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CHARACTERIZATION OF THE TOPOISOMERASE II-INDUCED CLEAVAGE SITES IN THE c-*myc* PROTO-ONCOGENE

IN VITRO STIMULATION BY THE ANTITUMORAL INTERCALATING DRUG mAMSA

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Abstract—In an attempt to get an insight into the activity of mAMSA (a DNA topoisomerase II-mediated drug) on the human proto-oncogene c-*myc*, an *in vitro* system consisting of purified calf thymus DNA topoisomerase II and a c-*myc* DNA inserted in λ phage was utilized. The occurrence of discrete bands, detected by hybridization of Southern blots with appropriate c-*myc* probes, indicated the presence of cleavage sites in the sole presence of DNA topoisomerase II. The band intensity increased in the presence of mAMSA, while no significant difference occurred in the cleavage pattern. The location of the cleavage sites along the c-*myc* locus revealed a striking correspondence with that of some DNase hypersensitive sites. These results indicate that DNA topoisomerase II is most certainly implicated in the mAMSA activity and that the drug stimulates the topoisomerase II cleaving activity at specific sites, which may be involved in the biological activity of the drug.

Type II DNA topoisomerases (Topo II)§ are enzymes which catalyse the concerted double-stranded breakage and rejoining of the DNA backbone. Their enzymatic properties have been well characterized and this has led us to investigate their biological functions concerning DNA replication and gene expression (for a general review, see ref. 1). Moreover, the role of Topo II in the process of cell proliferation has recently been emphasized: the level of activity of Topo II has been found to be increased in regenerating hepatocytes [2] and in virus-transformed tumor cells [3]. In the light of recent results [4, 5], an attractive model concerning the strand passing activity of the enzyme has been suggested: during the reaction, enzyme and DNA form an intermediate covalent complex named "cleavable complex", each of the two enzyme subunits being covalently linked to the 5′-phosphoryl end of the DNA strand. Treatment of this complex with protein denaturants results in DNA strand breaks. More recent studies have shown that some antitumor compounds stimulate *in vitro* and *in vivo* DNA cleavage by Topo II [6–8], suggesting that these enzymes may be suitable targets for drugs in rapidly proliferating cells.

Extensive studies have recently shown the role of certain proto-oncogenes in the control of cell proliferation. In the case of c-*myc*, one of the best investigated proto-oncogenes, experiments dealing with quiescent cells have demonstrated that cells leaving Go to re-enter the cycle exhibit a transient increase in c-*myc* mRNA content [9]. Conversely, proliferating HL-60 cells that have stopped dividing and have initiated a differentiation process contain less c-*myc* mRNA [10, 11]. Furthermore, the c-*myc* activation is generally assumed to play a crucial function in the initiation and/or progression of the malignancy [12–14], a higher stability of c-*myc* transcript being the possible basis of proto-oncogene activation [15, 16].

Since c-*myc* expression and Topo II activity seem to be involved in cell proliferation, one may wonder about the possible action of Topo II-mediated drugs on the c-*myc* gene. This has prompted us to study the *in vitro* cleavage activity of purified calf thymus Topo II on a complete human c-*myc* gene in the presence of mAMSA [4′-(9-acridinylamino)-methanesulfone-m-anisidide] [17], a DNA intercalating antitumoral drug.

MATERIALS AND METHODS

Drugs. mAMSA and oAMSA were kindly provided by Dr B. Baguley (Auckland Medical School, New Zealand). The drugs (20 mM) were dissolved in dimethyl sulfoxide and stored at −20°.

Purification of calf thymus Topo II. The purification of Topo II was carried out by a procedure already described for an enzyme of a different origin [3]. The decatenation of trypanosome kinetoplast DNA (kDNA) was used to detect Topo II through the different purification steps, as previously

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§ Abbreviations used: mAMSA, 4′-(9-acridinylamino)-methanesulfone-m-anisidide; oAMSA, 4′-(9-acridinylamino)-methanesulfone-o-anisidide; SDS, sodium dodecyl sulfate; Topo II, DNA topoisomerase II; Et-Br, ethidium bromide.

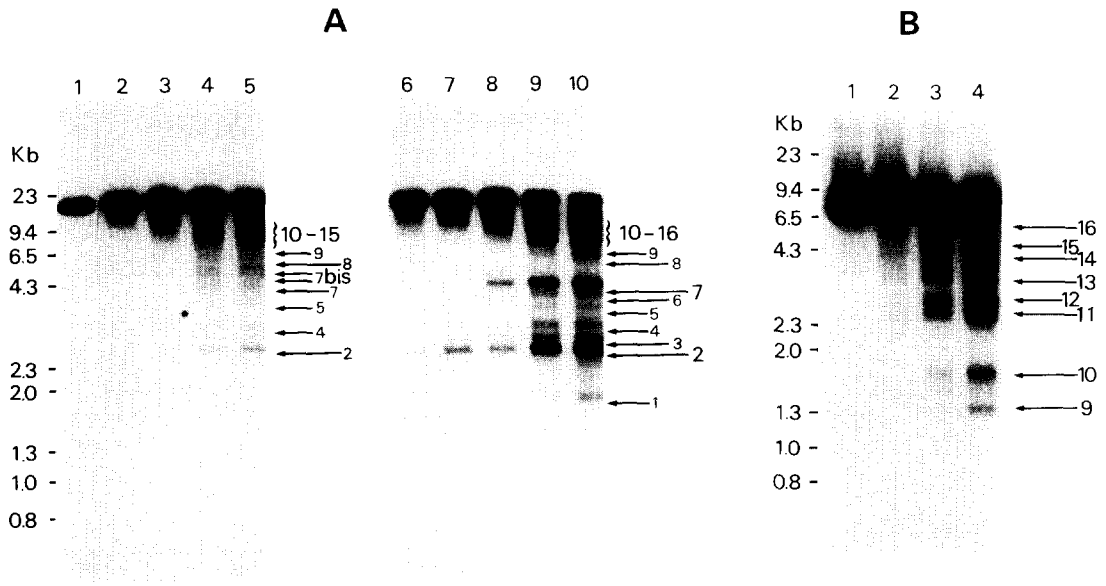

Fig. 1. Topo II cleavage products of the c-*myc* gene. Restrictions enzyme digests of λ K76 DNA (25 ng) were incubated with Topo II for 10 min, the reaction was stopped with SDS-proteinase K treatment, and the samples were analysed by electrophoresis and Southern blot hybridization, using probes A or B as described in Materials and Methods. Panel A: *Eco* RI digest of λ K76 DNA, using probe A. Lane 1, control DNA (no Topo II); lanes 2, 3, 4, 5, + Topo II, 50, 100, 200, 400 ng; lane 6, + Topo II, 100 ng; lane 7, same as lane 6 + 1 μM of oAMSA; lanes 8, 9, 10, same as lane 6 + 0.1, 1 and 5 μM of mAMSA. Panel B: *Xba* I digest of λ K76 DNA, using probe B. Lane 1, control DNA (no Topo II); lane 2, + Topo II 100 ng; lanes 3, 4, same as lane 2 + 0.5, 5 μM of mAMSA. The arrows indicate the cleavage products generated by Topo II alone or by Topo II and drugs. Their positions were reported with the same number on the map of c-*myc* (see Fig. 4). The sizes of the fragments, in kilobase pairs (kb), were deduced from the migration rate of phage λ *Hind* III and ØX174 *Hae* III DNA fragments used as gel calibration markers.

described [18]. After SDS-PAGE treatment, the purified enzyme gave two major protein bands of 140 and 120 K [19], representing about 80% of the protein content of the gel.

The specific activity of the enzyme preparation was 1.5×10^5 units/mg of protein, one unit being defined as the quantity of enzyme which fully decatenated 0.1 μg of kDNA in 30 min at 37°. Topo II was stored at −20° in a conservation buffer (100 mM Tris HCl, pH 7.9, 20 mM β-mercaptoethanol, 0.5 mM EDTA, 50% glycerol), without detectable loss of activity.

Double-stranded DNA cleavage assay. Topo II (100 ng, except where indicated) was incubated at 37° for 10 min in the absence or presence of the drug at various concentrations. The reaction mixture (20 μl) contained 20 mM Tris HCl, pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM EDTA, 0.5 mM dithiothreitol, 15 μg/ml of BSA and 25 ng of λ K76 DNA digested by either *Eco* RI, *XBa* I or *Cla* I. The reaction was stopped by addition of 5 μl of 5% SDS, 4 mg/ml proteinase K, 0.02% bromophenol blue, 40% glycerol. The mixture was incubated for 45 min at 50°. The samples were loaded on horizontal 1.2% agarose slab gels in 36 mM NaH_2PO_4, 1 mM EDTA, 30 mM Tris, pH 7.7 and run for 3 hr at 10 V/cm at room temperature. The gel was stained with Et-Br and photographed under u.v. light with a Polaroid 665 film. The reversion of double-stranded DNA cleavage was performed by addition of salt (0.5 M NaCl) to the reaction mixture before addition of a protein denaturant, as previously described [5].

Origin of the λ K76 clone. The λ K76 clone was obtained by screening a genomic DNA bank of a human lymphoblastic cell line (KE-37R cell line [20]). It was shown to consist in a 15 kb fragment, which included the three exons and both the 5′ and 3′ *Eco* RI sites of the normal c-*myc* gene (see the restriction map in Fig. 4).

Hybridization and probes. After gel electrophoresis, the λ K76 DNA fragments were denatured with NaOH and transferred to a GeneScreenPlus membrane (New England Nuclear) [21]. *Eco* RI digested λ K76 DNA fragments and *Xba* I or *Cla* I digested λ K76 DNA fragments were analysed using probe A and probe B respectively. Probe A is the *Eco* RI-*Cla* I fragment of the human c-*myc* gene (third exon) from pMYC-ECL recombinant plasmid [22]. Probe B was prepared by *Pvu* II excision of the first exon of the human c-*myc* gene from PP20 recombinant plasmid [23]. These probes were labeled with [^{32}P]dCTP (2–3000 Ci/mmole) at a specific activity of 2–4 10^8 cpm/μg DNA using the nick translation technique [24]. The hybridizations were performed under stringent conditions [25] and the hybrids were revealed by autoradiography (Kodak XAR 5 autoradiographic film). The sizes of

the DNA fragments were deduced from the migration rates using phage λ-*Hind* III and ØX174-*Hae* III fragments as markers.

RESULTS

mAMSA stimulates the Topo II-mediated double-stranded DNA breaks in c-myc *oncogene*

The DNA obtained from the λ K76 clone bearing a complete human c-*myc* insert, was digested with either *Eco* RI or *Xba* I prior to use as substrate for purified calf thymus Topo II. The resulting fragments were analysed by Southern blot hybridization with either an *Eco* RI-*Cla* I fragment (probe A) or a *Pvu* II fragment (probe B).

Figure 1A shows the results obtained with *Eco* RI-digested λ K76 DNA after hybridization with probe A. When compared with untreated DNA (lane 1), the Topo II-treated preparation shows a pattern of cleavage products. While the band intensities increased with Topo II concentrations (lanes 2–5), no qualitative changes of the pattern were observed, indicating that specific cleavage sites were involved in the reaction. The cleaving activity of Topo II was strongly stimulated by the acridine derivative mAMSA. The yield of the cleavage products varied as a function of the drug concentration (lanes 8–10). A careful examination of the autoradiographs showed that mAMSA stimulated the formation of most, but not all the cleavage products generated by Topo II alone. The generation of the cleavage bands 2, 7, and 9–15 (shown by arrows in Fig. 1A) was particularly stimulated by mAMSA. It can be noticed that the band 7 bis in the control Topo II pattern (lane 5) were observed only in the presence of the higher Topo II concentration (400 ng instead of 100 ng). In a few cases, mAMSA generated some cleavage products (arrows 1, 3, 6, 16) different from those determined by Topo II alone.

oAMSA, a biologically inactive stereoisomer of mAMSA [17], stimulated to a lesser extent the Topo II cleaving activity, as the same bands were observed with a much fainter intensity (lane 7). For example, the band 2 generated by Topo II alone (lane 6) was increased 8-fold in the presence of 1 μM of oAMSA (lane 7) and 28-fold in the presence of 1 μM of mAMSA (lane 9), as quantified by micro-densitometry.

In order to confirm these results, the λ K76 DNA was digested with other restriction enzymes, *Xba* 1 (Fig. 1B), and *Cla* I (not shown), and hybridized with probe B. The results were very similar: again discrete bands, with higher intensity than in the Topo II control pattern (lane 2), were observed in the presence of mAMSA (lanes 3, 4), indicating a specificity of the stimulated cleavage process.

Reversion by salt and inhibition by ethidium bromide

The reversion of the cleavable complex by salt allows the distinction between a double-stranded cleavage induced by Topo II and that due to a contaminant nuclease activity [5]. As shown in Fig. 2, the patterns of the DNA incubated with Topo II alone (lane 6) and variable concentrations of mAMSA (lanes 7–9) indicate a reversion of the cleavage process in 30 min (to be compared with

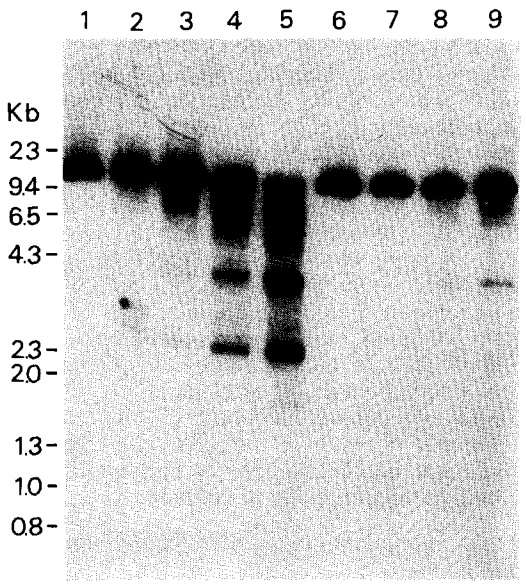

Fig. 2. Reversion by salt of Topo II cleavage in the c-*myc* gene. Double-stranded DNA cleavage was carried out as described in Materials and Methods, using *Eco* RI digest of λ K76 DNA (25 ng) and probe A. Lane 1, control DNA (no Topo II); lane 2, + Topo II 100 ng; lanes 3, 4, 5, same as lane 2 + 0.1, 1, 10 μM of mAMSA; lanes 6–9, reversion of DNA cleavage by addition of 0.5 M NaCl for 30 min at 37° before SDS and proteinase K (lanes 6–9 correspond to lanes 2–5 without NaCl).

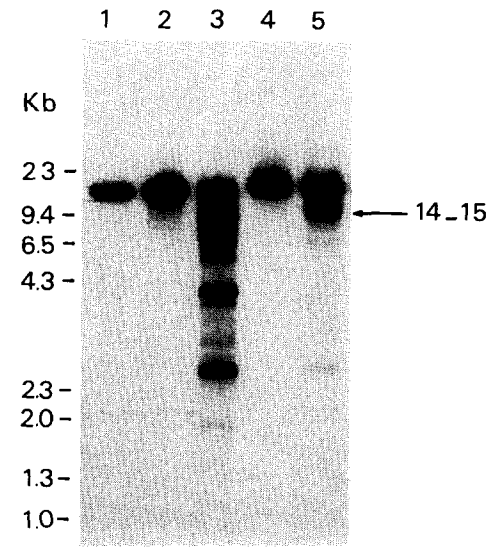

Fig. 3. Ethidium bromide inhibition of Topo II cleavage of the c-*myc* gene. Double-stranded DNA cleavage was carried out as described in Materials and Methods using *Eco* RI digest of λ K76 DNA (25 ng) and probe A. Lane 1, control DNA (no Topo II); lane 2, + Topo II 50 ng; lane 3, same as lane 2 + 25 μM of mAMSA; lane 4, same as lane 2 + 24 μM of Et-Br; lane 5, same as lane 2 but with simultaneous addition of 25 μM AMSA and 24 μM Et-Br.

lanes 2–5). At the highest concentrations of mAMSA (1 and 10 μM, lanes 8, 9), a complete reversion requires a longer incubation time (1 hr) (data not shown). These results exclude the presence of a significant nuclease contamination.

Figure 3 shows the inhibition by ethidium bromide (Et-Br) of Topo II-mediated DNA breaks in the absence or presence of mAMSA (*Eco* RI digested λ K76 DNA hybridized with probe A). The appearance of cleavage bands was suppressed in the reaction with Topo II alone (lanes 2 and 4). Furthermore, a strong decrease in cleavage products occurred when mAMSA (25 μM) and Et-Br (24 μM) were simultaneously added to the reaction mixture (lanes 3 and 5). A notable exception was the conservation of mAMSA stimulated bands 14 and 15 (Fig. 3, arrow). The absence of any new band in the presence of Et-Br indicates that the drug did not exert a qualitative inhibitory effect on DNA break induction.

Mapping of the Topo II-induced cleavage sites in the c-myc *oncogene*

The sizes of the DNA fragments generated by Topo II were used to map the cleavage sites in the c-*myc* locus. The Topo II cleavage sites do not seem to be arranged at random. With the exception of two minor sites located in the second exon of the gene (arrows 4, 5), the preferred Topo II cleavage sites generating the most intense bands are located in the 5′ end of the c-*myc* locus (arrows 9–15) and in the introns (arrows 2, 7, 7bis, 8) (Fig. 4A).

When mAMSA was used to stimulate the Topo II activity, the cleavage sites (Fig. 4B) were approximately the same as those induced by Topo II alone. Most of these sites were located at the 5′ end of the c-*myc* locus. Two other important cleavage sites were detected in the introns (arrows 2 and 7). When these sites are compared with the DNase I hypersensitive sites located in the human c-*myc* proto-oncogene, reported by Dyson *et al.* [26, 27], there seems to be a rather good correspondence between the major Topo II cleavage sites and the DNase I hypersensitive sites (Fig. 4).

DISCUSSION

This study was undertaken as a preliminary approach to assess the activity of Topo II-mediated pharmacological drugs on genes assumed to play a critical role in the process of cell proliferation. Proto-oncogene c-*myc* was chosen because the sequence of the whole gene is known [23], and the structural features of the c-*myc* flanking sequences, which may regulate gene expression, are currently under investigation in several laboratories [15, 16]. As a preliminary approach, the stimulation of Topo II activity by mAMSA was evaluated in an *in vitro* system. The results indicate four main points:

(1) The cleavage sites are not randomly generated by Topo II. This is indicated by the presence of discrete bands, whose intensity increases as a function of the enzyme concentration. Conversely, random breaks would result in the occurrence of more and more complicated patterns as a function of the mAMSA concentration.

(2) In the presence of mAMSA, the DNA patterns are not qualitatively modified but the intensity of certain bands is considerably enhanced. This is a direct confirmation that the drug activity is mediated *in vitro* by Topo II. Furthermore, the recognition of the sites by the enzyme does not change in the presence of the drug. At similar concentrations oAMSA is much less active. This is in agreement with the selective lack of cytotoxic activity of this analog in living cells [17].

(3) The inhibition of Topo II activity by Et-Br

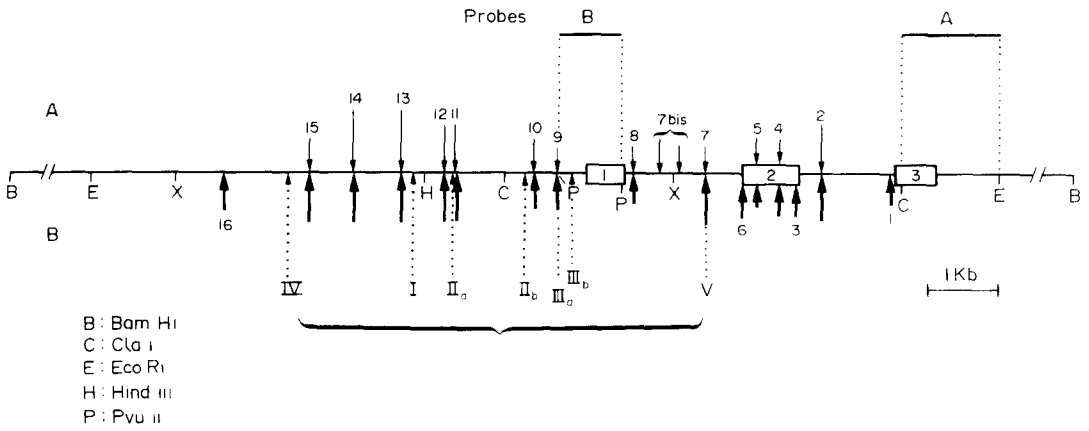

Fig. 4. Map of the Topo II cleavage site in the c-*myc* locus. The restriction map of the c-*myc* oncogene cloned in λ K76 was identical to that determined by Gazin *et al.* [23]. The three c-*myc* exons were positioned in boxes 1, 2 and 3. Panel A: The probes A and B used for hybridization are indicated at the top of the figure. Thin arrows in the c-*myc* locus indicate the position of the cleavage sites generated by Topo II alone. Panel B: thick arrows indicate the position of the Topo II cleavage sites stimulated by mAMSA. The thickness and the length of the arrows roughly indicate the importance of the cleavage product. The dotted arrows indicate the position of the DNase I hypersensitive sites in the c-*myc* locus, according to Dyson *et al.* [26, 27]. For clarity sake, not all the known restriction sites are shown in the diagram.

strongly suggests that Topo II is the specific target of mAMSA. This is in agreement with the inhibition of the cytotoxicity of Epipodophyllotoxin by Et-Br [28]. Moreover, Et-Br has been shown to interfere with Topo II-mediated cleavage *in vivo*, as well as *in vitro* [28]. It is not known whether Et-Br interferes with the nicking activity of Topo II on DNA by a direct interaction with the enzyme, or by a DNA intercalating interaction that would prevent the binding of Topo II on the DNA sites.

(4) The cleavage sites are not located in the coding sequences of c-*myc*. The majority of the most intense bands corresponds to sites located 5′ to exon 1 (arrows 9–16), and in introns flanking exon 2 (arrows 2, 7). In order to find out whether there is a correlation between the Topo II stimulation by the drug and its biological activity, these results should be extended to an *in vivo* system. This approach has been successfully utilised to characterize cleavage sites in DNA from simian virus 40-infected monkey cells treated with Epipodophyllotoxins and in SV40 chromatin of mAMSA-treated cells [29, 30]. In preliminary experiments on the mAMSA effect on NCI N417 [31] and HL-60 [32] cell cultures that contain multiple c-*myc* copies, we have found that the most important cleavage sites are located 5′ to the c-*myc* exon 1 and in good correspondence with most of the *in vitro* cleavage sites [33]. The most striking difference was the lack of the major *in vitro* site located between exon 1 and exon 2 (site 7), in *in vivo* experiments. These two sites are the same as, or very close to most of the DNase-hypersensitive sites described in c-*myc* [26, 27] (see Fig. 4). In agreement with these findings, we have observed that DNAse I hypersensitive sites in N417 cells exhibited good correspondence with cleavage sites induced by mAMSA in N417 cells [33]. An association between Topo II cleavage sites and DNase-hypersensitive sites has also been found in an *in vitro* system using the enzyme from *Drosophila melanogaster* and the 87A7 heat shock locus [34]. These findings favor the existence of common targets for DNase and Topo II, and are likely to be related to the chromatin structure of the gene.

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