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# Analysis of topoisomerase II-mediated DNA cleavage of the c-*myc* gene during HL60 differentiation

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We have investigated the effect of mAMSA, a potent topoisomerase II inhibitor, on the c-*myc* proto-oncogene of the acute promyelocytic leukemia HL60 cell line during its differentiation. When HL60 cells were induced by dimethylsulfoxide (DMSO) to terminally differentiate, a rapid drop in the level of c-*myc* mRNA was observed, followed by an arrest of cell proliferation. In contrast, the level of topoisomerase II mRNA was transiently increased with a maximum at 6 h after DMSO addition and was then completely abolished after 48 h, indicating that topoisomerase II is activated during the onset of HL60 differentiation. In exponentially growing cells, treatment by mAMSA results in the formation of topoisomerase II-mediated double strand DNA breaks in the c-*myc* gene at positions where topoisomerase II would normally nick and reseal the two strands. In HL60 cells treated with both mAMSA and DMSO, the sites in the c-*myc* gene at which mAMSA had induced cleavage were not altered. However, a DNA cleavage site located at the end of the first c-*myc* exon (position +3100), was strongly stimulated by mAMSA and DMSO treatment. This site fell within a DNase I hypersensitive region encompassing the MYC intron factor 1 (MIF1) binding site, where transcription elongation is normally blocked during differentiation. These data indicate that a change of topoisomerase II binding to critical regulatory region of the c-*myc* gene is associated with the downregulation of this gene during differentiation.

Topoisomerase II; c-*myc* gene; Differentiation; mAMSA; HL60 cell

## 1. INTRODUCTION

The c-*myc* proto-oncogene plays a central role in the control of cell proliferation and differentiation [1,2]. Large variations in c-*myc* RNA levels occur during terminal differentiation [1,2] and mitogenic stimulation of quiescent cells [2–5]. The deregulation of c-*myc* expression, frequently observed in cancer cells, can be caused by gene amplification, chromosomal translocation, retroviral insertion or point mutation [1,2]. c-*myc* expression is controlled by different mechanisms, including change in transcription initiation or elongation, and mRNA stability [4,6,7]. Several positive and negative control elements located in the 5′ flanking region and in the vicinity of the promoter region have been shown to regulate the transcription of c-*myc* [8–10].

DNA topoisomerases are enzymes that solve the DNA topological problems linked to transcription, replication and chromosomal segregation during cell division [11]. Eukaryotic topoisomerase II is the cellular target of a variety of anticancer drugs, including mAMSA and etoposide [12]. These drugs poison topoisomerase II by stabilizing enzyme–DNA cleavage complexes [12]. Previous studies, in which specific topoisomerase II inhibitors were used as probes, have indicated that in the c-*myc* gene the DNA cleavage mediated by topoisomerase II occurs at specific positions corresponding to important regulatory elements located in accessible regions of the c-*myc* chromatin [13–15]. DNA cleavage induced by mAMSA in the 5′ region is also increased with the c-*myc* mRNA steady state level, corresponding to increased gene transcriptional accessibility [16].

In the present study, we have mapped the cleavage sites of topoisomerase II in the c-*myc* proto-oncogene of terminally differentiated and undifferentiated HL60 cells. We have identified a new topoisomerase II cleavage site near the MIF1 binding site, suggesting that topoisomerase II participates in the c-*myc* transcriptional regulation which occurs during cell differentiation.

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## 2. MATERIALS AND METHODS

### 2.1. *Drugs*

mAMSA (4′-(9-acridinylamino)-methansulfon-*m*-anisidide) was obtained from Substantia Laboratory (Parke Davis Division, Courbevoie, France). Drug stock solution in DMSO at 10 mM was prepared immediately before use. Further dilutions were made in deionized water. DMSO was purchased from Sigma Chemicals.

### 2.2. *Cell lines and culture conditions*

The human promyelocytic leukemia cell line HL60 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in suspension culture at 37°C under 5% $CO_2$ in a humidified atmosphere, in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 2 mM L-glutamine and 50 μg/ml gentamycin as described previously [17]. Cell growth was determined by Trypan blue dye exclusion.

2.3. *Northern blot analysis*

About $10^7$ HL60 cells were treated for the indicated time with 1.25% DMSO. Total cellular RNA was prepared by the guanidinium isothiocyanate/CsCl density gradient fractionation method [18]. Ten $\mu$g of each RNA sample were electrophoresed on a 1% agarose gel containing formaldehyde transferred to a Hybond $N^+$ membrane (Amersham) and hybridized with the c-*myc*, topoisomerase II or $\beta$-actin probes. Membranes were washed under stringent conditions as described previously [18]. DNA/RNA hybrids were revealed by autoradiography on Amersham MP hyperfilms. Signals were quantified by densitometry (Pharmacia LKB ultroscan XL) and results normalized against $\beta$-actin.

2.4. *In vivo analysis of topoisomerase II cleavage sites*

About $10^6$ HL60 cells in the exponential phase of growth were treated for the indicated time with 1.25% DMSO and were exposed to 5 $\mu$M mAMSA for 30 min at the end of the DMSO treatment. 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 and cells were incubated for 12 h at 50°C. Lysates were then treated with phenol, phenol–chloroform and chloroform, dried and resuspended in Tris-HCl, pH 7.5, 0.5 mM EDTA at a concentration of 2 mg/ml. DNA (10 $\mu$g) were digested with *Eco*RI restriction endonuclease and electrophoresed in 1.2% agarose gel. DNA fragments were transferred onto a Hybond $N^+$ membrane (Amersham) and hybridized with the c-*myc* probe. Hybrids were revealed after 48 h by autoradiography on hyperfilm MP. Analysis of peak intensity was done by densitometry using the Pharmacia LKB Ultroscan XL apparatus, and band size was determined using a specific computer program of the Ultroscan XL, with fragments of phage I DNA digested with *Hin*dIII used as internal markers.

2.5. *Probes*

The probes used were the 1.4 kb *Cla*I-*Eco*RI insert of the human c-*myc* third exon [19], the 1.8 kb *Eco*RI insert of the human topoisomerase II$\alpha$ cDNA [20] and the 1.15 *Pst*I insert of the murine $\beta$-actin gene [21]. Probes were nick-translated with [$\alpha$-$^{32}$P]dATP and dCTP (3000 Ci/mmol, Amersham) to a specific activity of $5 \times 10^8$ dpm/$\mu$g.

## 3. RESULTS

HL60 cells consist of a majority of promyelocytes which differentiate into functionally and morphologically mature granulocytes in response to DMSO treatment. According to Collins et al. [17], HL60 cells treated for 48 h with 1.25% DMSO stop dividing, become strongly adherent on culture dish and undergo morphological changes, but 95% remain viable, when viability is assessed by Trypan blue dye exclusion.

To determine the level and kinetics of c-*myc* and topoisomerase II transcription during differentiation, HL60 cells were induced to differentiate with DMSO, and RNA was extracted at various times afterwards. Equal amounts of RNA were transferred to nitrocellulose filters and probed with c-*myc*, topoisomerase II and $\beta$-actin. The results, presented in Fig. 1, showed that 3 h after the induction of differentiation, the c-*myc* mRNA level had nearly disappeared and remained low 48 h following the DMSO treatment. Topoisomerase II mRNA level was found to be 2-fold increased at 6 h after the addition of DMSO and then was progressively decreased until a complete disappearance at 48 h (Fig. 1).

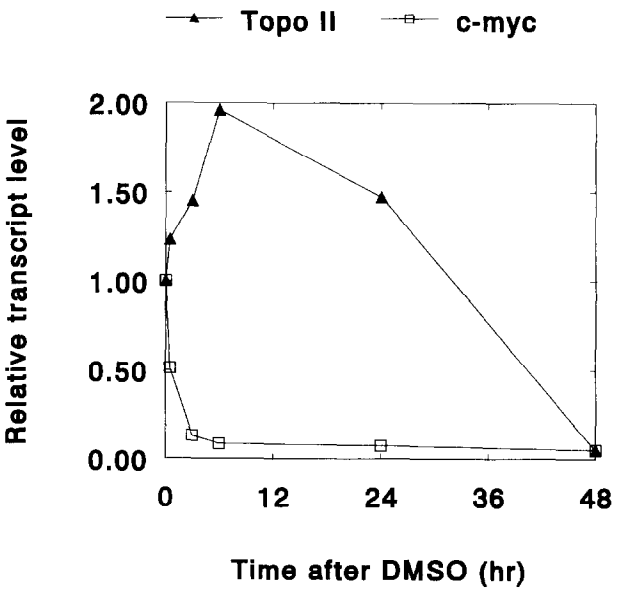


Fig. 1. Relative c-*myc* and topoisomerase II mRNA levels in HL60 cells during differentiation. HL60 cells were induced to differentiate with 1.25% DMSO for the indicated time and RNA extracted. Ten $\mu$g of RNA were electrophoresed, transferred on Hybond $N^+$ and hybridized with the c-*myc*, topoisomerase II and $\beta$-actin probes (see section 2). Peak intensity of the mRNA signals were quantified by densitometry on the autoradiograms (Ultroscan XL) and values were normalized with the $\beta$-actin values. Results are expressed relative to the mRNA level of DMSO-untreated HL60 cells (defined as 1).

In order to determine whether DMSO-induced downregulation of c-*myc* gene may alterate the in vivo topoisomerase II cleavage sites, we have examined here the c-*myc* DNA from HL60 cells treated with mAMSA as already described [13]. When DNA from untreated HL60 cells was digested with *Eco*RI and probed with the 1.4 kilobase pairs *Eco*RI-*Cla*I fragment of c-*myc*, a 13 kilobase DNA band was deteced (Fig. 2, lane 1) which is markedly amplified, as previously described [4]. The DNA pattern from mAMSA-treated HL60 cells during the exponential phase of growth (Fig. 2, lane 2) showed additional DNA bands of lower molecular weight, indicating the presence of topoisomerase II sites, as already described [13,14]. The c-*myc* DNA, prepared from HL60 cells at different times (0, 3, 6, 24 and 48 h) after DMSO treatment (Fig. 2, lanes 3–7), had no cleavage sites and were comparable to that of control untreated HL60 cells (lane 1). When HL60 cells were treated for 30 min with 5 $\mu$M mAMSA at different times after the addition of DMSO (3, 6, 24 and 48 h, see lanes 8, 9, 10 and 11, respectively), the c-*myc* DNA cleavage pattern was modified: mAMSA strongly stimulated DNA cleavage at a site located in the intro between exons 1 and 2 (indicated by an arrowhead). This cleavage site was barely detectable in non-differentiated cells, treated with mAMSA (lane 1).

In order to quantify and to localize more precisely the new site induced in c-*myc*, the autoradiogram shown in

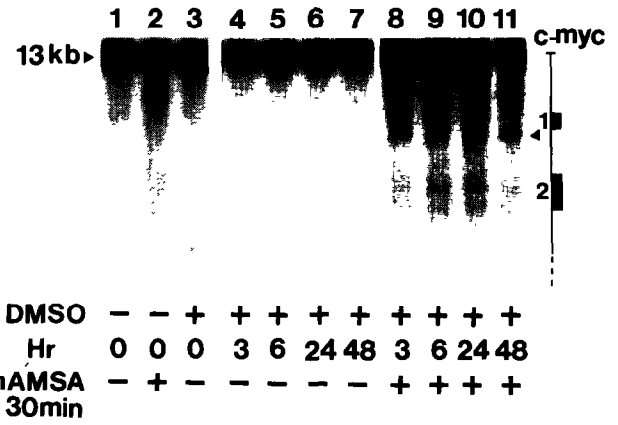


Fig. 2. In vivo DNA cleavage sites induced by mAMSA in the c-*myc* gene during differentiation. Control untreated HL60 cells (lane 1). Undifferentiated HL60 cells treated with 5 $\mu$M mAMSA for 30 min (lane 2). HL60 cells treated with 1.25% DMSO for the indicated time (0–48 h) are presented in lanes 3–7. HL60 cells differentiated with 1.25% DMSO for the indicated time (3–48 h) and treated with 5 $\mu$M mAMSA for 30 min at the end of the DMSO treatment are presented in lanes 8–11. After treatment, cells were lysed by SDS and DNA extracted (see section 2). DNA samples (10 $\mu$g) were digested with *Eco*RI, transferred on Hybond $N^+$ and hybridized with the c-*myc* probe. Arrowhead indicated the topoisomerase II cleavage site induced during differentiation. Schematic map of the c-*myc* gene is presented on the right.

Fig. 2 was scanned with a laser densitometer. The densitometric tracing of the c-*myc* gene obtained from untreated HL60 (lane 1), mAMSA- or mAMSA plus DMSO-treated HL60 cells (lanes 2 and 11, respectively) are shown in Fig. 3. The intensity of the cleavage was normalized relatively to the 13 kilobase c-*myc* DNA band from untreated HL60 cells. The mAMSA cleavage site stimulated by DMSO treatment was localized around position +3100 ± 200 base pairs from the upstream *Hin*dIII site, according to the sequence published by Gazin et al. [22]. The analysis also indicated that the intensity of cleavage induced by mAMSA at position +3100 was equal to 19.5% and 3.5% of the total c-*myc* gene cleavage generated for differentiated or undifferentiated HL60 cells, respectively. After DNA content normalization, the other DNA cleavage sites stimulated by mAMSA did not have intensities significantly modified by the DMSO treatment.

## 4. DISCUSSION

Earlier studies have shown that the activity of topoisomerase II is significantly affected by the growth state of the cell [23]. Differentiation can be induced chemically in several human and murine leukemia cell lines and is associated with a significant decrease in topoisomerase II activity [24,25] and mAMSA-induced DNA protein crosslinks [26,27]. Early events such as c-*myc* gene downregulation have also been described [3,4]. In accordance with these studies we found that treatment of HL60 cells with 1.25% DMSO for 48 h led to a complete disappearance of the c-*myc* and topoisomerase II gene transcripts. However, while c-*myc* downregulation occurs within 3 h after the differentiation induction, topoisomerase II downregulation appears to be a later event. Our results also indicated that the topoisomerase II mRNA level is transiently increased, with a maximum at 6 h after the start of the differentiation. Due to the important functions of topoisomerase II during replication, transcription and mitosis [11,12], the topoisomerase II decrease at 48 hours would probably correspond to the arrest of the cell proliferation, while the transient topoisomerase II increase would reflect transcriptional processes activated at the differentiation onset.

The present study also demonstrated that mAMSA altered the DNA cleavage patterns in the c-*myc* protooncogene during DMSO-induced differentiation. Interestingly, the pre-existing topoisomerase II cleavage sites in the c-*myc* gene, detected during the exponential phase of growth of HL60 cells, were not greatly modified by mAMSA during the time course of DMSO-induced differentiation. However, mAMSA induced cleavage at a distinct additional site which corresponds to the beginning of the first intron in the c-*myc* locus, 3100 base pairs from the start of the gene. Furthermore, in previous in vitro studies, a strong mAMSA-induced cleavage site had been mapped at this position by using purified

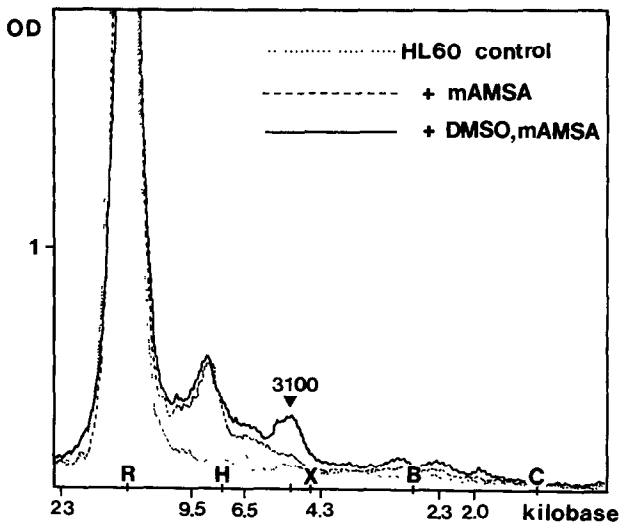


Fig. 3. Densitometric scan of the c-*myc* DNA electrophoretic pattern presented in Fig. 2: control untreated HL60 cells (.....), undifferentiated HL60 cells treated with 5 $\mu$M mAMSA (------), differentiated HL60 cells (48 h) treated with 5 $\mu$M mAMSA (—). The intensity of the curves were normalized relatively to the 13 kilobase pairs (*Eco*RI-*Eco*RI) c-*myc* DNA band from untreated HL60 cells. The *x*-Axis indicate the DNA marker scale in kilobase and the position of some restriction enzyme sites in the c-*myc* gene (R = *Eco*RI, H = *Hin*dIII, X = *Xba*I, B = *Bam*HI, and C = *Cla*I). The *y*-Axis indicate the optical density. Band size and peak intensity analysis were determined using the Pharmacia LKB Ultrascan XL programs. Arrow and number indicate the topoisomerase II cleavage site induced during differentiation and its position in base pairs relatively to the *Hin*dIII site, according to the sequence published by Gazin et al. [22].

topoisomerase II and c-*myc* DNA [15,28]. These data indicate that a potential topoisomerase II cleavage site, which is masked during the growth of untreated HL60 cells, is revealed by mAMSA during the differentiation process. In previous studies, we reported that the topoisomerase II cleavage sites located in the 5′ non-coding region of the c-*myc* gene are associated with the increased gene transcription, reflecting the accessibility of this portion of the chromatin to topoisomerase II [13–16]. Our present results indicate that a different topoisomerase II cleavage site is associated with the transcriptional downregulation of the c-*myc* gene during DMSO-induced differentiation. In fact, the first half of c-*myc* intron 1 has been implicated as a negative transcription regulatory region for this gene [8]. Interestingly, the topoisomerase II cleavage site identified here was located within the DNA binding site of the MIF1 (Myc intron factor 1) [29], which has been identified as a methylated DNA-binding protein [30]. This element was frequently found to be mutated in Burkitt's lymphoma and these mutations have been implicated in the deregulation of c-*myc* gene transcription [31,32]. A DNase I hypersensitive site located about 300 base pairs downstream from the 5′ end of the first intron (position +3200) also appears during HL60 differentiation, and the decreased c-*myc* transcription was shown to be due to a block of the elongation of the transcript in this region [4].

In conclusion, these data suggest that topoisomerase II is associated during differentiation with this negative regulatory region of c-*myc* and may participate to the its transcriptional regulation.

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