Megabase Pair Deletions in Mutant Mammalian Cells following Exposure to Amsacrine, an Inhibitor of DNA Topoisomerase II


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ABSTRACT

Amsacrine, 4'-[9-acridinylamino]-methanesulfon-m-anisidine, belongs to the class of cancer chemotherapeutic agents that target DNA topoisomerase II. We show that, over its cytotoxic range, amsacrine is a potent mutagen of the S1 phenotype in the A1 (human x hamster) hybrid cell line. By contrast, amsacrine induction of the Hprt- phenotype in A1 cells is at least two decades less frequent and is not concentration dependent. Such differential mutation frequencies are hypothesized to reflect the concomitant loss of essential genes neighboring the hprt locus. It may be that some amsacrine cytotoxicity is due to the inactivation of essential genes by large deletions. The A1 mutation system is well suited for the detection and mapping of mutations which are large deletions because its MIC1 locus, which controls the expression of the selectable cell surface antigen S1, is on a single human chromosome. This human chromosome 11 is in addition to the genome of the Chinese hamster ovary cell and is basically nonessential. Since there are no sister human chromosomes in A1 cells, deletions which extend beyond the MIC1 locus may be conveniently and unambiguously mapped. We have detected the presence or absence of 9 different chromosome 11 markers in 48 S1+ mutants cloned from amsacrine-treated cultures. We find that almost all (92%) of the mutants have deletions of at least 1.5–2 megabase pairs in length. The distribution of marker loss frequencies flanking the MIC1 locus does not appear symmetric with respect to distance from that locus. We speculate that amsacrine-induced deletions are mediated by a series of subunit exchanges between overlapping topoisomerase II dimers at the bases of replicons or larger chromosomal structures such as replicon clusters or chromosome minibands.

INTRODUCTION

Topoisomerase II is a major component of the nuclear matrix and the eukaryotic mitotic chromosome scaffold. It is a likely component of fixed replication sites located at the base of DNA replicon loops (1–5). In its role as an enzyme, eukaryotic topoisomerase II can decrease DNA supercoiling by introducing double strand breaks through which it passes duplex DNA; these double strand breaks are then religated (1–9). The basic step in the mechanism of action of many of the antitopoisomerase II class of chemotherapeutic agents, including amsacrine and epipodophyllotoxins (e.g., etoposide and teniposide), is the inhibition of DNA religation and the stabilization of DNA-topoisomerase II cleavage complexes (6–10). How this leads to cell killing has not been precisely established. The number of the stabilized cleavage complexes does not alone explain cytotoxicity (11–13).

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1 Supported by NIH Grants CA 36447 and CA 09236 and a grant from the Colorado State University Graduate School (Biomedical Research Science Grant-HHS).
2 Present address: 7427 Carroll Ave., Takoma Park, MD 20912.
3 The abbreviations used are: amsacrine, 4'-[9-acridinylamino]-methanesulfon-m-anisidine; CHO, Chinese hamster ovary fibroblasts; PCR, polymerase chain reaction; mbp, megabase pair; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; SSPFe, saline–sodium phosphate–EDTA buffer; SDS, sodium dodecyl sulfate; dT, thymidine kinase; hprt, Hprt, hypoxanthine phosphoribosyl transferase; mbp, megabase pairs of DNA.

Patients with acute lymphocytic leukemias or certain solid tumors can develop secondary acute myeloid leukemias as a result of treatment with the antitopoisomerase II agents etoposide or teniposide (14, 15). These secondary cancers often show characteristic chromosomal translocations. On the other hand, amsacrine is not considered mutagenic in bacteria, as measured by the Ames assay, although it may induce a small number of frameshift mutations (16). In cultured mammalian cells, amsacrine has been shown to be only weakly mutagenic for the hprt locus (10, 16–21). However, amsacrine is highly clastogenic (9, 17). The observation that amsacrine is a potent mutagen for the tk locus of L5178Y cells, inducing small-size colonies (18–21), has led to the hypothesis that amsacrine-induced tk mutations represent large deletions of DNA (18, 20). The extent of these deletions has not been determined, although molecular analyses of amsacrine-induced mutants consistently demonstrate the complete loss of the tk allele which expresses the marker phenotype (21).

Mutagens that cause large deletions in chromosomes (e.g., ionizing radiation) can be best studied using systems whose marker loci are not hostage to surrounding, essential genes (18–23). Typically, such clastogenic agents inefficiently induce mutation of the sex chromosome-linked hprt locus but are seen as much more mutagenic in autosomal, heterozygous systems (e.g., TK) where loss of neighboring essential genes can be offset by allelic genes on sister chromosomes. Unfortunately, the presence of two or more alleles complicates deletion mapping by obscuring the loss of relevant markers.

We have used the A1 system to study mutagens which produce very large deletions, such as ionizing radiation and asbestos (23–27). Agents like UV (24), chemical carcinogens (24), cigarette smoke condensate (28), and food mutagens (e.g., aflatoxins) (29) are also readily detected. The A1 cell line is a CHO × human cell hybrid which contains a single human chromosome 11 (29). This chromosome confers expression of new genes, including lethal cell surface antigens (30). Expression of one of these genes, S1 (formerly known as a1), is controlled by the MIC1 locus (31) and serves as a marker phenotype. The MIC1 locus maps to 11p13 in the same chromosomal regions as the Wilms' tumor gene (32, 33). Lesions involving the MIC1 gene result in loss of S1 expression. Mutated A1 cells no longer expressing the S1 antigen are not killed by specific antibody-mediated complement cytolyis and therefore can be selected from wild-type cells (22, 29).

Since the intact human chromosome 11 is not necessary for the survival of A1 cells, the MIC1 locus is not hostage to surrounding, essential loci. There is only a single human chromosome 11; therefore, losses of additional markers are not obscured by sister chromosomes. This allows the mapping of large deletions which might flank the MIC1 locus. We have measured deletion sizes in S1+ mutants from amsacrine-treated A1 cultures by employing Southern, PCR, and isoenzyme analyses of human chromosome 11 markers. We find that almost all (92%) of the examined mutants have deletions of at least 1.5–2 mbp in length.

2 C. Waldren, B. Schaeffer, A. Ueno, T. Puck, S. Wood, P. Sinclair, D. Doolittle, C. Smith, W. Harvey, M. Shibuya, and J. Sinclair. J. The food carcinogens 2-amino-3,4-dimethylnitrosamine (MeQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP) activated by cocultured chick embryo liver cultures (CELC) are mutagenic to mammalian A1 cells, submitted for publication.
MATERIALS AND METHODS

Chemicals. Ham’s F-12, fetal calf sera, penicillin G/streptomycin, and gentocin (G418) were purchased from Gibco Laboratories, Grand Island, NY. HEPES buffer was purchased from Boehringer Mannheim, Indianapolis, IN. Amsacrine (NSC 249992) originated from the Drug Synthesis Chemistry Branch, Division of Cancer Treatment, NCI, and was a gift from Dr. M. M. Elkind. It was stored frozen in dimethyl sulfoxide at a concentration of 10 mM. Monoclonal antibody specific for S1 antigen was prepared from hybridoma culture (34). Rabbit serum complement was purchased from Hazelton Research Products, Denver, PA and tested (26). \[\text{\textsuperscript{32}P}\]CTP for radiolabeling of DNA was purchased from Dupont NEN Research Products, Boston, MA.

Cell Culture. The \(A_6\) cell line is a hamster \(\times\) human hybrid in which there is a single human chromosome 11 against a CHO background (22-31). The AND-6 cell line is a variant of \(A_6\) cells produced by transfecting the neomycin resistance gene into the parental line (35). The neomycin resistance gene has integrated into the long arm of human chromosome 11. These cells were cultured as described previously (23, 27).

Amsacrine Treatment, Cytotoxicity, Mutation, and Cloning of Mutants. AND-6 cells were passaged in selective media containing geneticin for at least 1 week prior to experimentation in order to reduce background levels of S1 mutants. Cell cultures for experimentation were prepared by inoculating 5 \(\times\) 10\(^5\) AND-6 cells into 100-mm tissue culture dishes and incubating overnight. Log phase cell cultures were treated for 16 h (37\(^\circ\)C) with different concentrations (up to 40 \(\mu\)M) of amsacrine in media. The cells were then washed once with medium (37\(^\circ\)C); trypsinized; suspended in F-12 medium containing 7\% fetal calf serum, HEPES buffer, and penicillin/streptomycin; and counted. Some of these cells were immediately plated to ascertain cell survival following amsacrine treatment. The remainder of the cells were subcultured over a 9- to 12-day expression time. Cell cultures were passaged regularly to prevent overcrowding and inoculated at numbers greater than 2 \(\times\) 10\(^5\) cells/plate in order to avoid fortuitous enrichment of mutants.

Mutation assays were performed as described (24) and in duplicate. From each treated culture (two cultures per amsacrine concentration), 2 \(\times\) 10\(^5\) cells were seeded into each of at least two 100-mm culture dishes. Following cell attachment, the cultures were challenged for the expression of the S1 cell surface antigen with specific monoclonal antibody (34) plus rabbit complement mixed with 5\% (v/v) human serum. Two S1 challenges were performed for each separate experiment, and their results were weight averaged with respect to plating efficiency. Just prior to fixation, some S1- mutants were cloned at random from the cell cultures treated with 40 \(\mu\)M amsacrine. Each of these mutant colonies was expanded for molecular analyses to determine which other human chromosome 11 markers had also been lost. HPRT+ mutants were selected as described previously (24). Briefly, 2 \(\times\) 10\(^5\) cells were plated into each of at least five 100-mm dishes containing F-12 medium with 7\% fetal calf serum, HEPES buffer, and penicillin/streptomycin; then 6-thioguanine (10 \(\mu\)g/ml, final concentration) was added to these cultures to select HPRT+ mutants. Plating efficiencies in the presence or absence of complement were determined. All dishes were incubated for 10 days. Colonies were then fixed and stained. Averaged background mutant frequencies for S1- and HPRT- mutants were approximately 170 \(\times\) 10\(^{-5}\) and 3 \(\times\) 10\(^{-5}\), respectively.

Gene Probes. Table 1 lists the human chromosome 11 markers used to measure deletion sizes (33, 36-39). We estimated the distances of the MIC1 locus from the 11p13 markers (D16, P8, P5, and CAT) by comparing published physical maps of the Wilms’ tumor/aniridia/gonadal blastoma/retardation complex region (33, 36). The distances for the other markers were extrapolated from published cytogenetic assignments of their loci, given that the total DNA content of human chromosome 11 is approximately 140-150 mabps, i.e., 140-150 centimorgans (40). The D16, CAT, ACP, and FTH probes used in Southern blot analyses were obtained from the American Type Culture Collection (Rockville, MD). P5BE1.2 and P8B1.25 probes were gifts from Dr. V. Hult (University of Texas at Houston, Texas). Radiolabeled DNA probes for the detection of human chromosome 11 markers were prepared as described (27).

DNA Extraction, Restriction, and Southern Analyses. Southern blot analyses were performed according to the method of Maniatis et al. (41). Genomic DNA (20-40 \(\mu\)g) was prepared from S1- clones by phenol-chloroform extraction. Aliquots of these DNA samples were digested with EcoRI (Amersham Corp., Arlington Heights, IL) and run on a horizontal gel apparatus (Bio-Rad Laboratories, Richmond CA) for 16 h at 20 V in Tris-acetate/EDTA electrophoresis buffer. The DNA samples were then transferred onto charged Hybond N+ nylon membranes (Amersham) by alkaline Southern blotting using a BLOT apparatus (New Haven, CT). After one wash in 5X SSPE [an aqueous solution of 3.6% (w/v) NaCl and 0.5% (w/v) sodium citrate], the filters were loaded into hybridization tubes with 5X standard saline citrate [an aqueous solution of 3.6% (w/v) NaCl, 0.5% (w/v) NaH2PO4, and 0.1% (w/v) EDTA]. 5% Denhardt’s solution, 2.5% SDS, and 20 \(\mu\)g/ml denatured salmon sperm DNA and prehybridized at 65\(^\circ\)C for 1-4 h in a hybridization incubator (Robbins Scientific, Sunnyvale, CA). DNA probes were random prime labeled (kit purchased from Boehringer Mannheim) with \[\text{\textsuperscript{32}P}\]dCTP to greater than 10\(^{10}\) cpm/\(\mu\)g and added to a final concentration of 10\(^{9}\) cpm/ml in the hybridization tubes containing the filters. After hybridization for 16 h at 65\(^\circ\)C, the filters were washed two times in 2X SSPE plus 0.1% SDS for 10 min and then washed again at 65\(^\circ\)C for 15 min in 0.2X SSPE plus SDS and dried. X-ray films (Hyperfilm-MP, Amersham) were exposed through intensifying screens to these filters at \(\sim\)70\(^\circ\)C and then developed by standard procedures. The presence or absence of bands was judged visually. Comparative Southern analyses between AND-6 and CHO genomic DNA for each probe allowed discernment of human from hamster alleles.

PCR Detection of the RAS Marker. Oligonucleotide primers used in the PCR detection of \(H\)-ras were designed using the published gene sequence (42). One primer sequence, RAS-1, was 5’-ATG ACG GAA TAT AAG GTG GTG-3’ and the other, RAS-2, was 5’-TCT ATA GTG GGG TCG TAT TCG-3’. One primer sequence, RAS-1, was 5’-ATG ACG GAA TAT AAG GTG GTG-3’ and the other, RAS-2, was 5’-TCT ATA GTG GGG TCG TAT TCG-3’. PCR was performed as described (43) using an iced master mix of 1.25 mM deoxynucleotide triphosphate mixture (0.2 mM, final concentration), 20 \(\mu\)M of each primer (RAS-1 and RAS-2; 0.2 \(\mu\)M, final concentration), distilled water, 10X Stoffel fragment buffer (10 mM KCl-10 mM Tris-HC1, final concentration), 25 mM MgCl\(_2\) (2.5 mM, final concentration), and then washed again at 65\(^\circ\)C for 15 min in 0.2X SSPE plus SDS and dried. RAS probe was used in PCR detection of the RAS marker. Oligonucleotide primers used in the PCR detection of \(H\)-ras were designed using the published gene sequence (42). One primer sequence, RAS-1, was 5’-ATG ACG GAA TAT AAG GTG GTG-3’ and the other, RAS-2, was 5’-TCT ATA GTG GGG TCG TAT TCG-3’. PCR was performed as described (43) using an iced master mix of 1.25 mM deoxynucleotide triphosphate mixture (0.2 mM, final concentration), 20 \(\mu\)M of each primer (RAS-1 and RAS-2; 0.2 \(\mu\)M, final concentration), distilled water, 10X Stoffel fragment buffer (10 mM KCl-10 mM Tris-HC1, final concentration), 25 mM MgCl\(_2\) (2.5 mM, final concentration), and then washed again at 65\(^\circ\)C for 15 min in 0.2X SSPE plus SDS and dried. RAS probe was used in PCR detection of the RAS marker.

Table 1: Human chromosome 11 markers used in analyses of amsacrine-treated \(A_6\) cells. Distances are shown in megabase pairs from the MIC1 locus

<table>
<thead>
<tr>
<th>Marker</th>
<th>Locus</th>
<th>Chromosome location</th>
<th>Reference for chromosome location</th>
<th>Estimated distance from MIC1 (mbp)</th>
<th>Method for determining missing marker</th>
<th>Source</th>
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<tr>
<td>S1</td>
<td>MIC1</td>
<td>1p13</td>
<td>Rose et al., (33)</td>
<td>0</td>
<td>Antibody + complement</td>
<td>C. Walden</td>
</tr>
<tr>
<td>P5</td>
<td></td>
<td>1p13</td>
<td>Compton et al., (36)</td>
<td>1.5</td>
<td>Southern blot</td>
<td>V. Huff</td>
</tr>
<tr>
<td>D16</td>
<td>1D11S6</td>
<td>1D11S6</td>
<td>Rose et al., (33)</td>
<td>3</td>
<td>Southern blot</td>
<td>ATCC*</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase subunit A</td>
<td>1p14–p15</td>
<td>Yang-Feng et al., (38)</td>
<td>11</td>
<td>Isozyme typing</td>
<td>Commercial assay</td>
</tr>
<tr>
<td>RAS</td>
<td>HRAS</td>
<td>11p15.5</td>
<td>Koufos et al., (39)</td>
<td>34</td>
<td>PCR</td>
<td>C. Walden</td>
</tr>
<tr>
<td>PB1.25</td>
<td></td>
<td>1p13</td>
<td>Compton et al., (36)</td>
<td>0.5</td>
<td>Southern blot</td>
<td>V. Huff</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
<td>1p13</td>
<td>Rose et al., (33)</td>
<td>2</td>
<td>Southern blot</td>
<td>ATCC</td>
</tr>
<tr>
<td>ACP</td>
<td></td>
<td>1p11</td>
<td>Junien and McBride (37)</td>
<td>22</td>
<td>Southern blot</td>
<td>ATCC</td>
</tr>
<tr>
<td>FTH</td>
<td></td>
<td>1q13</td>
<td>Junien and McBride (37)</td>
<td>45</td>
<td>Southern blot</td>
<td>ATCC</td>
</tr>
</tbody>
</table>

* ATCC, American Type Culture Collection.
at 72°C; and 4°C soak. Four % gels (80 ml of 1X Tris-acetate/EDTA electrophoresis buffer, 2.4 g NuSieve GTG Agarose, and 0.8 g SeaKem Agarose; FMC BioProducts, Rockland, ME) were loaded with DNA samples which were electrophoresed at 70 V for 90 min, stained with ethidium bromide, and examined using light transilluminators.

**Lactate Dehydrogenase Isoenzyme Assay.** The presence or absence of lactate dehydrogenase A was detected in S1- mutants according to established procedure (30) using a commercial serum lactate dehydrogenase assay (Helena Laboratories, Beaumont, TX).

**RESULTS**

Amsacrine-induced Cytotoxicity and Mutation Frequencies. Fig. 1A shows a log-linear relationship of survival of log phase AND-6 cells exposed to amsacrine. The D0 (i.e., the mean lethal dose) was 24 nm. Net frequencies of S1- and HPRT - mutants per 10^5 survivors over the same amsacrine concentration range are shown in Fig. 1B. While the shape of the S1- mutant frequency curve is linear, there appears to be some quadratic tendency to it, with the curve bending up at the higher concentrations. The average preexisting S1- mutant fraction was 170 mutants per 10^5 survivors. At 40 nm, cell survival was approximately 20% with 375 net S1- mutants induced per 10^5 survivors. Concentrations greater than 40 nm amsacrine decreased cell survival below 10% and increased mutant frequency, but erratically, so that mutant quantitation was not reliable (data not shown). The preexisting HPRT - mutant fraction was approximately 1 per 10^5 survivors. At 40 nm (20% survival), there were approximately 2 mutants per 10^5 survivors. Amsacrine-induction of HPRT - mutants did not appear to be concentration dependent.

Mapping Deletions in A4 Cell Mutants from Amsacrine-treated Cultures. Fig. 2 shows a typical Southern blot used to detect the presence or absence of chromosome 11 markers D16, FTH, and P5 in twelve S1- clones and an S1- positive control. RAS was detected by PCR in all 48 S1- mutants cloned from amsacrine-treated cultures; this was expected since the tip of the short arm of the human chromosome 11 is required for survival of the A4, hybrid (32). Data for the detection of the LDH marker by isoenzyme analyses (30) is also summarized in Fig. 3.

Fig. 3A shows the deleted portions of chromosome 11 in 48 S1- clones. The deleted sections are shown as their minimum possible sizes. Retention of a marker always meant that additional markers in the same direction but more distant from MIC1 were also retained, i.e., deletions appeared to be continuous. Since the induced mutant fraction was three to four times background, there is a one-third to one-fourth chance that any particular clone arose spontaneously rather than through amsacrine treatment. Fig. 3B shows the cumulative percentage of lost markers. Because all clones were first selected for the S1- phenotype, 100% of the mutants were assumed to have lost the MIC1 locus. Previous cytogenetic and molecular analyses have demonstrated that induction of the S1- phenotype in A4 cells results from the disappearance of the MIC1 locus (32, 33). The declining percentage of S1- clones that had lost markers progressively distant from the MIC1 locus is further evidence that the induced S1- phenotype did, in fact, result from lesions of the MIC1 gene (Fig. 3B). Only 4 of the 48 mutant clones (8%) did not show any additional 11p13 marker loss besides that of the S1- phenotype.

Fig. 3B also shows that a large fraction (88%) of the S1- mutants had deleted the CAT locus, which is 2 mbp proximal (i.e., toward the centromere) to MIC1. ACP marker loss occurred in 45% of S1- mutants in amsacrine-treated cells; FTH, located at 1q13 on the long arm of chromosome 11, was lost in only 8% of S1- mutants. The P5 marker, which is distal (i.e., away from the centromere) to MIC1, was lost in 80% of clones. Approximately 50% of mutants had deletions that encompassed the D16 marker. The LDH marker, 11 mbp distal to MIC1, was absent in 20% of S1- mutants. Marker losses appeared to be asymmetric with a greater percentage of markers lost proximal to MIC1 (i.e., P8-CAT) than distal to the MIC1 (P5-D16) for about the same distance (2–3 mbp).

**DISCUSSION**

S1 versus TK and HPRT Marker Loss following Amsacrine Treatment. Our amsacrine-induced A4 mutant frequencies (Fig. 1) are generally comparable to those reported by others (Table 2). Amsacrine-induced HPRT - mutant frequencies in A4 cells were not concentration dependent and were approximately 100-fold less frequent than S1- mutants.

Because amsacrine is a clastogen (9, 10, 16, 18) and highly mutagenic for the tk locus of L5178Y/TK+/- cells, while only marginally mutagenic for the hprt locus (18), and because amsacrine-induced TK- colonies are small, it has been hypothesized that amsacrine-induced mutations result from large deletions of DNA (18, 20). Clive et al. (21) found that virtually all of amsacrine-induced TK- mutants had lost the active 6.3 kb allele, thus demonstrating that amsacrine-induced deletions are intergenic. The A4 mutation system allows us to estimate the sizes of the deletions in mutants from amsacrine-treated cultures. As predicted, they are large deletions with a prevalent minimum size of 1.5–2 mbp (Fig. 3A). Detailed physical maps of the Wilms’ tumor/aniridia/gonadoblastoma/retardation complex in the 11p13 band (33, 36) have afforded a high resolution of the distances between MIC1 and other 11p13 markers. Distances of MIC1 from chromosome 11 markers outside of 11p13 were estimated from cyt-

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Fig. 1. A. survival of AND-6 cells after incubation for 16 h at 37°C with increasing concentrations of amsacrine. The points shown were averaged from five different survival assays. The SEMs are depicted as error bars when larger than the symbols drawn. B. net frequencies of S1- and HPRT - mutants per 10^5 survivors. Averaged background mutant frequencies for S1- and HPRT - mutants were approximately 170 × 10^-5 and 3 × 10^-5, respectively, and have been subtracted. Two experimental sets are shown for S1- mutants. Points, the average of two challenges per experiment (see "Materials and Methods"). HPRT - mutant frequencies have been averaged.
genetic maps and, therefore, are less exact. The availability of markers dictated our use of widely spaced non-11p13 markers. Thus, a meaningful determination of an average minimum amsacrine-induced deletion beyond 11p13 is problematic. The large deletions we see may be due, in part, to the general lack of viability constraints on damage to the human chromosome 11 in AL cells and also suggests that deletions, located so as to damage essential genes, may contribute to amsacrine cytotoxicity in other systems and in vivo.

Comparing S1- Mutants from Cultures Treated with Amsacrine or Other Mutagens. Comparison of amsacrine-induced mutant frequencies with those induced at similar cytotoxicity by other known mutagens in the AL system indicates that amsacrine is a potent mutagen of the MIC1 locus. At its mean lethal dose (LD50), amsacrine induces approximately 160 mutants per 10^5 survivors compared to 165 mutants for chrysotile fibers (27), 130 mutants for γ-ray, and 120 mutants for ethyl methanesulfonate (24) and substantially exceeds the 45 mutants per 10^5 survivors found for UV (24). Thus amsacrine should be a carcinogen, as are other strong mutagens. Epipodophyllotoxins induce secondary cancers (14, 15), and it would be useful to demonstrate and characterize the mutagenicity of those agents in the AL system.

The deletion spectrum in S1- mutants from amsacrine-treated cultures differs from that of spontaneous AL mutants (25, 27). S1- mutants from amsacrine-treated cultures were more likely than spontaneous mutants to have deletions that exceed the MIC1 locus. Continuing studies on spontaneous mutants from zero treatment control cultures have shown that 24% of spontaneous S1- mutants lose only the S1 marker; in contrast, just 8% of S1- mutants from amsacrine-treated cultures lost only S1. Twenty-three % of spontaneous AL mutants lost at least part of the long arm of human chromosome 11. Few S1- mutants from amsacrine-treated cultures (8%) lost the long arm marker FTH. The extent of deletions proximal to MIC1 may be limited by the centromere. This might argue that amsacrine is a poor inducer of translocations in the AL system since deletions spanning the centromere are probably almost all translocations or that it induces translocations that result in multicentric chromosomes. Mutants from spontaneous, amsacrine-, γ-ray-, or iron ion-treated cultures seem to lose the ACP marker (22 mbp proximal to MIC1) more commonly (45%-69%) than the LDH marker (9%-20%), which is 11 mbp distal to MIC1. This may be due, in part, to the requirement for the retention of the tip of 11p in AL cells and its mutants (32).

Models of Topoisomerase II-mediated Mutation Based on AL Chromosome Deletion Maps. Since topoisomerase II is a homodimer, it has been hypothesized that illegitimate recombinations could result from subunit exchange between different topoisomerase II complexes; such recombinations have been shown to occur in prokaryotes and might mediate translocations, deletions, and amplifications in eukaryotes (44, 45). Topoisomerase II is thought to be a component of fixed replication sites located at the base of DNA replicon loops (2-5). Because 90% of the S1- mutants from amsacrine-treated cultures had deletions which were at least 1.5-2 mbp (Fig. 3B), it is unlikely that these large deletions were mediated by the loss of a single replicon (0.05-0.10 mbp). We speculate that amsacrine-induced deletions may involve a series of many topoisomerase-DNA loop complexes or, alternatively, fewer subunit exchanges between overlapping, large chromosome structures containing megabase pair quantities of DNA. There may be examples of such structures. Pienta and Coffey (2) postulate that there are 1.1-mbp “minibands” composed of 17 loops, in the condensed, mitotic chromosome. Nakamura et al. (46) report “replicon domains” of 1 mbp composed of multiple replicons during the S phase of the cell cycle.

Distribution of marker deletion frequencies are asymmetric with respect to the MIC1 locus (Fig. 3B). Even though CAT is 1.5 mbp proximal to P8, both are lost with the same frequency. Thus, most deletions span both P8 and CAT. This may suggest preferential loss of a 2-mbp structural unit proximal to MIC1. Amsacrine and epipodophyllotoxins do not suppress and often stimulate DNA strand scission at cleavage sites detected in the absence of drug, i.e., produced physiologically by topoisomerase II (47). Irregular spacing of topoisomerase II sites might contribute to deletion asymmetry. It has been suggested that the relatively low sequence specificity for topoisomerase II cleavage could indicate that different sites are used for different functions, such as replication, transcription, nuclear matrix organization, and chromomosome structure (47). Matrix-associated regions, which

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* C. A. Waldren, personal communication.
Fig. 3. Summary maps of molecular analyses of S1- mutants picked from cultures exposed to 40 nm amsacrine for 16 h at 37°C. Induced mutation fraction was three to four times background. S1+ colonies ring-cloned for molecular analyses were picked at random. Chromosome 11p markers are shown in bold. Distances are in megabase pairs from the MIC1 locus. Markers to the right of MIC1 map toward the tip of the short arm; those to the left are toward the telomer of the long arm. The position of the centromere (O; approximately 20 mbp from MIC1) is shown as a reference. A, minimum size of the deletions in 48 individual S1- mutants. Maps are arranged downward in order of increasing deletion size. Lines are drawn to the last deleted marker from the MIC1 locus. The first four mutants depicted have lost only the S1 marker. Only the last four mutants have deletions which extended beyond the centromere into the long arm; they have lost the FTH marker. All mutants retained RAS. B, the percentage of S1- mutants that have lost each of the various human chromosome 11 markers tested.

Table 2

<table>
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<tr>
<th>Cell line</th>
<th>Marker</th>
<th>Percentage survival</th>
<th>Mutants per 10^5 Survivors</th>
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<td>TK</td>
<td>10</td>
<td>280</td>
<td>16, 20</td>
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<td>20</td>
<td>600</td>
<td>19</td>
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<td>L5178Y LY-R$^a$</td>
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<tr>
<td>L5178Y/TK ^+/−</td>
<td>HPRT</td>
<td>13</td>
<td>64</td>
<td>21</td>
</tr>
<tr>
<td>A5</td>
<td>HPRT</td>
<td>20</td>
<td>56</td>
<td>17</td>
</tr>
</tbody>
</table>

$^a$LY-S cells and LY-R cells are L5178Y strains that are heterozygous for TK and are radiosensitive and radioresistant, respectively.

can be a feature of enhancer elements and recombination (48), have often been shown to contain strong topoisomerase cleavage sites (48, 49). First division metaphases of etoposide-treated human lymphocytes indicate that aberrations in R-band-rich regions are favored (50). A site of recurrent translocations in acute lymphocytic leukemia (TCL2) is located just proximal to the MIC1 locus (33). Our findings may support the hypothesis that production of mutations by agents that target chromosomal matrix components may be influenced by the particular arrangement of those components.

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Megabase Pair Deletions in Mutant Mammalian Cells following Exposure to Amsacrine, an Inhibitor of DNA Topoisomerase II


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