ABSTRACT

Amsacrine and demethylepipodophyllotoxins (etoposide and teniposide) are potent topoisomerase II inhibitors which have optimum activity in different cancers. To investigate whether these differences are due to different activity on cellular oncogenes, drug-induced topoisomerase II cleavage sites were mapped and sequenced in the human c-myc protooncogene. In the presence of purified murine L1210 topoisomerase II, amsacrine induces prominent cleavage in the P2 promoter (site 2499/2502). Footprinting experiments indicate that topoisomerase II binds to the entire promoter region (~20 base pairs on the sides of the P2 site). In the case of teniposide or etoposide, cleavage is more diffuse and markedly less at the P2 site. Mapping of cleavage sites in human small cell lung carcinoma cells (NCI-N417) also shows that cleavage in the P2 promoter region is induced preferentially by amsacrine but not by demethylepipodophyllotoxins. Thus, selective gene damage among topoisomerase II inhibitors may contribute to differential anticancer activity.

INTRODUCTION

The c-myc protooncogene appears to play a central role in the control of cell proliferation and differentiation (1–3). c-myc expression is controlled by several mechanisms, including change in transcription initiation and elongation, RNA turnover, and translation (3–6). The deregulation of c-myc expression by amplification, chromosomal translocation, retroviral insertion, or point mutation causes transformation and immortalization of normal cells (1, 3, 7, 8). The human c-myc protooncogene contains two promoters (P1 and P2) separated by about 165 base pairs and located near the 5′ end of the first exon (Fig. 1) (9). The transcription of c-myc is regulated by a composite of positive and negative elements located both upstream and downstream from these promoters (5, 10–17). However, many of the cellular factors involved in the c-myc expression have not been characterized.

Eukaryotic DNA topoisomerase II is the cellular target of a variety of anticancer drugs including m-AMSA2 and the epipodophyllotoxin derivatives, VP-16 and VM-26. These drugs poison topoisomerase II by stabilizing enzyme-DNA cleavage complexes (18, 19). Amsacrine and epipodophyllotoxins induce different cleavage patterns (20, 21), while VM-26 and VP-16 induce similar patterns; VM-26 is more potent than VP-16 (22–24). Previous studies have indicated that the c-myc oncogene may be 20-fold more sensitive to amsacrine-induced topoisomerase II inhibition than the overall genome, suggesting that cleavage complexes induced in the c-myc oncogene may be important for antitumor activity (25).

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3 To whom requests for reprints should be addressed, at Bldg. 37, Rm. 5C27, National Institutes of Health, Bethesda, MD 20892.
4 The abbreviations used are: m-AMSA, amsacrine; 4′-(9-acridinylamino) -methanesulfon-m-anisidide; VP-16, etoposide; VM-26, teniposide; SDS, sodium dodecyl sulfate; 1 × TBE, 89 mM Tris-89 mM boric acid-2 mM EDTA, pH 8.
5 m-AMSA was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. VM-26 and VP-16 were obtained from Bristol-Myers Co. (Wallingford, CT). Drug stock solutions were made in dimethyl sulfoxide at 10 mM immediately before use. Further dilutions were made in deionized water. Doxorubicin and 4-demethoxydaunorubicin (Idarubicin) were obtained from Farmitalia Carlo Erba (Milan, Italy). Stock solutions were made in deionized water at 0.2 mM and kept frozen at −20°C.
6 Preparation of End-labeled DNA Fragments. DNA fragments were 5′ end labeled as described previously (28, 29). Briefly, pJB327 DNA was first linearized with one of the restriction enzymes shown on Fig. 1, and then the 5′-DNA termini were dephosphorylated with calf alkaline phosphatase and labeled with [γ-32P]ATP using T4 polynucleotide kinase. Next, labeled DNA was cleaved with a second restriction enzyme in order to generate fragments of different lengths, which were separated by agarose gel electrophoresis and isolated by electroelution.
7 DNA sequence analysis, samples were precipitated with ethanol
and resuspended in 2.5 μl loading buffer (80% formamide-10 mM NaOH-1 mM EDTA-0.1% xylene cyanol-0.1% bromophenol blue). Samples were heated to 90°C and immediately loaded into DNA sequencing gels [8% polyacrylamide-acrylamide:bis (29:1)] containing 7 M urea in 1× TBE buffer. Electrophoresis was at 1500 V (60 W) for 2–3 h.

The gels were dried on 3MM paper sheets and autoradiographed with Kodak XAR-5 film. In some instances, dried gels were used to quantify DNA cleavage using a Betascope 603 blot analyzer (Betagen Inc.). Computer analysis was performed using the Excell spread-sheet program.

DNase I Digestion. The reaction buffer was the same as that used in the topoisomerase II reactions. For footprinting experiments, DNase I was added 15 min after the beginning of the topoisomerase II reactions. DNase I digestion was allowed to proceed for the indicated times, and reactions were terminated by adding 25 mM EDTA-1% SDS-0.1 mg/ml proteinase K (final concentrations). DNA was precipitated, and samples were processed for DNA sequencing as described in the above section.

Analysis of in Vivo Topoisomerase II Cleavage Site. Human small cell lung carcinoma NCI-N417 cells, which contain 40-50 copies of the c-myc protooncogene, were grown as floating aggregates in RPMI 1640 medium containing 10% fetal calf serum and antibiotics (31). About 100 cells in exponential growth phase were exposed to drugs for 1 h at 37°C in fresh culture medium. Cells were washed with 50 mM Tris-HCl, pH 7.5-1 mM EDTA and immediately lysed with 1% SDS-50 mM Tris-HCl, pH 7.5-25 mM EDTA. Proteinase K was added to a final concentration of 0.5 mg/ml for 12 h at 50°C. Lysates were then treated with phenol, phenol-chloroform, and chloroform, and DNA was precipitated with ethanol, dried, and resuspended in 10 mM Tris-HCl, pH 7.5-0.5 mM EDTA at a concentration of 2 mg/ml. DNA samples (10 μg) were digested with BglII restriction endonuclease and electrophoresed in 1.2% agarose gel. DNA fragments were transferred onto a Hybond N membrane (Amersham). Hybridization and washing were performed in stringent conditions as described previously (25). The probe used was the 414-base pair PstI fragment encompassing the greatest part of the second exon (see Fig. 1). The probe was labeled with [α-32P]dCTP and [α-32P]dATP (3000 Ci/mmol; Amersham) to a specific activity of 3–7×10^6 dpm/μg using the nick translation technique (25). Hybrids were revealed by autoradiography on Amersham hyperfilms MP.

Genomic Mapping of DNA Breaks. The genomic localization of the in vitro and in vivo drug-induced topoisomerase II-mediated DNA breaks was determined as described previously (28–30, 32). Briefly, autoradiography films were scanned with a DU-8B Beckman spectrophotometer set at 555 nm, and the position and absorbance values were used to transmit to a computer. 32P-end-labeled DNA fragments were run as markers and were used to determine the regression lines of the logarithm of the fragment size (in base pair) versus the migration distance of each fragment. Regression coefficients were consistently near 0.99. Each autoradiography lane was analyzed by using the same reference line, and the size of each DNA fragment induced by topoisomerase II was computed. The reproducibility of fragment size determinations in different gels was usually within 50 base pairs. A final correction was made to convert DNA fragment size to genomic position by taking the HindIII restriction site as position 1 (Fig. 1; 33, 34).

RESULTS

m-AMSA Induces a Major DNA Double-Strand Cleavage Site in the P2 Promoter of c-myc DNA. DNA double-strand breaks induced by purified murine leukemia topoisomerase II in the presence or in the absence of m-AMSA were mapped in the c-myc plasmid pJB327 (Figs. 1 and 2). Fig. 2, A and B, shows that m-AMSA induces cleavage in several discrete regions. The cleavage pattern induced by m-AMSA differs from that induced by topoisomerase II alone, implying that m-AMSA attacks certain topoisomerase II sites (20). Densitometer scanning of the autoradiographies was used to determine the position of the cleavage sites (20, 28–30, 32). A composite picture of the m-AMSA-induced cleavage sites was obtained by computer analysis to normalize the intensity distribution relative to a linear genomic position scale (Fig. 2C). Cleavage is most intense around position 2500, in the vicinity of the P2 promoter. A series of cleavage sites is also observed in the first intron, with a strong cleavage site around position 3200.

Differential Induction of DNA Double-Strand Breaks by m-AMSA and VM-26 in Purified DNA. Fig. 3 shows that VM-26 and m-AMSA induce very different cleavage patterns. Instead of inducing the P2 site as the case of m-AMSA (A), VM-26 (lane V) or VP-16 (cleavage pattern similar to VM-26, data not shown) induce a large number of breaks that result in a smear of fragments smaller than full-size DNA. This was also the case for topoisomerase II in the absence of drug (lane E), although cleavage was globally less intense than in the presence of drugs. Therefore, m-AMSA and VM-26 produce very different cleavage patterns in the 1500 base pairs corresponding to the 5' flank of the c-myc protooncogene.

Sequencing of the m-AMSA Cleavage Sites in the P2 Promoter. In order to sequence topoisomerase II cleavage sites in the P2 promoter, the DNA was labeled at the Xhol site of the coding strand (Fig. 1). Fig. 4 shows that the major m-AMSA cleavage site is at position 2499 of the DNA-coding strand, 10 nucleotides downstream from the promoter start (lane A). A weaker site is visible at position 2486, three bases upstream from the promoter start. Another cluster of sites is found immediately upstream from the TATAA box sequence and additional sites at specific locations (Fig. 4, lane A). Additional experiments using DNA labeled on the complementary strand at the RsrII restriction site (see Fig. 1) showed that the most intense cleavage site is at position 2502 on the transcribed DNA strand.

Fig. 1. Schematic representation of the 5' region of the human c-myc protooncogene. Open boxes, exons 1 and 2; P1 and P2, two promoters. The plasmid (pJB327) used for DNA cleavage reactions with purified DNA topoisomerase II is shown. Filled boxes, polylinker used to clone the XhoI-XbaI c-myc fragment into pBR327 (dashed lines). Restriction sites are indicated and numbers correspond to their genomic positions (HindIII restriction site is 1).* Solid line (top right), probe used for in vivo cleavage reactions.

Fig. 2. Position and absorbance values were used to determine the regression lines of the logarithm of the fragment size (in base pair) versus the migration distance of each fragment. Regression coefficients were consistently near 0.99. Each autoradiography lane was analyzed by using the same reference line, and the size of each DNA fragment induced by topoisomerase II was computed. The reproducibility of fragment size determinations in different gels was usually within 50 base pairs. A final correction was made to convert DNA fragment size to genomic position by taking the HindIII restriction site as position 1 (Fig. 1; 33, 34).
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Fig. 2. DNA double-strand breaks induced by DNA topoisomerase II in the presence and absence of m-AMSA. EcoRl/Xbal DNA fragments (see Fig. 1) that had been uniquely 32P-end labeled at one of their 5' termini (A, EcoRl; B, Xbal) were reacted as indicated to the left for 30 min at 37°C. Reactions were stopped by adding SDS and proteinase K (1% and 0.1 mg/ml final concentrations, respectively). Samples were run into 1% agarose gels in TBE buffer, and autoradiography was performed. Marker sizes are, from left to right, 1857, 1060, 929, 383, and 121 base pairs. Autoradiographies were scanned and genomic positions of the breaks computed (numbers in A and B). Actual cleavage sites are usually within 50 nucleotides of the computed values. C, composite graph of A and B in which intensity distribution is plotted against a linear genomic position scale.

strand (data not shown). The 4-base pair staggering of sites 2499 on the coding strand and 2502 on the transcribed strand indicates that these break sites correspond to the two cleavage sites of the DNA double-strand break that is detected under nondenaturing conditions around position 2500 (see Fig. 2). Thus, the major m-AMSA double-strand break site in the P2 promoter (Figs. 2 and 3) corresponds to a unique double-strand break at positions 2499 and 2502 on the coding and the transcribed strands, respectively.

Doxorubicin (lane D) and its more potent analogue, 4-demethoxydaunorubicin (Idarubicin, lane I) (32) induce prominent cleavage at the 2486 site and no cleavage at the 2499 position. These two drugs also stimulate cleavage near the TATAA box. In agreement with the results shown in Fig. 3, VM-26 (lane V) induces many more sites than the other drugs. Topoisomerase II induces several weak cleavage sites (lane E) that coincide with those induced in the presence of drugs. The 2499 site is the most intense site in the case of topoisomerase II in the absence of drug, indicating that the enzyme binds at or near the promoter and that drug-induced cleavage results from the stabilization of enzyme cleavage complexes.

Relative Intensity of the m-AMSA-induced Cleavage Sites in the P2 Promoter Region. The autoradiography shown in Fig. 4 had been overexposed in order to show all the cleavage sites induced in the presence and absence of drugs. In order to quantify cleavage at the m-AMSA-induced sites, a dried gel similar to that used to generate the autoradiography shown in Fig. 4 was processed by betascope analyzer. Such an analysis shows that cleavage intensity is markedly more at site 2499 (at least 10-fold) than at any other site within the 140 nucleotides analyzed (Fig. 5).

DNase I Footprinting of the 2499 m-AMSA Cleavage Site in the P2 Promoter. Fig. 6 shows that in the absence of topoisomerase II, DNase I produces a large number of cleavage sites of different intensities (lanes 1 and 2). Analysis of the protection conferred by topoisomerase II in the presence of m-AMSA, upstream from the 2499 cleavage site shows a 20-nucleotide protected region (lanes 3 and 4). This result is in agreement with an independent determination in another DNA fragment using Drosophila topoisomerase II (35). If we assume that similar protection would be seen on the other strand, then the
Fig. 3. Comparison of topoisomerase II-mediated DNA double-strand breaks induced in the absence of drug (lane E, enzyme alone) or in the presence of VM-26 (lane V) or m-AMSA (lane A). The EcoRI/XbaI fragment uniquely end labeled at the EcoRI site was used (lane C, control). Reactions were performed as described in Fig. 2. Lane M, molecular size markers (their size in base pairs is indicated at far right). P2, position of the P2 promoter which corresponds to the major m-AMSA-induced double-strand break site.


total binding region of topoisomerase II would be approximately 40 base pairs and would cover most of the P2 promoter.

In Vivo Cleavage Induced by m-AMSA and VP-16 in the c-myc Protooncogene of Human Small Cell Lung Carcinoma NCI-N417 Cells. NCI-N417 cells have 40-50 copies of the c-myc protooncogene/cell (31). In previous studies, the c-myc gene from cells treated with m-AMSA or epipodophyllotoxins has been found to be cleaved at specific positions (25, 36, 37). Most of these in vivo cleavage sites are located in the 5'-noncoding region of the gene and close to DNase I-hypersensitive sites. However, the mapping strategy previously used did not permit the localization of cleavage sites with a sufficient precision in the region encompassing the first exon of c-myc. Here, we used a strategy that enabled us to analyze this region (Figs. 1 and 7). DNA double-strand breaks are measured after cell lysis at neutral pH. When DNA from untreated NCI-N417 cells is digested with BglIII and probed with the 0.4-kilobase PstI fragment of c-myc, a DNA band of 6.5 kilobases is detectable (Fig. 7, left lane) that corresponds to the site predicted by the published sequence data (33, 34). The DNA pattern from drug-treated N417 cells reveals the presence of additional DNA bands of lower molecular weight that are not observed in untreated cells and presumably correspond to topoisomerase II cleavage sites. m-AMSA stimulates the formation of a strong cleavage site in the P2 promoter region in a concentration-dependent manner. By contrast, in the case of VP-16 (second lane from left) and VM-26 (not shown), cleavage is more diffuse and markedly less in the P2 promoter region (Fig. 7).

DISCUSSION

The present study demonstrates that m-AMSA and epipodophyllotoxins (VP-16 and VM-26) produce very different cleavage patterns in the human c-myc protooncogene. Both in cell-free systems and in NCI-N417 cells, m-AMSA selectively inhibits topoisomerase II at the c-myc P2 promoter site, while VM-26 or VP-16, but not m-AMSA, block topoisomerase II in many other regions. Hence, selective gene damage by m-AMSA and demethylipodophyllotoxins may contribute to the differential activity of these drugs in different tumors.

DNA sequence analysis and comparative compilation of a large number of drug-induced cleavage sites in simian virus 40 DNA has provided some insight into the molecular mechanisms responsible for differential topoisomerase II inhibition by different drugs at different sites (21, 38). m-AMSA preferentially enhances topoisomerase II sites that have an adenine at one of the 5' termini of a topoisomerase II double-strand break, while VM-26 or VP-16 generally enhance the sites which have a cytosine at one of the 3' termini of the breaks (21). Preference for one of the bases immediately flanking the drug-induced cleavage site is also found for anthracyclines, in which case adenine is always present at one of the 3' termini of the breaks (21). Therefore, local base sequence selectivity of drugs together with chromatin structure (29) probably contribute to cleavage specificities of m-AMSA and epipodophyllotoxins in the human c-myc protooncogene.

The strong m-AMSA-induced cleavage and binding site of topoisomerase II in the P2 promoter was found both in purified enzyme-DNA systems and in drug-treated cells. This indicates that the enzyme binds to the P2 promoter region in chromatin. At this site, topoisomerase II may act as a negative regulator of transcription by blocking the accessibility of the promoter region. Another negative regulatory factor (myc-binding protein 1, Fig. 4) has been hypothesized to act in this way (16). Alternatively, topoisomerase II binding may facilitate transcrip-
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Fig. 5. Quantitative analysis of m-AMSA-induced cleavage in the P2 promoter region of the c-myc gene. A dried gel similar to that used to generate the autoradiography shown in Fig. 4 was scanned with a betascope analyzer, and the data were computed with the Excell spread-sheet program. Numbers above each peak: cleavage sites indicated in Fig. 4 (sites 2565 and 2610 correspond to the two bands at the right end of lane A in Fig. 4). Positions 2440 and 2680 do not correspond to cleavage sites but to the boundaries of the examined region.

This possibility is attractive because topoisomerase II cleavage sites in the β-globin gene locus coincide with DNase I-hypersensitive sites. During chicken erythrocyte maturation, these two types of sites appear and disappear at the same time.

Fig. 6. Footprinting of the major m-AMSA cleavage site in the P2 promoter. DNA was 32P-end labeled at the Xhol site (same as in Fig. 4). Topoisomerase II reactions were for 30 min at 37°C prior to DNase I addition (0.01 μg/ml for 1, 3, or 10 min). Reactions were stopped by adding simultaneously 25 mM EDTA and 1% SDS (final concentrations). Autoradiography lanes with comparable DNase I activity are as follows: lane 1, DNase I alone, 0.01 μg/ml, 1 min; lane 2, DNase I + topoisomerase II (no drug), 0.01 μg/ml, 10 min; lane 3, DNase I + topoisomerase II + m-AMSA, 0.01 μg/ml, 10 min; lane 4, DNase I + topoisomerase II + m-AMSA, 0.01 μg/ml, 10 min; lane 5, topoisomerase II + m-AMSA (no DNase).

Fig. 7. In vivo cleavage sites induced by m-AMSA and VP-16 in the c-myc protooncogene of human small cell lung carcinoma NCI-N417. Cells were treated for 60 min at 37°C as indicated. The DNA was extracted, electrophoresed, transferred to nylon filter, and hybridized to the second exon probe shown in Fig. 1. The size (in kilobases) of DNA markers is indicated to the far right. The full-size DNA band at the gel origin is 6.5 kilobases.

In conclusion, genetic machinery involved in cell proliferation control is regulated by various DNA-binding proteins which are often deregulated in cancer cells, some of them identified as oncogenes. Their sequence recognition requirements are unique, and it is now accepted that heterogeneity among various types of tumors is associated with different patterns of deregulation of these DNA-binding proteins. The functional significance of the cleavage site differences among topoisomerase II upstream from transcribed genes (40). In addition, topoisomerase II preferentially forms cleavage complexes in the nuclear matrix-associated region of simian virus 40 (30) and has been shown to be a major component of the nuclear matrix (41) where actively transcribing genes are concentrated. In the case of the human c-myc promoter, its binding to topoisomerase II may facilitate its association with transcription factors. Enzyme activity at this site may play a critical role in relaxing the negative superhelical tension that is associated with transcription (42–45). Further experiments will be necessary to elucidate whether topoisomerase II forms a multiprotein complex at promoter sites and the exact role of the enzyme in transcription.
inhibitors at the gene level may contribute to differential anticancer activity.

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