Stimulation of the Topoisomerase II Induced DNA Cleavage Sites in the c-*myc* Protooncogene by Antitumor Drugs Is Associated with Gene Expression

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ABSTRACT: The antitumor drugs mAMSA and VM26 were shown to stimulate the topoisomerase II (Topo II) cleavage activity on the c-*myc* protooncogene in several human tumor cell lines (N417, HL60, EJ, H146, CaSki, A431, IGROV1, and CAL18A) and human peripheral lymphocytes. The mAMSA-induced gene cleavage was found to increase with the steady-state levels of c-*myc* transcripts in cell lines while no cleavage could be evidenced in the other genes so far tested. In mAMSA-treated N417 cells, the overall genomic DNA cleavage detected by alkaline elution was found to be about 20 times lower than the c-*myc* gene cleavage. Topo II mRNA levels were associated with the nuclear Topo II decatenating activity in cell lines and increased with c-*myc* cleavage. Topo II decatenating activity was found to be 3 times lower in quiescent than in exponentially growing N417 cells, but the c-*myc* cleavage induced by mAMSA was found as intense in quiescent as in growing cells. Thus, our data seem to indicate that c-*myc* gene cleavage is not related to cellular Topo II content but rather to c-*myc* gene transcription. Therefore, we suggest that only a small fraction of the Topo II is able to react with drug on the c-*myc* gene in relation to its transcriptional accessibility. Since c-*myc* overexpression is frequently found to be related to human cancer progression, we suggest that this gene could be an important target for Topo II related antitumor drugs.

The antitumor drugs 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (mAMSA)[1] and VM26, an acridine and an epipodophyllotoxin derivative, respectively, have been shown to interfere with mammalian topoisomerase II (Topo II), an enzyme that alters DNA conformation by transiently breaking and rejoining both DNA strands (Ross, 1985; Rowe et al., 1984). These drugs trap the enzyme in vitro in an intermediary complex with DNA, the cleavable complex, preventing the final rejoining step of the reaction (Nelson et al., 1984; Chen et al., 1984). Treatment of the complex with a protein denaturating agent such as SDS leads to a single- or double-strand DNA breakage and to the covalent linking of each Topo II subunit to both 5′-phosphoryl ends of the broken DNA strand (Liu et al., 1983; Sander & Hsieh, 1983). In vivo, these drugs were also shown to interact according to that mechanism. DNA breaks were revealed in overall genomic DNA using alkaline elution (Kohn, 1987) or in specific regions of particular genes using the Southern blot technique (Riou et al., 1986a; Rowe et al., 1986). These data and other studies on cell lines resistant to these drugs (Bakic et al., 1986; Glisson et al., 1986; Pommier et al., 1986; Per et al., 1987) suggested that the formation of Topo II–DNA complexes could be one of the major causes of their cytotoxic action in vivo. In previous reports, we have shown from experiments performed both in vitro and in vivo that the cleavable complex induced by mAMSA and VM26 preferentially occurs in the 5′ noncoding end of the c-*myc* gene close to DNAse I hypersensitive cleavage sites (Riou et al., 1986a,b). However, in analyzing the drug effects in untranscribed genes from N417 cells, a cell line derived from a human small cell lung carcinoma (Riou, J. F., et al., 1987), we detected neither drug-induced Topo II cleavage site (drug ITCS) formation nor DNase I hypersensitive cleavage sites. The 5′ end of the c-*myc* gene is assumed to be important for its regulation, and several transcription control elements described by Chung et al. (1986) have been localized near the mAMSA and VM26 ITCS (Riou, J. F., et al., 1987).

Amplification and/or transcriptional activity is an important mechanism through which the c-*myc* gene is activated in the most aggressive cancers (Alitalo et al., 1987; Taya et al., 1987; Riou, G., et al., 1987). In addition, Topo II activity has been reported to be increased in cells induced to proliferate (Duguet et al., 1983; Miskimins et al., 1983; Taudou et al., 1984) and in transformed cells (Riou et al., 1985; Heck & Earnshaw, 1986; Nelson et al., 1987). Therefore, these data lead us to assume that the amplified and/or overexpressed c-*myc* gene in cancer cells could be an important target for antitumoral drugs in relation to their Topo II activity. To test that hypothesis, we have analyzed the mAMSA- and VM26-induced cleavage activity in the c-*myc* gene from different cell lines and from peripheral blood lymphocytes in relation to their c-*myc* expression, their amplification, and their Topo II content.

Materials and Methods

Cell Lines. Small cell lung carcinoma NCI N417 and NCI H146 cell lines were grown as floating aggregates in RPMI 1640 medium containing 10% FCS (Carney et al., 1985). Promyelocytic HL60 (Collins et al., 1978) and ovarian adenocarcinoma IGROV1 (Bénard et al., 1985) cell lines were grown in RPMI 1640 medium containing 10% FCS. Vulva epidermoid carcinoma A431 (Fabricant et al., 1977), uterine cervix squamous cell carcinoma CaSki (American tumor and cell collection), bladder carcinoma EJ (American tumor and cell collection), and breast adenocarcinoma CAL18A (Gioanni et al., 1985) cell lines were cultured as a monolayer in DMEM containing 10% FCS. Peripheral blood lymphocytes from

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[1] Abbreviations: Topo II, topoisomerase II; kDNA, kinetoplast DNA; ITCS, induced topoisomerase II cleavage sites; FCS, fetal calf serum; DMEM, Dulbecco's minimum essential medium; RPMI, Roswell Park Memorial Institute medium; mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; VM26, 4′-demethylepipodophyllotoxin 6-thenylidene-β-D-glucoside; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

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Table I: Cloned Genes Used in the Analysis of Tumor Cell Line DNA Preparations

genes	size of DNA probe (kb)	enzyme cleaving tumor cell DNA	size of genomic DNA fragment(s) detected (kb)	ref
c-*myc*[a]	1.4 (*Eco*RI–*Cla*I)	*Eco*RI	13	Modjtahedi et al. (1986)
c-*fos*[a]	1.3 (*Bgl*II–*Bgl*II)	*Eco*RI	10	Curran et al. (1982)
c-*mos*[b]	2.5 (*Eco*RI–*Eco*RI)	*Hin*dIII	4.9	Prakash et al. (1982)
c-*erb*B[a,d]	1.03 (*Eco*RI–*Eco*RI)	*Hin*dIII	11	Ullrich et al. (1984)
		*Eco*RI	7.8	
c-*erb*B2/*neu*[c]	1.6 (*Eco*RI–*Eco*RI)	*Eco*RI	6.6 and 4.4	Kraus et al. (1987)
β_1 globin[b]	1.7 (*Bgl*II–*Xba*I)	*Eco*RI	7.2	Fritsch et al. (1980)
c-*myb*[c]	2.6 (*Eco*RI–*Eco*RI)	*Hin*dIII	7.9 and 6.3	Salmon et al. (1986)

[a] Transcribed. [b] Untranscribed. [c] Weakly transcribed in the cells studied here. [d] Highly transcribed in A431 cells.

healthy donors were fractionated in a Ficoll-Hypaque gradient.

Drug Action on Cells and DNA Preparation. Cells in exponential growth phase and lymphocytes, as indicated, were exposed to mAMSA for various periods of time at 37 °C in fresh culture medium. Cells were washed with 50 mM Tris, pH 7.5, and 1 mM EDTA and immediately lysed with 1% SDS, 50 mM Tris, pH 7.5, and 25 mM EDTA. Proteinase K was added to a final concentration of 1 mg/mL for 6 h at 50 °C. Lysate was then treated twice with phenol and DNA extracted with chloroform, precipitated with ethanol, dried, and then resuspended in 10 mM Tris, pH 7.5, and 0.5 mM EDTA.

DNA samples (10 μg) were digested with *Eco*RI or *Hin*dIII restriction endonuclease and electrophoresed in a 1.2% agarose gel, and DNA fragments were transferred onto a GeneScreen Plus membrane (New England Nuclear). mAMSA (Dr. B. Baguley, New Zealand) and VM26 (Sandoz laboratory) were dissolved in dimethyl sulfoxide (10 mM) and then in distilled water to 1 mM.

RNA Preparation. Total RNAs were extracted from untreated cells by using the guanidinium–isothiocyanate CsCl buoyant density gradient procedure (Maniatis et al., 1982).

Hybridization and Probes. DNA cleaved with appropriate restriction enzyme and total RNA (10 μg) were analyzed by Southern and Northern blot, respectively. Hybridizations were performed in stringent conditions using specific probes described in Table I. The human Topo II probe is the 1.8-kb *Eco*RI fragment of the λh Top2-Z2 cDNA clone (Tsai-Pflugfelder et al., 1988). Probes were labeled with [^{32}P]dCTP (3000 Ci/mmol) to a specific activity of (2–4) × 10^8 cpm/μg using the nick translation technique (Maniatis et al., 1982). Hybrids were revealed by autoradiography on Kodak XAR5 films. Transcript levels were measured by serial dilutions of RNA (5, 2.5, 1.25, 0.62 μg) applied onto a nitrocellulose filter, using a slot blot apparatus (Schleicher & Schuell), and hybridized.

Assay for Topo II Activity. About 10^7 cells were harvested and washed in 1 mL of buffer (A) containing 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 10 mM β-mercaptoethanol, and 1 mM PMSF. Nuclei were obtained by addition of 1.75% Nonidet P40 for 15 min in ice, then washed in 1 mL of buffer A, and counted in the presence of trypan blue (v/v). Topo II extraction was performed by addition of 0.5 mL of buffer A containing 0.33 M NaCl for 15 min in ice. Nuclei and DNA were discarded by centrifugation (1500*g* and 20000*g*), and the supernatant (crude extract) was analyzed for its protein content (Bio-Rad reagent). Decatenating activity was measured by using kDNA prepared from *Trypanosoma cruzi* (Riou & Yot, 1975; Riou et al., 1986c). Briefly, serial dilutions of crude extract were applied in a reaction mixture containing 5 μg/mL kDNA, 160 mM KCl, 15 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, 0.5 mM DTT, 20 mM Tris, pH 7.5, and 10 μg/mL BSA for 30 min at 37 °C. The reaction performed in 20 μL was stopped by the addition of 5 μL of a solution containing 0.4% Bromophenol blue, 20% glycerol, 1 mM EDTA, and 0.1% SDS. Samples were loaded onto a 1.5% agarose gel for electrophoresis and stained by ethidium bromide. One unit of Topo II represents the quantity of crude extract which decatenates 50% of the kDNA in the conditions of the assay. Specific Topo II activity (units per cell) was calculated from at least two separate experiments.

Results

mAMSA- and VM26-Induced Topo II Cleavage in the c-myc Gene from Different Human Cell Lines. In a previous study, the c-*myc* gene from N417 and HL60 cell lines was shown to be cleaved by mAMSA (Riou, J. F., et al., 1986a, 1987). Since a high number of c-*myc* copies were present in the genome of these cell lines (Little et al., 1983; Collins & Groudine, 1982), it seemed of interest to test the drug effect in other cell lines (H146, CAL18A, EJ, CaSki, IGROV1, and A431) containing only a single or very few c-*myc* gene copies. Topo II cleavage induced by mAMSA was detected in the c-*myc* gene from all the human tumor cell lines so far tested and also in normal peripheral blood lymphocytes. The DNA pattern from drug-treated H146 cells (Figure 1, lane 5) shows the 13-kb germline band and some discrete bands of lower sizes not observed in untreated cells (data not shown). Genomic DNAs from N417, HL60, CAL18A, and CaSki cells were diluted to $^1/_{47}$th, $^1/_{30}$th, $^1/_3$rd, and $^1/_3$rd, respectively, to reduce to 1 the c-*myc* gene copy number (undiluted N417 genomic DNA is presented in lane 1). The c-*myc* gene cleavage bands obtained after mAMSA treatment of the different cell lines are shown in Figure 1. A quantitative measurement of cleavage band intensities was performed by densitometric scanning (Chromoscan 3, Joyce Loebl) of autoradiographic films at different exposure times. Values of relative c-*myc* gene cleavage were normalized against the c-*myc* germline DNA values (Table II) and expressed as multiples of the c-*myc* cleavage detected in lymphocytes arbitrarily defined as 1. The c-*myc* cleavage bands were always found more intense in cell lines than in human peripheral lymphocytes. Since N417 cells contained about 47 copies of the c-*myc* gene per haploid genome (Little et al., 1983), data showed that the c-*myc* cleavage accounts for about 150 times that detected in lymphocytes (compare lanes 1 and 8 of Figure 1). Moreover, the c-*myc* cleavage patterns of all cell lines so far tested exhibited the major cleavage sites previously described in N417 and HL60 cells (Riou, J. F., et al., 1986a, 1987).

H146, IGROV1, EJ, and A431 cells were also treated with VM26. Sizes and intensities of c-*myc* cleavage bands corresponded to those observed when the cells were treated with mAMSA (data not shown).

mAMSA Does Not Induce Topo II Cleavage Sites in Other Genes. We have previously reported that the untranscribed

Table II: Association between c-*myc* Cleavage and Biological Parameters in Cell Lines

cell lines and lymphocytes	no. of c-*myc* gene copies/haploid genome	relative levels of			Topo II act. (10^{-4} units/nucleus) ± SD[b]
		c-*myc* DNA cleavage[a]	c-*myc* mRNA	Topo II mRNA ± SD[b]	
N417	45	3.4	40	50 ± 2.5	14 ± 4
HL60	30	2.9	15–16	23 ± 2	ND[c]
H146	1	2.5	11	52 ± 13	ND
CAL18A	2–3	2.2	6–7	15 ± 2.5	7 ± 1
EJ	1	1.8	3	6 ± 2.3	3.5 ± 1
CaSki	2–3	1.8	3	16 ± 10	11.5 ● 3.5
A431	1	1.4	2	12 ● 9	ND
IGROV1	1	1.3	2	8 ● 2.4	8.5 ● 0.5
lymphocytes	1	1	1	1 ± 0.05	0.25 ± 0.15

[a] Cleavage was obtained after mAMSA treatment of cells (see text and Materials and Methods). Values are given relative to a single c-*myc* gene copy. [b] SD, standard deviation. [c] ND, not determined.

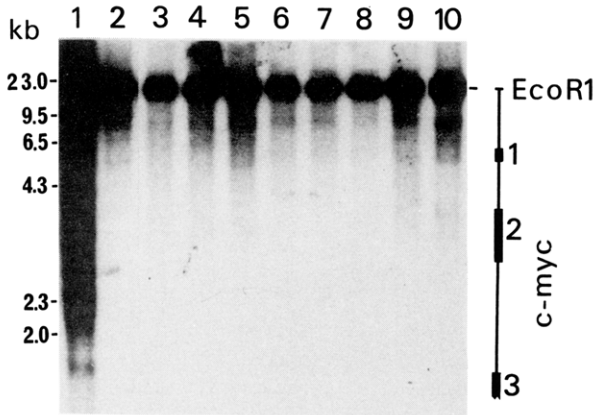

FIGURE 1: mAMSA-induced DNA cleavage in the c-*myc* gene from human tumor cell lines and lymphocytes. Cells were treated for 3 h at 37 °C with 20 μM mAMSA before cell lysis and DNA extraction under protein denaturant conditions (see Materials and Methods). DNAs were digested with *Eco*RI and analyzed by Southern blot hybridization using the human c-*myc* probe (third exon) (see Table I). The blot was exposed to Kodak XAR5 film for 8 days. DNA from treated cells: lane 1, N417 (10 μg); lane 2, N417 (0.20 μg); lane 3, IGROV1 (10 μg); lane 4, CaSki (3.3 μg); lane 5, H146 (10 μg); lane 6, EJ (10 μg); lane 7, A431 (10 μg); lane 8, human lymphocytes (10 μg); lane 9, CAL18A (3.3 μg); lane 10, HL60 (0.33 μg). Cell DNA specimens (lanes 2–10) were diluted in order to obtain the equivalent of a single c-*myc* gene copy. The DNA cleavage bands induced by mAMSA in c-*myc* are represented by discrete bands of lower molecular weight than the 13-kb DNA band. Introns and exons of the c-*myc* protoncogene map are represented according to the distances of electrophoretic migration as indicated by the DNA size of markers.

c-*mos* gene and β_1-globin pseudogene were not cleaved by mAMSA (Riou, J. F., et al., 1987). In the present study, N417 cells were treated with drug for longer exposure times (3, 6, or 24 h, 10 μM mAMSA). No cleavage bands could be detected (Figure 2A). Identical results were obtained in the other cell lines (data not shown). Our analysis was extended to the study of transcribed genes (Table I) known to play important role in cell growth or differentiation (Bishop, 1987). These genes (c-*erb*B, c-*fos*, c-*erb*B2/*neu*, and c-*myb*) were never found to be cleaved after cell treatment with mAMSA or VM26, even the c-*erb*B gene which is amplified and overexpressed in the A431 cell line (Ullrich et al., 1984) (Figure 2B,C).

Effect of mAMSA on c-myc Gene Cleavage and Transcription. In order to determine the specificity of the Topo II cleavage induced by mAMSA in the c-*myc* gene, the c-*myc* DNA cleavage was compared to overall genomic DNA cleavage obtained by alkaline elution. To relate the mAMSA-induced c-*myc* gene cleavage to the cytotoxic effect of the drug, N417 cells were treated at the IC_{50} of mAMSA (1 μM for 30 min) (Multon et al., 1989) and analyzed for c-*myc* gene cleavage. Data from densitometer scanning indicate that mAMSA induces the cleavage of 18 ± 3.4% (mean value ± SD of seven independent determinations) of the 13-kb *Eco*RI DNA band from the c-*myc* gene. Such cleavage would correspond to 1 Topo II cleavage site per 7.2×10^4 nucleotides. Cleavage frequencies increase with mAMSA concentration to reach a plateau at 20 μM corresponding to 1 Topo II cleavage site per 4.2×10^4 nucleotides. Assuming that 1000 rad yield 1 double-strand break (DSB) per 5×10^6 nucleotides (Kohn et al., 1981), the 300 rad-equiv produced by 1 μM mAMSA in N417 cells (Multon et al., 1989) would correspond to 1 DNA Topo II cleavage site per 1.5×10^6 nucleotides. Thus, the action of mAMSA at IC_{50} appears to be 20 times more efficient on the c-*myc* gene than on the overall genome. If we assume that other genes, transcriptionally active or inactive, are cleaved by mAMSA with the same frequency as overall genomic DNA, less than 1% of a 10-kb gene fragment would be cleaved in one or several bands. Such cleavage seems too low to be clearly detected by using our present methodology.

Table III: Variations of c-*myc* mRNA Levels in mAMSA-Treated N417 Cells[a]

time of drug incubation (h)	% of c-*myc* mRNA levels	time of drug incubation (h)	% of c-*myc* mRNA levels
0	100	6	52.2
1	67.0	12	46.5
3	47.7	24	56.8

[a] Total RNA was prepared from exponentially growing N417 cells treated with 10 μM mAMSA for various periods of time and analyzed by Northern blot hybridization (see Materials and Methods). c-*myc* mRNA levels were measured by densitometer scanning of autoradiograms (Chromoscan 3, Joyce Loebl). Values were normalized against 28S rRNA values obtained by scanning the UV photograph from ethidium bromide colored gels.

We have also analyzed the levels of c-*myc* gene transcripts in exponentially growing N417 cells treated with 10 μM mAMSA for different periods of time. As shown in Table III, the c-*myc* mRNA levels decreased by about 2-fold after 3 h of drug treatment. Longer exposure time (24 h) with drug do not affect this 50% drop.

mAMSA-Induced Cleavage Increases with c-myc Transcript Levels of Cell Lines. Total RNAs prepared from the different cell lines in exponentially phase of growth and lymphocytes were analyzed by Northern blot hybridization using the human c-*myc* probe (third exon) (Table I). As expected, a transcript of 2.4 kb was detected. The steady-state level of c-*myc* RNA was analyzed by slot blot hybridization. In order to provide a control for the amount of RNA on the filter, the c-*myc* signal was removed from the same filter by boiling for 10 min in distilled water and rehybridized to a probe for the

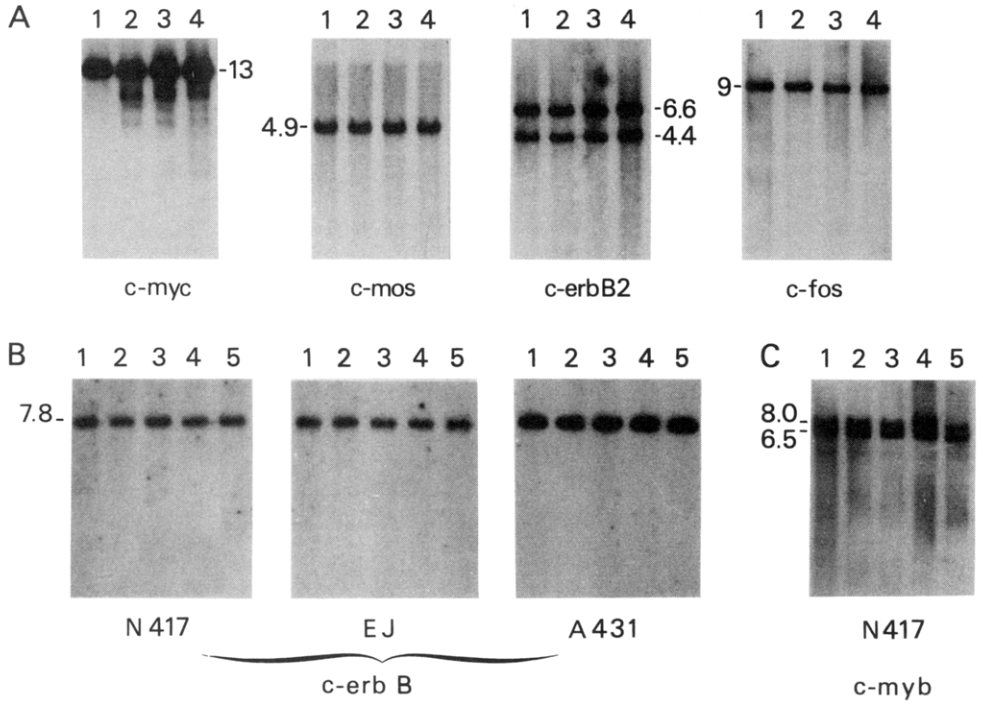

FIGURE 2: Absence of mAMSA-induced DNA cleavage in transcribed and untranscribed genes other than the c-*myc* gene. (A) DNA from N417 cells: lane 1, untreated cells; lanes 2–4, cells treated for 3, 6, and 24 h with 10 μM mAMSA, respectively. DNA samples (10 μg) were digested with *Eco*RI and analyzed by Southern blot hybridization using sequentially c-*myc*, c-*erb*B2/*neu*, and c-*fos* probes or were digested with *Hin*dIII and analyzed with c-*mos* probe (see Table I). Blots were exposed to Kodak XAR5 film for 6 h (c-*myc*) or 8 days (c-*mos*, c-*fos*, c-*erb*B2/*neu*). (B) DNA from N417, EJ, and A431: lane 1, untreated cells; lanes 2 and 3, cells treated for 3 h with 5 and 20 μM mAMSA, respectively; lanes 4 and 5, cells treated for 3 h with 5 and 20 μM VM26, respectively. DNA samples (10 μg) were digested with *Eco*RI and analyzed by Southern blot hybridization using the c-*erb*B probe (Table I). Blots were exposed to Kodak XAR5 film for 8 days. (C) DNA from N417 cells: lane 1, untreated cells; lanes 2 and 3, cells treated for 3 h with 5 and 20 μM mAMSA, respectively; lanes 4 and 5, cells treated for 3 h with 5 and 20 μM VM26, respectively. DNA samples (10 μg) were digested with *Hin*dIII and analyzed by Southern blot hybridization using the c-*myb* probe (Table I). Blot was exposed to Kodak XAR5 film for 8 days.

β-actin gene (Kvist et al., 1981) which is assumed to be uniformely expressed in cells (Figure 3). Relative levels of c-*myc* expression, normalized against actin values, are expressed as multiples of the c-*myc* RNA levels detected in lymphocytes, arbitrarily defined as 1 (Figure 3, Table II). Our results indicate that the c-*myc* gene is transcribed in all the cell lines but at different levels. Data of Table II indicate that the intensity of the c-*myc* cleavage increases with the c-*myc* transcript levels found in the different cells. Such a relationship was not found with c-*myc* gene amplification (Table II).

Stimulation of c-myc Gene Cleavage by mAMSA Is Partially Related to the Topo II Cell Content. We have analyzed Topo II enzymatic activity and Topo II gene transcription in cell lines at their exponential phase of growth. We have used the kDNA decatenation assay to determine the Topo II activity in nuclear extracts. Results presented in Table II show that the Topo II activity varies in function of the cell line and is much higher than in lymphocytes. Topo II gene expression was analyzed in total RNA preparations by Northern blot and slot blot hybridization using the human Topo II cDNA probe (Tsai-Pflugfelder et al., 1988). A 6.1-kb Topo II transcript band was revealed in all the RNA preparations from the different cell lines (not shown). Topo II transcript levels (Figure 3) were normalized against actin transcripts used as control. Results are expressed as multiples of the Topo II mRNA levels detected in lymphocytes, arbitrarily defined as 1 (Table II). Data indicate that Topo II mRNA levels significantly varied in the different cell lines and are associated with the nuclear enzymatic activity in cell lines. Levels of both Topo II activity and Topo II mRNA do not increase linearly with c-*myc* cleavage but roughly correlate when the standard deviation is taken into account (Table II).

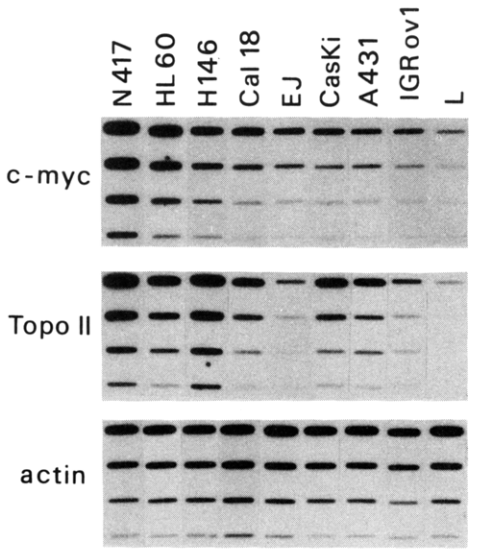

FIGURE 3: c-*myc* and Topo II RNA levels in human tumor cell lines and lymphocytes. Total RNA prepared from untreated cell lines in the exponential phase of growth and lymphocytes were analyzed by slot blot hybridization using sequentially c-*myc*, Topo II, and β-actin probes; 5, 2.5, 1.25, and 0.62 μg of total RNA preparation from each cell line were spotted onto the filter. The β-actin signal provides a control for the amount of RNA on the filter. Relative levels of c-*myc* and Topo II mRNA were measured by densitometric scanning (Chromoscan 3, Joyce Loebl) of the autoradiogram and expressed as multiples of the mRNA values detected in lymphocytes (Table II).

Topo II enzymatic activity, Topo II transcripts, c-*myc* transcripts, and c-*myc* gene cleavage induced by mAMSA were analyzed in exponentially growing and in quiescent N417

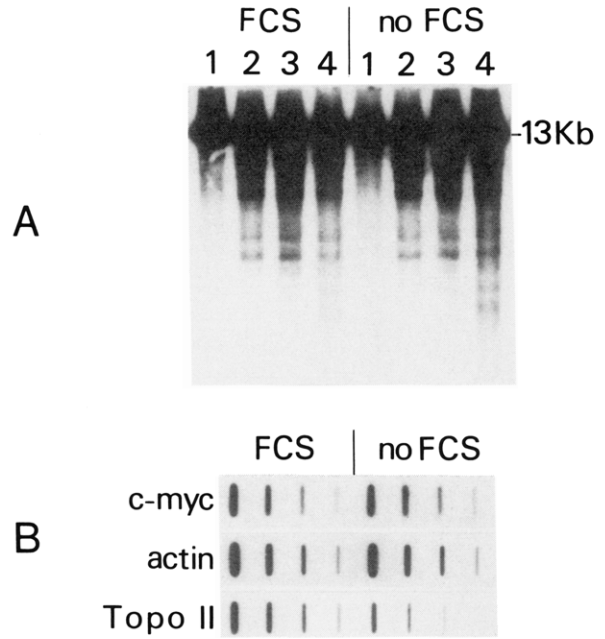

FIGURE 4: mAMSA-induced c-*myc* gene cleavage and c-*myc* and Topo II mRNA levels in exponentially growing and quiescent N417 cells. (A) Southern blot hybridization with the c-*myc* probe of *Eco*RI-digested DNA (10 μg) from exponentially growing (FCS) or quiescent (no FCS) N417 cells: lane 1, untreated cells; lanes 2–4, cells treated with 10 μM mAMSA for 3, 6, and 24 h, respectively. The blot was exposed to Kodak XAR5 film for 3 days. (B) Slot blot analysis of total RNA (5, 2.5, 1.25, and 0.62 μg) from exponentially growing (FCS) or quiescent (no FCS) N417 cells. The blot was sequentially hybridized with c-*myc*, Topo II, and the β-actin probes (see Materials and Methods). Values of c-*myc* and β-actin expression in growing or quiescent cells are identical.

cells. Quiescent cells were obtained after 48 h of serum deprivation which does not affect cell viability since more than 95% of the cells reenter division 24 h after serum addition. Topo II activity and Topo II transcript levels were found to be about 3 times lower in quiescent than in exponentially growing N417 cells while the c-*myc* transcript levels (Figure 4B) as well as gene cleavage intensity (Figure 4A) were unchanged. By contrast, when other cell lines (HL60, CAL18A) are deprived of serum, the c-*myc* transcripts drastically decreased as previously observed in other cell models (Kelly et al., 1983; Campisi et al., 1984; Blanchard et al., 1985). In quiescent HL60 cells, Topo II and c-*myc* transcript levels decrease while a low diminution of c-*myc* gene cleavage was observed (data not shown). These results suggest that in the N417 cell line, variations of the Topo II activity have no direct incidence on the c-*myc* gene cleavage activity.

Discussion

Our previous studies have shown that drug ITCS occur in the 5′ end of the amplified c-*myc* gene from N417 and HL60 cells (Riou, J. F., et al., 1987). In the present study, we have clearly demonstrated that mAMSA ITCS occur in a large range of human cells including normal peripheral lymphocytes which contain a single copy of the c-*myc* gene. Qualitatively, the c-*myc* gene cleavage pattern is identical in cell lines and lymphocytes with major cleavage bands located in the 5′ end of the gene (Riou et al., 1986a). This result indicates that the c-*myc* chromatin structure involved in the drug-induced Topo II cleavage activity is likely similar in all cells. However, cleavage intensity varies with the cell lines and is much higher than that found in lymphocytes.

Interestingly, our major finding indicates that the relative c-*myc* gene cleavage induced by mAMSA is not associated with gene amplification but rather with c-*myc* steady-state mRNA level; i.e., cell lines with high c-*myc* mRNA levels exhibited a more intense cleavage. This could be related to the proliferative state of the cell. The c-*myc* gene cleavage increases with its expression.

On the other hand, several reports using alkaline elution have indicated that overall genomic DNA cleavage induced by mAMSA was increased in proliferating or in transformed cells, explaining the cytotoxic effect of the drug (Sullivan et al., 1986; Potmesil et al., 1988). It was suggested that cleavage was directly related to the cellular Topo II activity which increased in proliferating cells (Duguet et al., 1983; Riou et al., 1985; Heck et al., 1986; Nelson et al., 1987). In cells resistant to VP16, the cytotoxicity was also shown to be roughly related to the Topo II activity (Ferguson et al., 1988). In this report, we show that the Topo II activity, and also the Topo II transcripts are higher in exponentially growing cells than in quiescent cells or in nondividing lymphocytes. We also found a rough relationship between the nuclear Topo II activity or Topo II mRNA levels and the c-*myc* cleavage.

Variations in growth conditions are known to modulate Topo II activity (Sullivan et al., 1986) and c-*myc* expression (Kelly et al., 1983). In quiescent N417 cells, the Topo II activity decreases but not the c-*myc* expression. However, the mAMSA-induced c-*myc* gene cleavage is qualitatively and quantatively unchanged. Moreover, experiments of sensitivity to DNase I performed on the c-*myc* chromatin in quiescent and growing N417 cells have shown that hypersensitive site patterns were identical (data not shown). These studies suggest that only a fraction of Topo II reacts in the presence of mAMSA with the c-*myc* chromatin, independently of the variations of Topo II activity.

Another hypothesis is that the c-*myc* gene cleavage observed in vivo depends on the chromatin accessibility to nuclear proteins associated with the transcriptional activity of the gene, thus explaining the relation between c-*myc* transcription and cleavage activity induced by mAMSA. It is quite intriguing that only the c-*myc* gene was shown to be cleaved. Indeed, no cleavage band could be evidenced in several other genes even if they are highly amplified and overexpressed as the c-*erb*B gene in the A431 cell line after a longer treatment of cells with mAMSA or VM26. Our results indicate that at IC_{50} the mAMSA-induced c-*myc* gene cleavage frequency is about 1 DSB per 7.2×10^4 nucleotides, corresponding to about 20 times the frequency (1 DSB per 1.5×10^6 nucleotides) found by alkaline elution on overall genomic DNA (Multon et al., 1989). Therefore, no definitive conclusions can be drawn about the lack of cleavage detection of these active genes. The Topo II mediated cleavage for these genes could also be located outside of the restriction fragments used for the drug ITCS analysis. In the c-*myc* gene, most of the drug ITCS occur in a 5-kb DNA region located at the 5′ end of the coding region of the gene (Riou, J. F., et al., 1986a, 1987). This cleavage region corresponds to the region of gene regulation (Chung et al., 1986) and is accessible to DNase I action (Dyson et al., 1985; Riou, J. F., et al., 1986a, 1987). By comparison, control elements and promotors of the other active genes analyzed here could be located far away from the regions involved in cleavage. This hypothesis is supported by results indicating that no DNase I hypersensitive cleavage sites could be evidenced in these genes using our analysis strategy (not shown).

In another attempt to detect drug ITCS, we analyzed the action of mAMSA in the VX2 cell line originating from a carcinoma induced by Shope virus in rabbit. This cell line contained a high number of viral DNA copies integrated in

the cellular genome and actively transcribed (George et al., 1985). Preliminary experiments indicated a high rate of drug-induced viral DNA cleavage occurring in specific positions of the viral genome (J.-F. Riou, unpublished results). The restriction fragments tested for the drug ITCS analysis encompassed the whole viral sequence.

Topo II, by its nicking–closing function, can modify the topological conformation of the chromatin and thus control chromatin transcription as reported in yeast in rRNA transcription (Brill et al., 1987). Drug cleavage activity could be directly related to the biological activity of the Topo II involved in RNA synthesis. According to this hypothesis, our results would indicate that when c-*myc* transcription increased, more Topo II molecules are trapped by the drug in a cleavable complex on the c-*myc* locus. In that respect, mAMSA, at cytotoxic concentration, decreases by 2-fold the rate of c-*myc* transcription in N417 cells (Table III). However, this decrease is maximum within 3 h of drug treatment, and 50% of the gene transcription is maintained even when the treatment is prolonged for 24 h. This result suggests that c-*myc* transcription is also partially under the control of a factor other than Topo II. That factor could be Topo I, since recent reports have shown that in yeast, Topo I can substitute Topo II for its activity in RNA synthesis (Brill et al., 1987) and also for the in vivo relaxation of the 2-μm plasmid (Saavedra et al., 1986). As shown in yeast, the segregation of sister chromatid at mitosis is the only essential function of Topo II (Holm et al., 1985). On the other hand, the c-*myc* gene region in which drug ITCS occurs is assumed to represent, by its DNase I hypersensitive cleavage sites, an accessible part of the gene chromatin structure. However, the biological role of these Topo II cleavage sites is not yet known; there is no evidence for an in vivo interaction of mAMSA or other antitumor drugs with Topo II on the DNA sites involved in Topo II function.

Finally, several reports have recently underlined the importance of the c-*myc* protein in the DNA replication process (Studzinski et al., 1986; Iguchiu-Ariga et al., 1987). Since activated c-*myc* gene (amplified and/or overexpressed) is often found associated with cancer progression (Little et al., 1983; Riou, G., et al., 1987), we suggest that this gene together with other genes having an important role in cellular proliferation might represent powerful targets for mAMSA or other related antitumor drugs such as anthracyclines, epipodophyllotoxins, and ellipticines in relation to their common mechanism of action on Topo II.

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