 probes (kindly provided by Dr. Mark Meuth, University of Utah, Salt Lake City, UT; Ref. 14) were directly labeled with the fluorophores Spectrum Green and Spectrum Orange, respectively, using a nick translation kit (Vysis). A mixture consisting of 500 ng of each labeled probe plus 10 μ g of repetitive *Cor1* DNA (15) from D422 cells was precipitated, resuspended in hybridization buffer (Vysis), denatured at 70°C for 5 min, and suppression-hybridized for 1 h at 37°C. *In situ* hybridization was performed as described previously (13), except that the slides were first denatured at 70°C for 70 s and then hybridized with the probes at 37°C for 16 h. Twenty metaphases were analyzed per cell line.

Results

Treatment of confluence-arrested CHO D422 cells for 2 days with 1 μ M *m*-AMSA resulted in 3% survival and a mutation frequency of $28 \pm 5 \times 10^{-6}$ compared to $0.5 \pm 0.1 \times 10^{-6}$ for untreated cells. The mutations included base substitutions, small deletions and insertions,

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³ The abbreviations used are: CHO, Chinese hamster ovary; *m*-AMSA, amsacrine.

⁴ Unpublished results.

and large-scale rearrangements, with rearrangements accounting for about 10% of the mutants (a full description of the spectrum will be presented elsewhere). Preliminary PCR-based mapping data have indicated that most of the rearrangements involved the deletion of large segments of the *aprt* sequence (data not shown). However, for one of the mutants, designated Ip8a, the mapping indicated that most or all of *aprt* was still present, but that there was a small segment of the gene centered near bp 2300 across which no PCR products could be generated, even when the primer binding sites were still present (*i.e.*, other PCR products could be generated using each of the primers or its complement). These results suggested a large-scale rearrangement of the gene, either a large insertion or the linkage of the two halves of *aprt* to distant genomic sites. Nested ligation-mediated PCR was used to amplify and sequence the upstream and downstream rearrangement junctions, along with ~100 bp of non-*aprt* sequence beyond the junctions. The junction sequences revealed that the upstream and downstream breakpoints in the *aprt* gene were nearly coincident, but with a 5-bp overlap (CAGTA) that was present at both junctions (Fig. 1). Otherwise, the sequences beyond the breakpoints had no homology to *aprt*.

To determine whether the rearrangement was a reciprocal exchange, PCR was performed with template DNA from the D422 parent line and a primer from each of the two non-*aprt* sequences, directed toward each breakpoint. The predominant product had a length corresponding to the sum of the distances from each of the primers to the breakpoint. The sequence of this product (128 bp) was determined in both directions using the same two primers, and it was found to correspond exactly to the non-*aprt* sequence beyond each breakpoint. Thus, the exchange was indeed reciprocal, and it occurred without any net gain or loss of DNA sequence (Fig. 1).

As part of a comprehensive study of *m*-AMSA-induced damage and mutagenesis in *aprt*, all sites of cleavable complex formation by *m*-AMSA plus topoisomerase II α in all exons of the gene have been mapped, and the relative cleavage frequencies have been determined. As shown in Fig. 1, a prominent site of apparent double-strand cleavage occurred precisely at the site of the reciprocal exchange, with the 4-base cohesive 5' overhang (AGTA) being part of the CAGTA

sequence common to both novel junctions. This was the third-strongest site of *in vitro* cleavage within the coding regions of *aprt*.

To determine whether the breakpoint in the non-*aprt* sequence also corresponded to a site of topoisomerase II-mediated cleavage, the 128-bp PCR product was generated in each of two separate reactions, one with each of the primers being 5' end-labeled with ³²P. Sites of *m*-AMSA-stimulated cleavage were mapped, and the extent of cleavage at each site was determined. Cleavage in the fragment was limited almost exclusively to a cluster of sites in the area of the reciprocal exchange, with by far the strongest site of apparent double-strand cleavage occurring in precise register with the strong cleavage site in the parental *aprt* sequence (Fig. 2). The ACTA sequence at the 4-base stagger of this cleavage site, however, did not occur in either of the newly formed junctions but was replaced by AGTA in both (Fig. 1).

As shown in Fig. 3, this rearrangement is consistent with a model involving formation of *m*-AMSA-stabilized cleavable complexes in each of the two parental sequences, followed by reciprocal exchange of topoisomerase subunits between the two complexes, religation of the exchanged ends, and, finally, correction of mismatches in both of the newly formed junctions to AGTA•TACT.

To determine whether the reciprocal exchange was inter- or intra-chromosomal, metaphase spreads of the Ip8a mutant cell line as well as nonmutant CHO K1 and D422 cells were hybridized with fluorescent probes corresponding to sequences 50 kb upstream (λ DM2, green fluorescence) and 10 kb downstream (λ B21, red fluorescence) from *aprt* (Ref. 14; Fig. 4). CHO K1 cells showed colocalization of the two flanking probes (*green* + *red* = *yellow*) at two sites. As expected (16), these were the CHO Z4 chromosome, which is a pericentric inversion of the normal Chinese hamster chromosome 3 (on which *aprt* resides), and the Z7 chromosome, which is thought to be a translocation between normal chromosomes 3 and 4 (Ref. 17; chromosomes were identified by GTG-banding; data not shown). Also as expected, the D422 strain, which is hemizygous for the *aprt* locus and surrounding sequences, showed only one of these coincident signals, that on Z4. In the Ip8a mutant, however, the upstream (*green*) and downstream (*red*) flanking probes hybridized to separate chromosomes, implying a chromosomal translocation at the *aprt* locus.

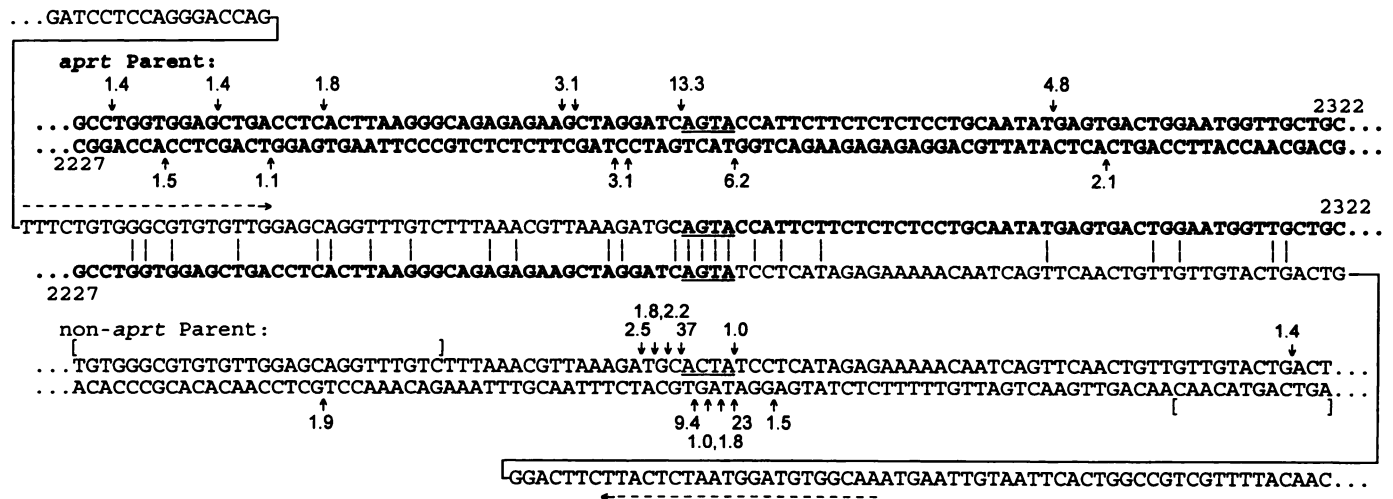


Fig. 1. Sequence analysis of the Ip8a *m*-AMSA-induced *aprt* gene rearrangement. Upper (**bold**) duplex shows the *aprt* parental sequence and the sites of *in vitro* cleavage by topoisomerase II α plus *m*-AMSA. Numbers above and below the arrows show the cleavage frequencies, normalized to the average cleavage frequency/base in the fragment (10). Just below the *aprt* duplex are shown the two rearrangement breakpoints, with **bold type** showing the extent of retention of the parental *aprt* sequence; *lightface type* indicates the non-*aprt* sequences beyond each breakpoint, with a continuation of those sequences shown at the very top and bottom of the figure. Vertical lines show base matches between the normal *aprt* sequence and each rearranged sequence. Underlines show the AGTA sequence retained in both newly formed junctions, which would correspond to the putative 4-base overlap of topoisomerase-mediated cleavage in *aprt*. The duplex just below the breakpoint sequences shows the non-*aprt* parental sequence, with sites of *in vitro* *m*-AMSA-stimulated cleavage (see Fig. 2). The non-*aprt* sequence contains ACTA, rather than AGTA, at the site of the reciprocal exchange with *aprt*. Dashed arrows show the PCR primers that were used to recover the non-*aprt* parental sequence from nonmutant cells and to generate the labeled fragment. Brackets indicate regions of each strand that were too close to the labeled end to determine cleavage sites. Only sites with a relative cleavage frequency of at least 1.0 are included.

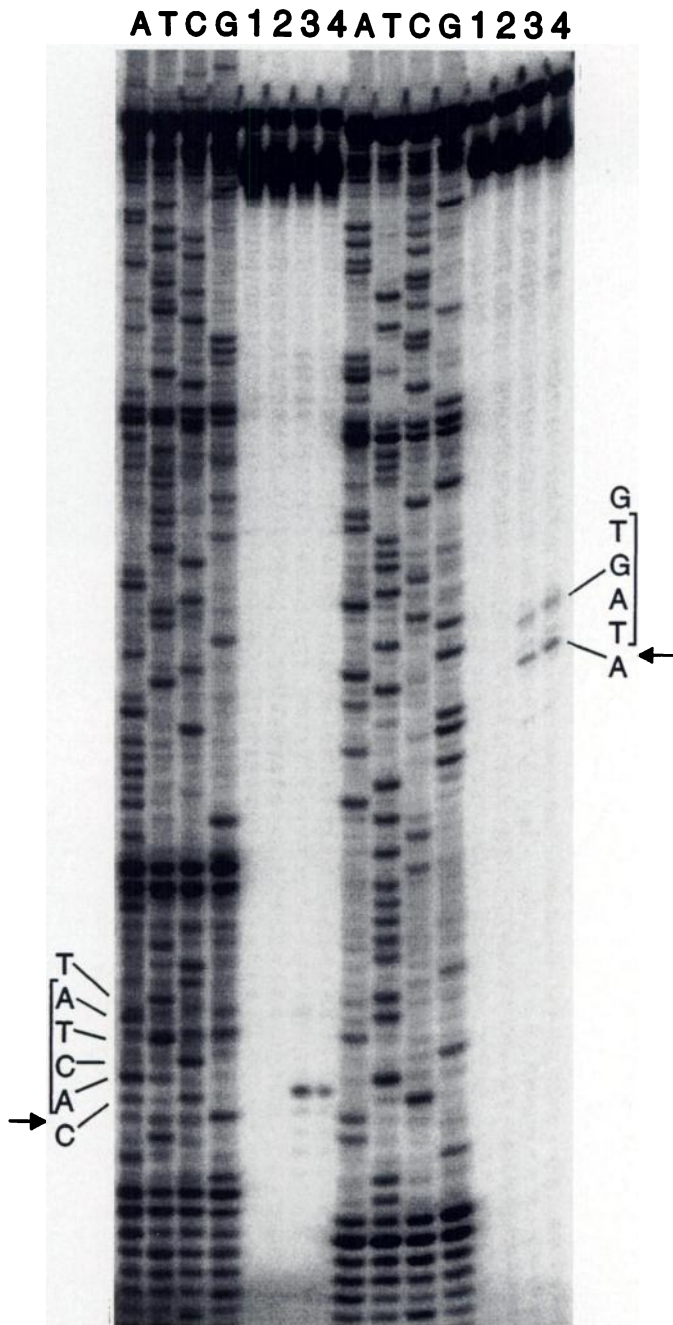


Fig. 2. *m*-AMSA-stimulated topoisomerase-mediated cleavage *in vitro* in the non-*aprt* parental fragment. PCR products 5' end-labeled in the top strand (left) or in the bottom strand (right; as indicated in Fig. 1; ~10 ng/sample) were treated with 10 (Lane 3) or 20 (Lane 4) μ M *m*-AMSA in the presence of 4 units of topoisomerase II α or treated with *m*-AMSA alone (Lane 1) or topoisomerase alone (Lane 2). The products were separated on a sequencing gel and subjected to phosphorimager analysis. A, G, C, and T represent dideoxy sequencing of the same (unlabeled) PCR products, using the same labeled primers and an Epicycle sequencing kit. Arrows show the locations of the predominant topoisomerase-mediated breaks in each strand. Brackets correspond to the complementary 4-base 5' overhangs and to the underlined ACTA in Fig. 1.

Presumably, this chromosomal translocation, which must have occurred within a 60-kb region encompassing the *aprt* gene, was a consequence of the same reciprocal exchange indicated by the sequencing data. In support of this assumption, molecular and cytogenetic analysis of *aprt* mutations induced in the same cells by other double-strand cleaving agents (bleomycin and neocarzinostatin) has indicated that ~70–80% of mutants that show reciprocal exchanges in *aprt* at the sequence level also show chromosomal separation of the

flanking probes. In contrast, with one exception, *aprt* mutants containing only point mutations consistently retain colocalization of flanking probes.⁴ Thus, the cytogenetic results (Fig. 4) strongly suggest that the two parental sequences originated from different chromosomes. However, it was not possible to determine unambiguously what other chromosomes were involved in the *aprt* translocation, because the changes in the GTG-banding patterns were complex, implying that additional chromosome rearrangements had also occurred in the Ip8a mutant.

Discussion

The remarkable fidelity with which the mammalian genome is maintained, despite the many millions of breakage/rejoining events effected by topoisomerase II with every cell generation, implies that topoisomerase subunit exchange under normal conditions must be extremely rare. Likewise, biochemical studies (18) indicate that topoisomerase II dimers are extremely stable. However, many of the genotoxic effects of topoisomerase II poisons, such as sister chromatid exchange, chromosome rearrangements, and production of large deletions, imply breakage and rejoining of DNA and thus could be explained in theory by a marked stimulation of subunit exchange (1, 4). On the other hand, these effects could be equally well explained by models involving dissociation of cleavable complexes to yield frank double-strand breaks, followed by inaccurate repair of those breaks. Thus, whereas there is ample evidence for topoisomerase II subunit exchange *in vitro* (5, 6), the evidence that subunit exchange ever occurs in intact cells is circumstantial at best.

At the molecular level, subunit exchange models impose rather stringent requirements on the specificity of any induced sequence changes; in particular, the breakpoints in both parental sequences must correspond to sites of drug-stimulated cleavage, and the register with which the two sequences are joined must correspond precisely to the 4-base overlap of the predicted 5' overhangs. In the few cases in which putative topoisomerase-mediated sequence changes have been determined, they have not conformed to those predictions. For example, small deletions and duplications could be ascribed to subunit exchange between cleavable complexes in the two daughter helices in the wake of a replication fork. However, although the deletions and duplications induced by teniposide in the CHO *aprt* gene (7) nearly always encompassed a prominent potential cleavage site (as assessed *in vitro*) and often occurred between

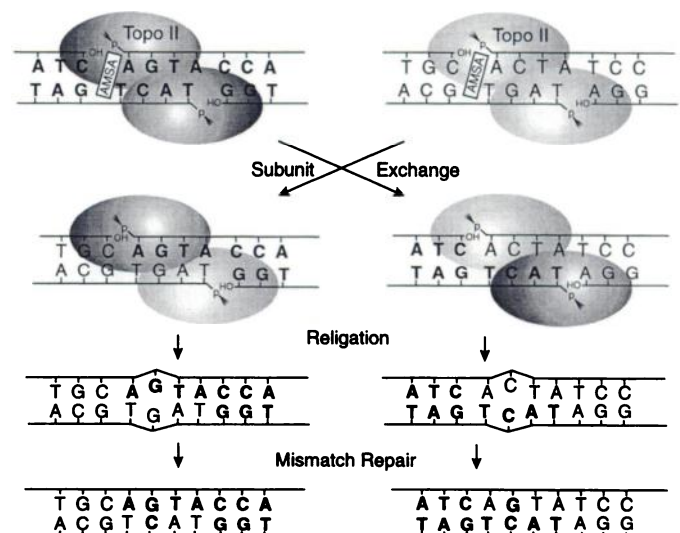


Fig. 3. Subunit exchange model for generation of the Ip8a rearrangement. Bold type shows the *aprt* parental sequence.

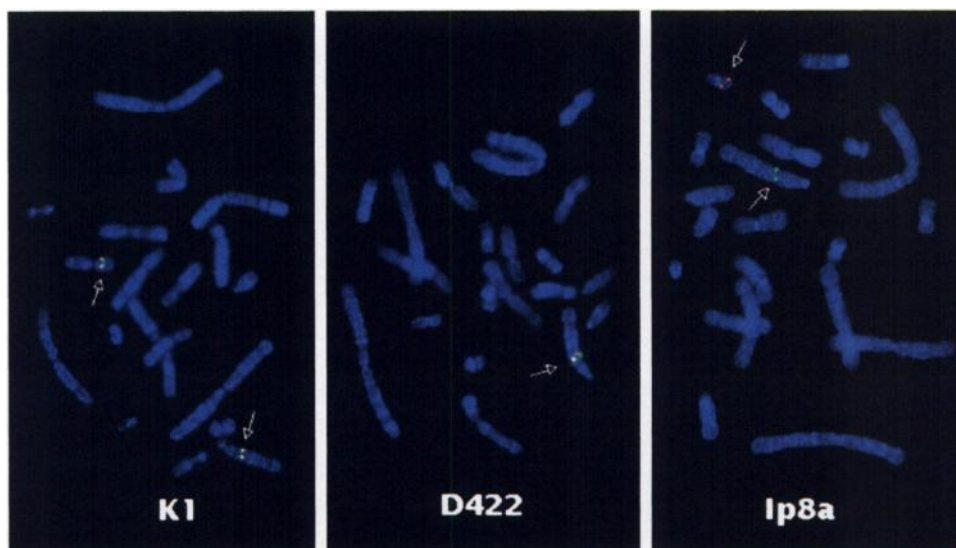


Fig. 4. *In situ* hybridization of sequences immediately upstream (ADM2, green fluorescence) and downstream (AB21, red fluorescence) of *aprt*. Arrows show the hybridization signals. In CHO K1, there are coincident (yellow) signals on both chromosome Z7 (upper left) and chromosome Z4 (lower right). In the *aprt* hemizygote CHO D422, only the signal on Z4 is present. In the Ip8a mutant, the single ADM2 and AB21 signals have become separated, indicating a chromosomal translocation at the *aprt* locus.

short direct repeats, not one of the deletion/duplication events was consistent with subunit exchange between two cleavage sites. A similar pattern was seen more recently for t(9;11) translocation breakpoints in various sporadic leukemias (8). Again, although the breakpoints were consistently located in the immediate vicinity of strong potential sites of topoisomerase-mediated cleavage, the two never corresponded exactly. The results in both systems thus imply that if topoisomerase-mediated cleavage was in fact involved, there must have been some trimming and/or patching at the ends before religation, a conclusion that rules out the simple subunit exchange model.

Indeed, the rearrangement described in Fig. 1 is, to our knowledge, the only reported instance of a breakage/reunion event occurring precisely between two prominent sites of topoisomerase-mediated cleavage in intact cells. The fact that both novel junctions of the exchange conform to this specificity makes it extremely unlikely that this correspondence is a mere coincidence. Even here, however, the possibility remains that the actual religation was effected not by topoisomerase II but by cellular repair enzymes after the removal of topoisomerase subunits from the ends. The use of cohesive overhangs (even those with only partial complementarity) for sequence alignment in end-joining repair pathways has been well documented (19); thus, such repair might yield precisely the same joining specificities as would a pure subunit exchange mechanism. It will therefore be difficult, if not impossible, to rigorously distinguish between these two mechanisms, at least for rearrangements occurring in intact cells.

Although no meaningful assessment of the frequency of apparent subunit exchange events can be made from the present data, it should nevertheless be noted that the target for mutation in these experiments, the *aprt* genomic sequence, is only ~2 kb, or about one-millionth of the genome. The apparent scarcity of reciprocal exchanges within *aprt* is thus expected and does not rule out the possibility that the incidence of *m*-AMSA-stimulated subunit exchange in the overall genome may be sufficient to account for a substantial fraction of *m*-AMSA-induced chromosomal translocations.

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