Drug-specific Sites of Topoisomerase II DNA Cleavage in Drosophila Chromatin: Heterogeneous Localization and Reversibility

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ABSTRACT

DNA cleavage stimulated by different topoisomerase II inhibitors shows in vitro a characteristic sequence specificity. Since chromatin structure and genome organization are expected to influence drug–enzyme interactions and repair of drug-induced DNA lesions, we investigated topoisomerase II DNA cleavage sites stimulated by teniposide (VM-26), 4-demethoxy-3'-deamino-3'-hydroxy-4'-epi-doxorubicin (dh-EPI, a doxorubicin derivative), 4'-(9-acridinylamino)-methanesulfon-m-anisidide, and amonafide in the histone gene locus and satellite III DNA of Drosophila cells with Southern blotting and genomic sequencing by primer extension. VM-26 stimulated cleavage in the satellite III DNA, whereas the other studied drugs did not. All four drugs stimulated cleavage in the histone gene cluster, but they yielded drug-specific cleavage intensity patterns. Cleavage sites by dh-EPI and VM-26 were sequenced in the histone H2A gene promoter and were shown to be distinct. DNA cleavage analysis in cloned DNA fragments with Drosophila topoisomerase II showed that drugs stimulated the same sites in vivo and in vitro. Strand cuts were in vivo staggered by 4 bases, and base sequences at major dh-EPI and VM-26 sites completely agreed with known in vitro drug sequence specificities. Moreover, DNA cleavage reverted faster in the satellite III than in the histone repeats. While stimulating similar levels of DNA breakage in bulk genomic DNA, dh-EPI and VM-26 markedly differed for cleavage extent and reversibility in specific chromatin loci. The results demonstrate a high heterogeneity in the localization, extent, and reversibility of drug-stimulated DNA cleavage in the chromatin of living cells.

INTRODUCTION

The chromatin of eukaryotic cells is organized into dynamic structural units, the DNA loops, the size and properties of which can vary among distinct genomic regions and during the cell cycle (1, 2). The complex topological problems that thus continuously arise are solved by DNA topoisomerases. These are widespread enzymes that regulate DNA topology through concerted DNA breakage and religation activities (3). An important function of DNA topoisomerases consists in relieving the torsional stress generated during DNA replication and transcription (4). Type II DNA topoisomerase has an essential role in the decatenation of daughter DNA helices at the end of replication and in the segregation of intertwined chromosomes (5, 6). Moreover, topoisomerase II is the target of several antitumor agents, such as the anthracyclines and the demethylepipodophyllotoxin VM-264 (7—9). These compounds stimulate enzyme-mediated DNA cleavage in vitro in a sequence-selective manner, resulting in drug-specific cleavage intensity patterns in sequencing gels (7—9).

In past years, much attention has been focused on chromatin sites of topoisomerase II activity in living cells. This enzyme has been suggested to regulate chromatin structure by specific interactions with SARS (1, 10—14) and ssc regions (elements that determine domain boundaries; Ref. 15). Moreover, topoisomerase II cleavage sites often colocalize with DNase I-hypersensitive regions (16—19), which raises the possibility that the enzyme may function to establish or maintain the local topology at hypersensitive regions. In all of these studies, the topoisomerase II inhibitor VM-26 has been used as a tool to enhance enzyme-mediated DNA cleavage. This approach was based on the assumption that VM-26 is able to stimulate DNA cleavage at all in vivo sites of topoisomerase II activity (14, 15, 17—19) and that chromatin structure is the primary determinant of cleavage site selectivity observed in vivo (16). Nevertheless, other groups have described differences in cleavage patterns in viral (20), epimodal (21), and cellular chromatin (22) following cell treatments with structurally unrelated inhibitors, mAMSA and VM-26, suggesting that the in vivo site selectivity is determined, at least in part, by the inhibitor.

Thus, to establish whether the drug sequence specificity is a determinant of drug-enhanced DNA cleavage in the chromatin of living cells, we have examined at a sequence level the localization of drug-stimulated topoisomerase II DNA cleavage in Drosophila melanogaster Kc cells. We selected two regions of the Kc cell genome (the satellite III DNA and the histone gene cluster) since their chromatin structures have been well characterized (see below); thus, the results may provide significant information on enzyme and drug activities also in human malignant cells. Our analysis has been focused on VM-26, dh-EPI (a potent doxorubicin derivative), mAMSA, and amonafide, since these four agents showed quite different sequence specificities in in vitro studies (23—26). The results demonstrate that each drug maintains in vivo a high degree of DNA sequence specificity, which corresponds to that established in vitro (9, 23, 27, 28), and suggest that the in vivo site selectivity of DNA cleavage is determined by several factors, including the chromatin structure as well as the inhibitor used. Moreover, a high degree of genomic heterogeneity was observed concerning cleavage sites, levels, and reversibility that might play a role in the drug antitumor activity.

MATERIALS AND METHODS

Drugs and Other Materials. VM-26 and dh-EPI were obtained from Bristol Italiana (Latina, Italy) and Pharmacia-Farmitalia (Milan, Italy), respectively. Amonafide and mAMSA were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). Drugs were freshly prepared in DMSO and then diluted in deionized water. Drosophila topoisomerase II was purchased from USB (Cleveland, OH). T4 polynucleotide kinase and polyclayamide were purchased from Life Technologies, Inc. (Basel, Switzerland). Restriction enzymes were from BioLabs (Taunus, Germany). [γ-32P]ATP was obtained from Amersham (Milan, Italy).

In Vivo Topoisomerase II Cleavage Assays. Exponentially growing Kc cells (3–4 × 10^6 cells/ml at 25°C) were treated with VM-26, dh-EPI, mAMSA, or amonafide for 30 min at the indicated concentrations. When indicated, cells were first treated with 25 μM distamycin for 30 min before the addition of topoisomerase inhibitors. Heat shock was performed at 37°C for 30 min before
the addition of drugs and continued cell incubation at 37°C. Aliquots of 2 x 10^6 cells were pelleted for 5 min at 800 x g and lysed in 5 ml of 20 mM Tris-Cl (pH 8)-1% SDS, followed by the addition of EDTA to 15 mM and proteinase K to 400 μg/ml. The lysate was incubated at 37°C for 16 h with gentle shaking, and DNA was then purified by standard procedures (29). To measure cleavage reversibility, cells were resuspended in fresh drug-free medium after drug treatments and incubated at 25°C for the indicated time. Cells were then collected and lysed as described above. Purified DNA samples were digested with HindIII and electrophoresed through 1.2% agarose gels overnight at 65 V in TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8). Southern blots were then performed according to standard protocols (29). Filters were prehydrized in 10 ml of 6X SSC (1X SSC = 150 mM NaCl, 50 mM sodium citrate), 5X Denhardt’s solution, 0.5% SDS, and 0.1 mg/ml denatured salmon sperm DNA for 4 h at 65°C. Cleavage products in the histone gene cluster were detected with indirect end-labeling by using either a 300-bp HindIII-III (HP probe) or a 530-bp HindIII-II (HP probe) fragments spanning the 5’ or 3’ coding regions of the HI gene, respectively. Hybridization probes generated by the random primer labeling procedure (29) were added to the filters, together with fresh salmon sperm DNA, and hybridizations were performed overnight at 65°C. To detect cleavage in the satellite III repeat, genomic DNA samples were electrophoresed onto 1.2% agarose gels without prior digestion with restriction enzymes. Hybridization to a cloned satellite III 359-bp repeat was as described above. Dried gels were routinely exposed to phosphorimager screens to measure cleavage levels at the different sites with a Molecular Dynamics phosphorimager 425 model and then were exposed to Amersham MP films.

**In Vitro Topoisomerase II Cleavage Assay.** A 5744-bp I DNA fragment containing 16 repeats of satellite III DNA from D. melanogaster was cloned into the AccI site of pUC18. The 5744-bp fragment was uniquely 5’-end-labeled as described previously and purified by agarose gel electrophoresis and electroelution (25, 26). DNA cleavage reactions were performed in 20 μl of 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 1 mM ATP, 15 μg/ml BSA with 10 units of Drosophila topoisomerase II (USB, Cleveland, OH), and 50 μM of VM-26 or 10 μM of dh-EPI at 30°C for 20 min. Reactions were stopped with SDS and proteinase K (1% and 0.1 mg/ml, respectively) and further incubated at 42°C for 45 min. DNA cleavage was examined by agarose gel electrophoresis (25, 26). Dried gels were exposed to Amersham MP films.

**Genomic Sequencing of Cleavage Sites.** Histone gene sequence information used for the selection of primers was obtained from the EMBL Nucleotide Sequence Database (accession number X14215; D. melanogaster histone gene cluster). Primers D10-1 (5’-CTAATCAGCTTAAGCTTTCCAGC-3’), from 160 to 180 of the upper strand, and D10-2 (5’-CCTTTGCAAGGATCTCCAC-3’), from 429 to 409 of the lower strand, were used for extension through the H2A-H2B spacer region to sequence site 10. DNA sequencing of site 10 and further extension of the orientation was achieved by primer walking with MboI for primers D10-1 and D10-2 and with HindIII or DdeI for primers V-1 and V-2, respectively. Primer extensions were carried out as described (31) using 1 μg of digested DNA and 1–5 x 10^6 cpm of labeled primer per sample. Taq DNA polymerase (1.2 unit; Perkin-Elmer-Cetus or Promega) or Ultima DNA polymerase (0.8 unit; Perkin-Elmer-Cetus) were added to 50-μl reactions in the buffer recommended by the manufacturer supplemented with 2.5 mM MgCl2 and 10 μM (final concentrations) of each deoxynucleotide (Boehringer Mannheim). Samples were subjected to 30 cycles of 1 min denaturation at 94°C, 2 min hybridization at 58°C, and 3 min extension at 72°C in a Perkin-Elmer-Cetus DNA thermal cycler. Sequence ladders were generated from similarly digested, appropriately diluted, cloned DNA templates; reaction mixtures were prepared exactly as above, except that extensions were carried out in the presence of 400 nM ddATP, 300 nM ddCTP, 100 nM ddGTP, or 600 nM ddTTP (Boehringer Mannheim). Reaction products were separated on a 6% or 8% sequencing gel (29). Dried gels were exposed to Amersham MP films.

**Filter Elution Technique.** DNA DSBs in intact cells were determined by the filter elution method (32, 33). Briefly, Kc cells were labeled with [3H]thymidine (0.025 μCi/ml; Amersham International, Milan, Italy) for 20 h. Cells were then chased in fresh radioactive-free medium for at least one cell cycle (18–20 h) before drug treatments (30 min at 25°C). Untreated cells were irradiated on ice with γ-rays and kept at 0°C until lysis on the filter. Control and drug-treated cells (5 x 10^6/sample) were layered on polycarbonate membranes of 25-mm diameter and 2-μm pore size (Nucleopore, Pleasanton, CA) and lyzed with 2% SDS, 0.1 M glycine, 25 mM EDTA (pH 9.6), and 0.5 mg/ml proteinase K (Merck, Darmstadt, Germany). DNA on the filter was eluted with 1% SDS, 20 mM EDTA (free-acid form), and tetrapropylammonium hydroxide (Eastman Kodak, Rochester, NY), pH 9.6, for 15 h. A calibration curve was derived from elution values of untreated control cells. 2000-rad, 5000-rad, and 8000-rad irradiated cells. DNA DSBs in drug-treated cells were then calculated as described (32) and expressed in rad-equivalents.

**RESULTS**

**Cleavage of Satellite III DNA Is Stimulated by VM-26 Only.** Satellite III DNA (1.688 g/cm^3) is a highly repetitive heterochromatologic DNA (about 5% of the whole genome) of the centromeric region of D. melanogaster X chromosomes. The repeat unit is a SAR-like, AT-rich sequence of 359 bp (30). Two nucleosomes are accommodated by each repeat, and a strong VM-26-stimulated topoisomerase II cleavage site has been mapped to one of the two nucleosomal linkers (19).

Exponentially growing Kc cells were exposed to VM-26, dh-EPI, mAMSA, or amonafide for 30 min at the indicated concentrations, and DNA cleavage stimulation was assayed by Southern blotting. Since topoisomerase II cleaves this satellite once per repeat, hybridization of DNA extracted from VM-26-treated Kc cells to a satellite III probe results in a DNA ladder of 359-bp unit length (Ref. 19; Fig. 1A). In striking contrast to VM-26, dh-EPI, amonafide, and mAMSA did not stimulate detectable cleavage products in this genomic locus, either in

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**Fig. 1. Topoisomerase II DNA cleavage in the centromeric satellite III DNA in vivo (A) and in vitro (B). A. Kc cells were treated with 25 μm distamycin for 30 min and then incubated with or without topoisomerase II inhibitors for an additional 30 min at 25°C. Lane C, no inhibitor; Lane V, 50 μM VM-26; Lane D, 10 μM dh-EPI; Lane A, 25 μM mAMSA; Lane N, 50 μM amonafide. DNA samples were electrophoresed on a 1.2% agarose gel without prior restriction enzyme digestion and hybridized to a cloned satellite III repeat. B. A cloned 5744-bp DNA fragment containing 16 repeats of the satellite III DNA was uniquely 5’-end-labeled and then incubated with 10 units of Drosophila topoisomerase II without or with drugs. Lane C, no inhibitor; Lane T, topoisomerase II alone; Lane V, 50 μM of VM-26; Lane D, 10 μM of dh-EPI. * a contaminating fragment.
the presence or absence of distamycin (Fig. 1A and data not shown). The addition of distamycin (25 \mu M) to Kc cells has been shown to markedly increase VM-26-stimulated cleavage levels in SAR regions (19), probably by displacing H1 histone from chromatin, thus increasing DNA accessibility to topoisomerase II (34).

Cleavage intensity patterns were drug specific in a cloned satellite III fragment of 16 repeats in the presence of purified Drosophila topoisomerase II (Fig. 1B). Interestingly, VM-26 stimulated a very strong cleavage site once per repeat that appears to be the same as that observed in vivo. The dh-EPI-stimulated cleavage intensity pattern was markedly distinct from that of VM-26, although levels of cleavage stimulation were lower than those of VM-26 (Fig. 1B). The present findings strongly indicate that the in vivo VM-26 site is also the target for VM-26 stimulation in vitro. Therefore, although satellite III repeats were accessible to topoisomerase II in the chromatin of living cells as shown by VM-26-stimulated cleavage, the other studied drugs were unable to trap the covalent DNA-enzyme complex in the heterochromatic satellite III DNA.

Drug-specific Patterns of Topoisomerase II DNA Cleavage in the Drosophila Histone Repeat. To determine whether differences might be observed in another locus, we extended our analysis to the histone gene cluster, a transcriptionally active region whose chromatin structure has been well characterized (35). The major histone repeat spans a 5-kb DNA region that contains a SAR localized in the intergenic spacer between the H1 and H3 genes (see map in Fig. 2) and is repeated about 110 times in the Drosophila genome (35).

DNA samples from the same experiments of Fig. 1A were digested with HindIII and analyzed by Southern blotting using the indirect end-labeling technique with probes from the 5' or 3' portions of the H1 gene (HP and HB probes, respectively, in Fig. 3). Drug-stimulated cleavage sites were numbered according to the convention used previously for VM-26-stimulated sites (19). Similarly to VM-26, dh-EPI stimulated DNA cleavage at several sites in the Drosophila histone repeat (Fig. 3); however, cleavage intensity patterns were clearly different between the two compounds. A striking cleavage of dh-EPI at site 10 was mapped at about 100 bp from the ATG codon of the H2A gene (Fig. 3, middle panel). VM-26 could stimulate cleavage in this region only after heat shock of Kc cells (Ref. 19 and see below). Site 10 is located in one of the three DNase I-hypersensitive regions that were previously mapped in the promoter regions between the H2A and H2B genes, the H3 and H4 genes, and at the 5' end of the H1 gene (35). We detected no cleavage stimulation by dh-EPI at the 5' end of the H1 gene and only weak stimulation between the H3 and H4 genes (sites \( a_3 \) and \( b_3 \) in Fig. 3). Weaker sites stimulated by dh-EPI were observed in the H4-H2A intergenic region (Fig. 3, site 9). In the H1-H3 spacer, dh-EPI sites were located within the SAR and at the 3' end of the H3 gene (Figs. 2 and 3). The H1-H3 spacer contains a number of phased nucleosomes (35), and several dh-EPI sites were localized in nucleosomal DNA linkers (Fig. 2), in agreement with published data (19, 36). Nevertheless, dh-EPI-stimulated sites \( a_3 \) and \( a_6 \) mapped in nucleosomal DNA (Fig. 2). DNA cleavage levels were measured at each site with a

![Fig. 2. Relative levels of in vivo topoisomerase II DNA stimulated by VM-26 or dh-EPI cleavage in the histone gene cluster. The histone gene cluster is represented as a 5-kb HindIII DNA fragment: thin lines, noncoding regions; thick horizontal arrows, coding sequences and relative direction of transcription; O between the H1 and H3 genes represent phased nucleosomes; a SAR is indicated by a line below the nucleosomes. In vivo topoisomerase II DNA cleavage sites, determined by experiments similar to those shown in Fig. 3, are indicated by arrowheads and columns above (VM-26) and below (dh-EPI) the histone repeat. The percentage of cleaved DNA at each site relative to total cleavage in the repeat is reported on the x-axis; the heights of columns correspond to the cleavage level at each site. Radioactive determinations were carried out with a Molecular Dynamics phosphorimager 425 model.](cancerres.aacrjournals.org)
phosphorimager and were expressed as percentages of cleaved DNA at that site over the total cleaved DNA in the histone repeat (Fig. 2). VM-26 stimulated 60–65% of DNA cleavage at sites 5 and 9, whereas 50–55% of dh-EPI-stimulated cleavage occurred at site 10 (Fig. 2).

Site specificity of drug-stimulated DNA cleavage was not restricted to VM-26 and dh-EPI. Amonafide and mAMSA also stimulated topoisomerase II DNA cleavage in the histone repeat, although to a lower extent than the other two drugs (Fig. 3, right panel). In this case, strong cleavage stimulation was detected in the intergenic region between the H4 and H2A genes at sites 9a and 9b (Fig. 3). Cleavage stimulated by amonafide and mAMSA in the histone SAR region was low, even in the presence of 25 μM distamycin (data not shown), which enhanced VM-26-stimulated cleavage in SAR linker sites (19).

In summary, all four drugs yielded specific cleavage intensity patterns in the Drosophila histone gene repeat. Together with the results shown above for the satellite III DNA, the present data demonstrate that topoisomerase II inhibitors maintain a high degree of site selectivity in the chromatin of Kc cells.

**Drug-specific Topoisomerase II DNA Cleavage in the H2A Histone Gene Promoter.** We next analyzed at a nucleotide level the sites of cleavage by dh-EPI and VM-26 in the intergenic H2A-H2B region, where the major cleavage site stimulated by dh-EPI was mapped (Figs. 2 and 3). Sequencing of topoisomerase II cleavage sites was carried out by primer extension techniques with thermostable DNA polymerases (31).

To map a break site correctly, it must be noted that thermostable DNA polymerases have been shown to add an extra, template-independent nucleotide at 3' ends of extended DNA fragments (28, 37, 38). Such an activity should be counteracted by the proofreading activity of DNA polymerases (38). Thus, to test DNA polymerases under our conditions, we sequenced the *Hinfl* restriction site and the VM-26-stimulated topoisomerase II DNA cleavage site in satellite III DNA using DNA polymerases without (Taq) or with (Ultma) 3' to 5' proofreading activity (Fig. 4). In the case of *Hinfl*-digested DNA, Taq polymerase added an extra nucleotide in 93% of the extended molecules, whereas Ultma polymerase did so only in 25% of molecules, as determined by phosphorimager analyses of gels (data not shown). Consistently, at the VM-26-stimulated cleavage site, a one-nucleotide difference was observed between DNA fragments extended by Taq and Ultma polymerases (Fig. 4). These findings have to be taken into account when interpreting data based on primer extension techniques; using Taq polymerase, a strand break would be assigned to the phosphodiester bond immediately 5' to the actual cleaved bond, resulting in an apparent stagger of 6 bases instead of 4 for topoisomerase II DNA cleavage, as reported previously (19).

The strong dh-EPI-stimulated cleavage site 10 was then sequenced using Taq DNA polymerase since it was the most efficient enzyme (Fig. 5). Two bands could be observed for the extension products of the upper strand that comigrated with an A and a T corresponding to positions —81 and —80 relative to the first ATG codon of the H2A gene (Fig. 5, A and B). By analogy to the results described above, the DNA cut was assigned to the 5' side of the T at —80 on the upper strand (Fig. 5C). Only one band was detected in the case of extension of the lower strand (Fig. 5A), corresponding to the T at —76; thus, cleavage in the upper strand was mapped to the 5' side of the T at —77 (Fig. 5C). dh-EPI-stimulated DNA cleavage was also investigated in a cloned histone DNA fragment with purified topoisomerase II, and the results showed that the in vivo site 10 was also a target for dh-EPI stimulation in vitro (data not shown). Interestingly, a close examination of the DNA sequence showed that the "dh-EPI" site mapped exactly to the TATA box of the H2A gene promoter (Fig. 5), suggesting that topoisomerase II might be bound to this sequence motif in vivo.

Since VM-26 strongly stimulated cleavage in the H2A-H2B spacer only in heat-shocked cells (19), we compared VM-26 and dh-EPI sites following heat shock of the cells. Kc cells were cultured at either 25°C or 37°C for 30 min and then exposed to VM-26 or dh-EPI for 30 min. The cleavage site stimulated by dh-EPI was not significantly influenced by heat shock (compare d lanes at 25°C and 37°C in Fig. 5B). In contrast, VM-26-stimulated DNA cleavage only after heat shock (at 37°C) but at a distinct site that was mapped to the A at the —109 position, exactly 29-bp apart from the dh-EPI site (compare v lanes at 25°C and 37°C in Fig. 5B). These results thus demonstrate that the two drugs stimulate site-selective cleavage also at nucleotide levels.

**Base Sequences at the in Vivo Sites of Drug-stimulated Topoisomerase II DNA Cleavage.** The above sequence data provide evidence that topoisomerase II double-stranded DNA cleavage occurs with a 4-base stagger also in vivo, in contrast to a previous report, which did not take into account quantitative nontemplated nucleotide addition by Taq polymerase (19). This prompted us to re-evaluate the base sequences at 10 sites of VM-26-stimulated DNA cleavage (Table 1), including those of nine sites published previously (19), as well as the new site in the H2A gene promoter region (Fig. 5C). A cytotoxic appears to be preferred at the —1 position (Table 1), in agreement with in vitro sequence preferences of VM-26 (23). The dinucleotide 5’-TA-3' was instead present at both the 3' termini of the major in vivo dh-EPI-stimulated site of the histone repeat (Fig. 5C). Again, this observation was in complete agreement with the in vitro sequence specificity of doxorubicin and dh-EPI (24, 25). These findings, together with the cleavage analysis after heat shock, showed that topoi-
Fig. 5. Sequencing of in vivo topoisomerase II DNA cleavage sites stimulated by dh-EPI and VM-26 in the H2A-H2B intergenic region. A. DNAs were from control cells (Lane c) or from cells treated with 10 μM dh-EPI (Lane d). Extension was performed with Taq polymerase from primer D10-1 (left panel, upper strand) or primer D10-2 (right panel, lower strand) labeled at 5' ends. Sequencing ladders were obtained from extension with the same primer and the appropriate dideoxynucleotide. *, a pause site; arrowheads, extended cleavage products. B, cleavage sites stimulated by dh-EPI and VM-26 under normal or heat-shocked conditions. DNAs were from control cells (Lane c) and from cells treated with 10 μM dh-EPI (Lane d) or 10 μM VM-26 (Lane v) at the indicated temperatures. Sequencing was performed with Taq polymerase from primer D10-1. Arrowheads, the extended cleavage products. C, base sequence of the region analyzed. Cleavage sites stimulated by drugs are shown. Numbers indicate the nucleotides covalently linked to topoisomerase II subunits at the cleavage site. Bases are numbered from the start codon of the H2A gene. Arrowheads, the observed cleaved bonds. The “VM-26” cleavage in the upper strand (−106 nucleotide) was assigned based on the cleaved bond observed in the lower strand and a 4-bp stagger. A line indicates the TATA box of the H2A gene promoter.

Heterogeneity of Cleavage Levels at Different Genomic Loci.

The observation that dh-EPI, mAMSA, and amonafide in vivo did not stimulate cleavage in the satellite III DNA, whereas VM-26 did (Fig. 1A), suggested that drug-stimulated cleavage levels might be markedly heterogeneous in different genomic loci. However, since the drug potency in stimulating DNA cleavage might be different among the studied inhibitors, we therefore measured the DNA breakage levels in the whole genome of Kc cells with the filter elution technique (32). The results showed that similar concentrations of VM-26 and dh-EPI produced nearly the same amounts of DNA DSBs (Fig. 6A). At the same time, overall cleavage levels in the histone gene locus stimulated by VM-26 and dh-EPI displayed different dose-response curves; the doxorubicin analogue was able to stimulate 2-3-fold higher levels of cleavage than VM-26, as determined by a phosphorimager analysis of Southern blots (Fig. 6B). Thus, unrelated inhibitors may promote about the same amount of DNA breakage in the genome as a whole.
yet the extent of DNA cleavage can substantially diverge in specific chromatin regions.

Heterogeneous Reversibility of Topoisomerase II DNA Cleavage in the Drosophila Genome. We then asked whether DNA cleavage reversion was also heterogeneous among genomic loci or structurally unrelated drugs. Cleavage reversibility was first investigated in the histone gene and satellite III repeats after cell treatments with VM-26 (Fig. 7A). These two regions displayed different behaviors; in the satellite III DNA, cleavage was completely reversed within 30 min from drug removal, whereas in the histone gene region, some cleavage still persisted at 30 min, and overall cleavage was as low as 10% of initial levels after 1 or 2 h (Fig. 7A). Reversion kinetics of VM-26-stimulated DNA cleavage were instead similar in cloned histone and satellite III DNA repeats with purified Drosophila topoisomerase II (data not shown). It is interesting to observe that not all cleavage sites in the histone gene cluster reversed with the same rate (Fig. 7A). Only 40-50% of DNA cleavage at sites 4 and 5 was reversed after 30 min, whereas cleavage reversal was almost complete at the other sites. We then analyzed the reversibility of DNA cleavage stimulated by dh-EPI in the histone gene repeat (Fig. 7B). Forty % of DNA cleavage persisted for up to 2 h after removal of dh-EPI at sites 6a and 10, whereas some dh-EPI-stimulated sites reversed completely after only 30 min (Fig. 7B, site 7b, •). Interestingly, the three closely positioned sites 6a, 6b, and 7b (Figs. 2 and 3) reversed with distinct rates (Fig. 7B). Overall, DNA cleavage stimulated by dh-EPI appeared to persist for longer periods of time than that stimulated by VM-26 (Fig. 7). No relation could be observed between in vivo cleavage levels and reversibility. In brief, DNA cleavage tended to be reversible at the majority of sites following drug removal but with different kinetics that were clearly dependent upon the drug, the genomic locus, and the particular site studied.

DISCUSSION

Our results demonstrate a high degree of heterogeneity in the genomic localization and reversibility of drug-stimulated topoisomerase II DNA cleavage in the chromatin of living cells. Among dh-EPI, VM-26, mAMSA, and amonafide, only VM-26 could stimulate cleavage in the centromeric satellite III DNA, whereas all four drugs did at the histone gene repeat. In this locus, however, cleavage intensity patterns markedly differed among the four drugs. Using mAMSA and VM-26, some differences in cleavage patterns have been reported previously in viral (20), episomal (21), and cellular chromatin (22).

Moreover, our study now shows that, in the case of VM-26 and dh-EPI, base sequences at specific drug-stimulated sites conform to the known drug sequence specificities established in vitro with purified topoisomerase II (9, 23, 24).

Thus, the DNA sequence specificity of the studied topoisomerase II inhibitors plays an important role in determining the sites of cleavage in the chromatin of living cells. This is best seen in the case of dh-EPI and VM-26, which stimulate cleavage at a different subset of sites in the histone gene repeat (Figs. 2 and 3) and exert all-or-none effects in the satellite III DNA (Fig. 1). The observed high degree of in vivo cleavage site selectivity is likely due to a combination of several factors, including: (a) the sequence specificity imparted by the inhibitor; (b) the local chromatin structure; (c) the loose sequence selectivity of the enzyme itself; and (d) possibly other protein factors. A likely hypothesis is that histones and other nuclear proteins modulate DNA accessibility, and drugs then select among the accessible sites where topoisomerase II can bind to and cleave DNA. For instance, since dh-EPI requires a relatively AT-rich sequence at the cleavage site, which appear to be GC-rich instead (Table 1). Sequencing of additional in vivo drug-enhanced cleavage sites will establish this point more definitively.

The identification of genomic sites of topoisomerase II activity is under intense investigation in several laboratories (14-19). Although sites of in vivo drug-enhanced cleavage and sites of enzyme activity and/or DNA binding are certainly related, nevertheless our data...
clearly demonstrate that cleavage site analysis can miss certain sites of enzyme activity, and this may lead to inaccurate conclusions with regard to topoisomerase binding sites in nuclear chromatin. The common use of VM-26 to detect sites of enzyme activity is based on the following key assumptions: (a) the inhibitor increases cleavage uniformly at physiological sites; and (b) sites of enzyme binding to DNA are always sites of DNA cleavage (14–19). However, the present results demonstrate that this is not always the case. In in vitro investigations, VM-26 (or any other inhibitor) did not uniformly stimulate cleavage sites in any given DNA fragment, even if, to our knowledge, this drug was the least sequence-specific inhibitor (9, 23).

Since in vivo cleavage sites are not detectable without drugs, cleavage intensity in the chromatin of intact cells is likely determined in large part by the particular drug used. Thus, it is not possible to derive even semiquantitative information on chromatin sites of enzyme binding/activity from cleavage patterns stimulated by any single inhibitor. In the extreme case, an enzyme binding site can be missed because the drug used cannot stabilize the cleavable complex at that particular site (see the opposite effects of VM-26 and dh-EPI in satellite III repeats and at site 10 of the histone gene locus in Figs. 1, 2, and 5). Moreover, it is possible that important functions of DNA topoisomerase II might be independent from enzyme catalysis (39, 40).

The high degree of heterogeneity we observed in the localization of topoisomerase II cleavage sites stimulated by unrelated drugs is accompanied by an equally heterogeneous reversibility of cleavage, which varies as a function of the drug, the genomic locus, and the particular cleavage site studied. In contrast, DNA cleavage reversibility in cloned DNA fragments in the presence of purified Drosophila topoisomerase II was similar between the histone and satellite III repeats. The heterogeneity in the reversibility of dh-EPI- and VM-26-enhanced cleavage may be due to different drug interactions with DNA or different cellular pharmacokinetics (33, 41). However, it is more difficult to explain the heterogeneity among sites stimulated by the same drug. It is possible that either particular aspects of chromatin structure or ongoing metabolic processes may influence the persistence of DNA cuts. In this respect, it is interesting to note that the reversibility of topoisomerase II DNA cleavage was much faster in the silent satellite III repeats than in the transcriptionally active histone gene repeats (Fig. 6). It remains to be established whether a relation between transcription and lack of complete cleavage reversion is a general event.

An unexpected finding of this work is that the striking topoisomerase II DNA cleavage stimulated by dh-EPI at site 10 of the histone cluster coincides precisely with the TATA box of the H2A gene promoter (42). This indicates that topoisomerase II may bind to this sequence motif in the chromatin of living cells, suggesting that TATA boxes of promoter regions might be in vivo sites of topoisomerase II activity. Although the functional significance of this result remains to be fully established, one possibility is that topoisomerase II binding to the TATA box may be related to the proposed activity of this enzyme as a general repressor of transcription (39). However, we do not know at present whether all of the histone gene repeats are equally cleaved by the enzyme or if cleavage occurs only in a subpopulation of the repeats; a functional assay will then be needed to determine whether topoisomerase II may play a direct role in gene expression.

The present findings raise the question as to whether the extensive genomic heterogeneity in DNA cleavage localization, extent, and reversibility has any role in the biological activity of topoisomerase II inhibitors. Although drug cytotoxic potency correlates well with the extent of drug-stimulated DSBs when considering closely related derivatives, this relation is lost when topoisomerase II inhibitors of different chemical classes are compared (7, 8, 41). Therefore, we suggest that the genomic site and reversion kinetics of in vivo DNA cleavage might be important factors influencing drug cytotoxic potency. Interestingly, VM-26 has been shown to stimulate higher cleavage levels in regions surrounding the c-myca and β-globin genes than in the α-satellite and L1 repeat DNAs in human cells, indicating that certain DNA regions may be preferentially damaged by VM-26 (43).

In conclusion, the present findings demonstrate that the studied drugs maintain, in the chromatin of living Kc cells, a high degree of sequence specificity that may correspond to that established in vitro (9, 27). In addition, the present data provide strong evidence that drug-stimulated topoisomerase II DNA cleavage is highly heterogeneous in the chromatin of intact cells, in terms of the extent (at specific genomic loci), localization, and reversibility. The present information should be taken into account in investigations on the genomic sites of enzyme activity, the chromatin structure, and the antitumor activity of topoisomerase II inhibitors.

ACKNOWLEDGMENTS

We thank M. Binasci for help in the sequencing experiments and L. Poljak and M. Binasci for critical comments on the manuscript.

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Reversibility

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