Transfection of human topoisomerase IIα into etoposide-resistant cells: transient increase in sensitivity followed by down-regulation of the endogenous gene

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We have investigated the possibility of overcoming the resistance of human brain tumour cells (HBT20) to etoposide by transferring the normal human topoisomerase IIα (H-topo II) gene into these cells. H-topo II in a mammalian expression vector containing a glucocorticoid-inducible mouse mammary tumour virus (MMTV) promoter was transfected into etoposide-resistant HBT20 cells (HBT20-hTOP2MAM). HBT20 cells transfected with pMAMneo vector alone served as control cells (HBT20-MAM). These were stable transfections. Following a 2 h dexamethasone treatment, H-topo II mRNA expression, protein production, etoposide-induced DNA–protein complex formation and sensitivity to etoposide were increased in HBT20-hTOP2MAM cells compared with control HBT20-MAM cells and with HBT20-hTOP2MAM cells not treated with dexamethasone. However, mRNA and protein levels and cell sensitivity returned to baseline when incubation with dexamethasone was continued for 24 h. This decrease from the 2 h values could not be explained by a loss of the MMTV promoter response to dexamethasone. (H-topo IIα promoter)–(chloramphenicol acetyltransferase) constructs containing regions −559–0 and −2400–0 were significantly down-regulated in HBT20-hTOP2MAM cells treated for 24 h with dexamethasone compared with dexamethasone-treated control cells. H-topo II mRNA stability after 24 h of dexamethasone treatment was not altered compared with that in control cells. Our data indicate that the exogenously produced H-topo II may have a negative-feedback effect on the endogenous topoisomerase II promoter, causing down-regulation of the endogenous gene.

INTRODUCTION

Topoisomerase II is an essential enzyme for cell division [1]. It is also a major target for a variety of active anti-neoplastic agents [2]. The anti-cancer mechanism appears to reside in the ability of the anti-neoplastic drug to inhibit re-ligation of the DNA cleavage produced by topoisomerase II as it performs its essential task of passing double strands of DNA through other double strands [3]. The signature of this drug-induced inhibition is the production of protein-associated DNA breaks that represent stabilization of the topoisomerase II–DNA complex by the drugs [4]. Resistance to topoisomerase II-reactive anti-neoplastic drugs involves quantitative or qualitative differences between topoisomerase II in drug-sensitive cells and the enzyme in drug-resistant cells. Low levels of topoisomerase II in quiescent cells lead to decreased formation of drug-stabilized cleavable topoisomerase II–DNA complexes [2,4,5]. Mutant topoisomerase II enzyme, which is resistant to stabilization by the drugs in a complex with DNA, also leads to drug resistance [6–9].

Several systems have been reported that use transfection with the topoisomerase II gene to investigate the effect of this gene on cellular drug sensitivity [10–12]. We have shown that gene transfer of *Drosophila* topoisomerase II (D-topo II) to *de novo* etoposide-resistant tumour cells partially circumvents their drug resistance, but the time frame of this sensitization was short [13]. In the present paper we demonstrate that transfection of the human topoisomerase IIα (H-topo II) gene into brain tumour cells and expression of this transfected gene also increases both cell sensitivity to etoposide and the total amount of topoisomerase II. However, this increased sensitivity was once again short-lived. Furthermore, renormalization of the levels of total (endogenous plus exogenous) H-topo II mRNA and protein occurs shortly after induction of the exogenous topoisomerase II gene. Our data suggest that the transient nature of the increased sensitivity to etoposide may be due to down-regulation of the endogenous gene, resulting in an overall decrease in cellular H-topo II protein (the molecular target of etoposide). This down-regulation appears to be at the transcriptional level through an effect on the promoter region.

MATERIALS AND METHODS

Reagents and drugs

Dulbecco's modified Eagle's medium, Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}, fetal calf serum, gentamicin and L-glutamine were purchased from Whittaker Bioproducts, Inc. (Walkersville, MD, U.S.A.). Anti-(H-topo II) polyclonal antibody was obtained from TopoGen, Inc. (Columbus, OH, U.S.A.). Etoposide was a gift from Dr. B. Long and Dr. J. H. Keller (Bristol-Myers Co., Syracuse, NY, U.S.A.) or was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and was solubilized in DMSO.

Cell line

The human brain tumour cell line HBT20 was kindly provided by Dr. F. Ali-Osman (Department of Experimental Pediatrics, M. D. Anderson Cancer Center). This glioblastoma multiform cell line was established from a human brain tumour specimen resected from a patient who had received no previous chemo-

Abbreviations used: CAT, chloramphenicol acetyltransferase; H-topo II, human topoisomerase IIα; D-topo II, *Drosophila* topoisomerase II; Dex, dexamethasone; MMTV, mouse mammary tumour virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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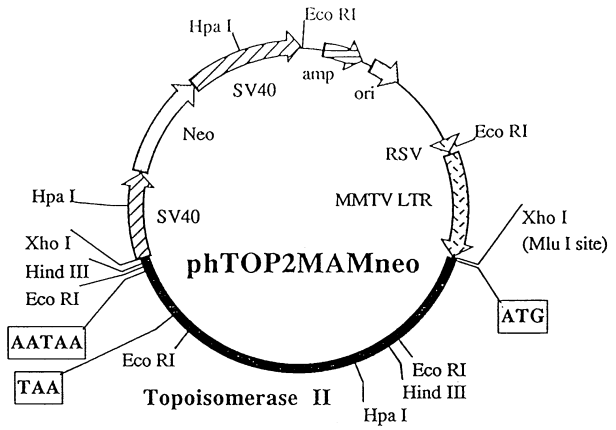

Figure 1 Plasmid pMAMneo with the H-topo IIα gene inserted into an *Xho*I site

SV40, simian virus 40.

therapy. These cells were cultured in Dulbecco's modified Eagle's medium with 10% (v/v) fetal calf serum, 2 mM glutamine and 50 μg/ml gentamicin (D10 medium). All cells were free of mycoplasma as screened by Gen-Probe (San Diego, CA, U.S.A.) or the American Tissue Culture Collection (Rockville, MD, U.S.A.).

Transfection of tumour cells

pMAMneo, a mammalian expression vector containing a glucocorticoid-inducible mouse mammary tumour virus (MMTV) promoter, was obtained from Clontech Laboratories (Palo Alto, CA, U.S.A.). Normal H-topo II cDNA [14] in pBluescript KS+ was obtained from American Type Culture Collection. No mutations were found in 10% of the 5′ end of this H-topo II cDNA by sequencing using Sequenase Version 2.0 (Amersham Life Science, Cleveland, OH, U.S.A.) (results not shown). H-topo II cDNA was obtained by digestion with *Mlu*I and *Xho*I. *Xho*I linkers were ligated on each end, and this DNA was ligated into the *Xho*I site of the pMAMneo vector to create phTOP2MAMneo (Figure 1). Sense or antisense orientation was determined by *Hpa*I, *Bgl*II, *Bam*HI, *Eco*RI and *Hin*dIII digestion. Transfection was performed 24 h after seeding of 10^5 cells in a T-75 flask (Costar, Cambridge, MA, U.S.A.) by calcium phosphate co-precipitation with 20 μg of phTOP2MAMneo or pMAMneo (transfection control). After 24 h of exposure at 37 °C the medium was removed, and cells were maintained in D10 medium for 3 days. Cultures were then selected in G418 (0.8 mg/ml; Gibco, Grand Island, NY, U.S.A.), and expanded. These cells were pooled and do not represent a single clone. Because topoisomerase II is suppressed in confluent cells (results not shown), all experiments were performed with cells at < 70% confluence.

Northern blot analysis

Samples of 20 μg of total RNA were extracted and size-fractionated using electrophoresis gels. The RNA was then transferred and hybridized with a H-topo II gene probe (a gift from Dr. L. Liu, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ, U.S.A.) [4] or a β-actin probe [15]. Because the inserted H-topo II gene has a translational termination and a potential poly(A) consensus sequence (Figure 1 and [14]), we could not discriminate transfected exogenous H-topo II mRNA from endogenous H-topo II mRNA.

For analysis of mRNA half-life, tumour cells were incubated with or without dexamethasone (Dex) for 24 h, and then the cells were washed to remove Dex. Actinomycin D (Sigma) at 10 μg/ml was then added to the culture medium. At the indicated times (0, 4, 8 and 24 h), total RNA was extracted as described above [15]. Densitometric analysis of Northern blots was performed using a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.), and values were normalized to the expression of β-actin.

Immunoblotting

For H-topo II protein detection, a modification of the method of Kauffmann et al. [16] was employed. Cells (1×10^7) were incubated with or without 10 μM Dex, then sonicated by 40 bursts at 60% power (Heat Systems-Ultrasonics sonicator) in alkylation buffer (6 M guanidine/HCl, 250 mM Tris, pH 8.5, 10 mM Na_2EDTA) containing 1% 2-mercaptoethanol and 1 mM PMSF. The reaction mixtures were allowed to reduce overnight, after which 100 μl of 1.5 M iodoacetamide in alkylation buffer was added to each 1.02 ml sample, followed by incubation for 1 h at room temperature; 10 μl of 2-mercaptoethanol was then added. Each sample was dialysed for 90 min against 4 M urea/50 mM Tris, pH 7.4, 4×90 min against 4 M urea and 3×90 min against 0.1% SDS, and then lyophilized for storage. Samples were solubilized in SDS sample buffer (4 M urea, 2% SDS, 62.5 mM Tris/HCl, pH 6.8, 1 mM Na_2EDTA) and electrophoresed in a 6% polyacrylamide gel. Immunoblotting was performed using an ECL® Western blotting analysis system (Amersham, Arlington Heights, IL, U.S.A.) according to the manufacturer's instructions with a 1:500 dilution of anti-(H-topo II) polyclonal antibody. India Ink staining was used to check the loading of protein in each lane [17].

SDS/KCl precipitation assay

Cells (4×10^5) from each of the transfectants (with or without Dex pretreatment) were radiolabelled with [^{3}H]thymidine deoxyribose (ICN Biomedicals, Irvine, CA, U.S.A.) and [^{14}C]leucine (Amersham) for 24 h at 37 °C. The cells were then washed and chased with medium for 1 h prior to incubation with DMSO or with various concentrations of etoposide for 1 h. The cells were lysed and the DNA–protein complexes precipitated as previously described [6].

Colony formation assay

Portions of 300 cells were plated in 35 mm 6-well plates (Costar). Expression of the H-topo II gene was induced by exposure to 10 μM Dex for 2 or 24 h prior to 2 h of etoposide treatment. After drug treatment, cells were washed twice with PBS and re-fed with D10 medium. Colonies were allowed to form for 12 days; they were then stained with 0.04% Crystal Violet in methanol and counted. Results were expressed as the survival fraction compared with the colony-forming efficiency of the untreated control.

Nuclear run-on transcription assay

HBT20-hTOP2MAM or HBT20-MAM cells [$(5–7) \times 10^7$] were incubated with or without Dex for 24 h, and then washed three times with cold PBS and suspended in hypotonic buffer (20 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM NaCl, 0.5 mM dithiothreitol, 0.3 M sucrose, 0.25% Nonidet P40) on ice for 5 min. The detergent-treated tumour cells were then layered on

to an equal volume of isolation buffer (20 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM NaCl, 0.5 mM dithiothreitol, 0.6 M sucrose) and centrifuged at 500 ***g*** for 10 min. The supernatant was aspirated, and the pelleted nuclei were gently resuspended in 500 μl of transcription buffer (10% glycerol, 20 mM Hepes, pH 7.8, 1 mM $MgCl_2$, 2 mM $MnCl_2$, 142 mM KCl) containing 0.25 μM each of CTP, ATP and GTP, 1.25 μM dithiothreitol, 0.75 μM spermidine, 5 μl of RNasin (Promega Biotech, Madison, WI, U.S.A.) and 100 μCi of [α-^{32}P]UTP (Amersham), and incubated at 30 °C for 30 min with gentle shaking. The reaction mixture was treated with 12.5 μg of RQ1 RNase-free DNase (Promega Biotech) and incubated at 30 °C for 5 min, followed by addition of 100 μg of proteinase K, 4 μl of 0.2 M EDTA, 17.5 μl of 10% SDS and 20 μg of yeast tRNA. The mixture was incubated at 40 °C for 45 min, extracted with phenol/chloroform (1:1, v/v) and precipitated in ethanol/sodium acetate, and the pellet was dissolved and precipitated in 10% trichloroacetic acid, 30 mM sodium pyrophosphate and 1 mM UTP. Finally, the mixture was precipitated in ethanol/sodium acetate. The nuclear run-on transcript was resuspended in 500 μl of hybridization buffer (50% formamide, 6 × SSPE, 5 × Denhardt's solution, 0.1% SDS and 200 μg/ml denatured salmon sperm DNA) and hybridized with DNA immobilized filters at 44 °C for 4 days. The procedure allowed the incorporation of (5–10) × 10^6 c.p.m. (total) into (5–7) × 10^7 isolated nuclei.

DNA immobilized filters were prepared as follows: 40 μg of linear DNA containing the target sequence {H-topo IIα gene probe and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [15]} was denatured with 0.8 M NaOH for 30 min at room temperature. The filters were then neutralized with 200 μl of 10 × SSC, and the denatured DNA solution was filtered immediately through a nylon filter membrane and UV cross-linked. After hybridization, the filters were washed with 0.1% SDS, 2 × SSC and 2 mM EDTA at room temperature for 3 × 30 min and then with 0.1% SDS, 0.1 × SSC and 2 mM EDTA at 50 °C for 3 × 30 min. The filters were exposed at −70 °C for 7–10 days. Scanning density was determined as above and values were normalized for differences in GAPDH scanning densities. Relative transcriptional activity is calculated as follows: relative transcriptional activity = density of the H-topo II probe/GAPDH density on the same filter.

Chloramphenicol acetyltransferase (CAT) assays

The (H-topo II gene promoter)–CAT constructs were generously provided by Dr. I. D. Hickson (Imperial Cancer Research Fund, University of Oxford, U.K.) [18]. The (MMTV promoter)–CAT construct was obtained from Clontech. Transfection of CAT constructs was carried out by calcium phosphate precipitation with 20 μg of CAT constructs. At 48 h after the transfection at 37 °C, the cells were harvested by scraping, suspended in 200 μl of 0.25 M Tris/HCl (pH 7.9) and lysed by three cycles of freezing and thawing. The protein concentration in each sample was measured using the Bio-Rad Detergent-Compatible Protein Assay (Bio-Rad, Hercules, CA, U.S.A.), and an identical amount of protein from each sample was used for the CAT assay. Aliquots of each sample (total 30 μl) were heated to 65 °C for 10 min to inactivate endogenous deacetylases and then incubated for 2 h at 37 °C with 3 mM acetyl-CoA (Sigma) and 0.05 μCi of [^{14}C]deoxychloramphenicol (Amersham). The products were extracted with ethyl acetate, and acetylated products were suspended on TLC plates (Eastman-Kodak, Rochester, NY, U.S.A.). The plates were then exposed to X-ray film. For quantification of the percentage acetylation of chloramphenicol, densitometric analysis was performed using a Personal Densitometer® as described above.

RESULTS

Effect of H-topo II transfection on H-topo II expression and protein level in HBT20 cells

Northern blot analysis of HBT20-hTOP2MAM cells showed that increased expression of H-topo II mRNA was observed following 2 h of Dex treatment (Figure 2). HBT20-Parent and HBT20-MAM (transfected with the pMAMneo vector as a control) cells showed no alteration in H-topo II mRNA following 2 h of Dex treatment (Figure 2). In contrast, after 24 h of Dex treatment, H-topo II mRNA expression in HBT20-hTOP2MAM cells was decreased compared with that in HBT20-Parent or HBT20-MAM cells, or in HBT20-hTOP2MAM cells without Dex treatment (Figure 3). HBT20-hTOP2MAM cells without Dex treatment showed similar H-topo II mRNA expression to

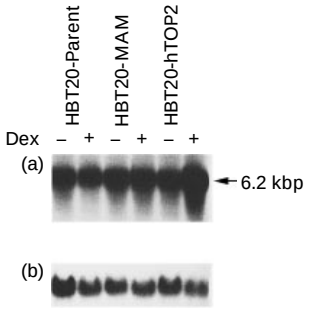

Figure 2 Northern blot analysis of HBT20-Parent, HBT20-MAM (control) and HBT20-hTOP2MAM cells after 2 h of Dex treatment

After a 2 h incubation with (+) or without (−) 10 μM Dex, total RNA was harvested and hybridized with the H-topo IIα probe (**a**) and a β-actin probe (**b**). HBT20-MAM cells are HBT20 cells transfected with the pMAMneo vector; HBT20-hTOP2 cells are HBT20 cells transfected with phTOP2MAMneo.

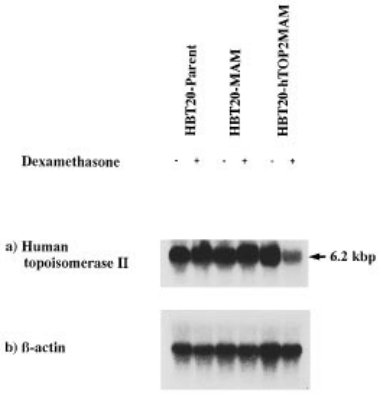

Figure 3 Northern blot analysis of HBT20-Parent, HBT20-MAM (control) and HBT20-hTOP2MAM cells after 24 h of Dex treatment

After 24 h incubation with (+) or without (−) 10 μM Dex, total RNA was harvested and hybridized with the H-topo IIα probe (**a**) and a β-actin probe (**b**).

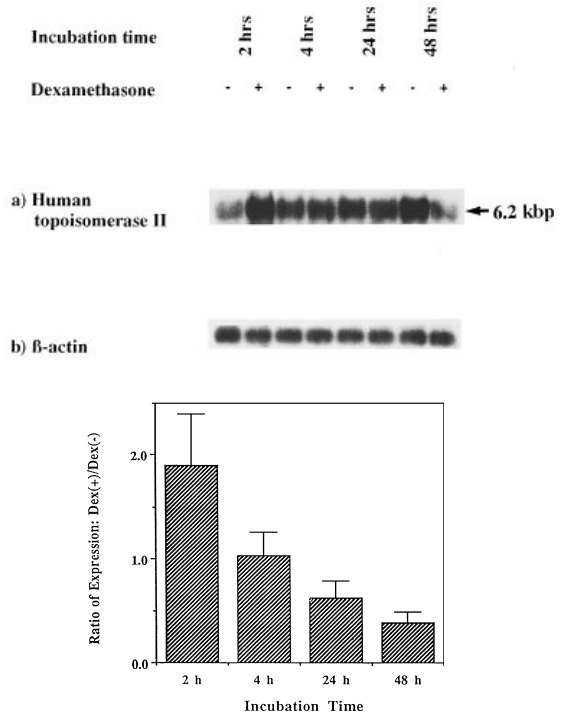

Figure 4 Northern blot analysis of HBT20-hTOP2MAM cells: effect of duration of Dex treatment

After 2, 4, 24 and 48 h of incubation with (+) or without (−) 10 μM Dex, total RNA was harvested and hybridized with the H-topo II α probe (**a**) and a β-actin probe (**b**). The bar graph shows densitometric analysis of H-topo II gene expression; values are means ± S.D. from three independent experiments.

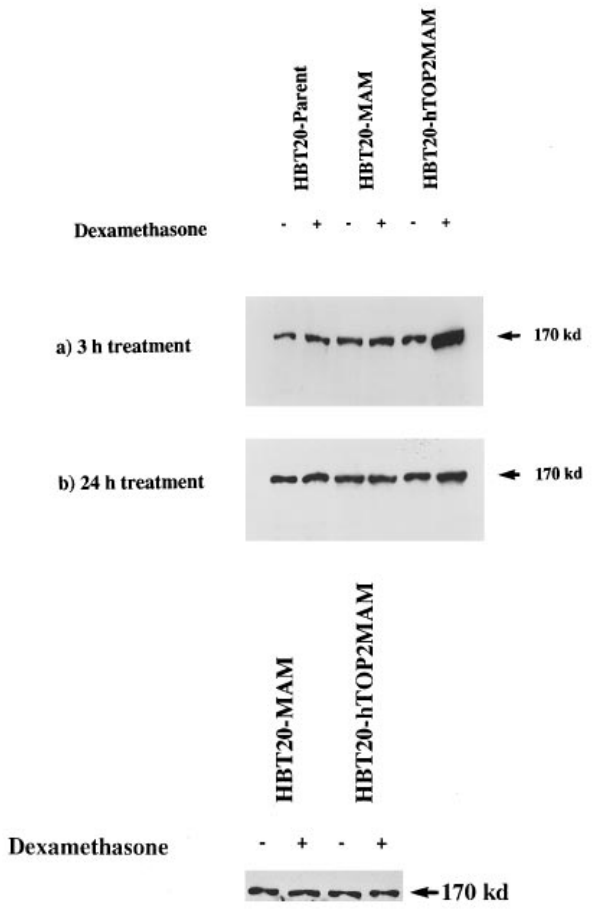

Figure 5 Immunoblotting in HBT20-Parent, HBT20-MAM and HBT20-hTOP2MAM cells

After treatment with or without Dex for 3 h (top), 24 h (middle) or 48 h (bottom), the cells were harvested and protein was extracted as detailed in the Materials and methods section. Samples (20 μg of protein) were electrophoresed in 6% polyacrylamide gels and stained with rabbit anti-(H-topo II) antibody. The position of H-topo II at 170 kDa (kd) is indicated. Protein loading was checked using the India Ink method [17]. All lanes showed equivalent staining.

that in HBT20-Parent and HBT20-MAM cells. We determined the kinetics of H-topo II mRNA expression in HBT20-hTOP2MAM cells. As shown in Figure 4, H-topo II mRNA was increased after 2 h of Dex treatment, similar at 4 h and decreased at 24 and 48 h compared with that in HBT20-hTOP2MAM cells without Dex treatment. By contrast, Dex treatment had no effect on the H-topo II mRNA expression of HBT-Parent and HBT-MAM cells (results not shown).

We quantified the H-topo II protein levels in HBT20-Parent, HBT20-MAM and HBT20-hTOP2MAM cells with or without Dex treatment for 3 and 24 h. Following 3 h of Dex treatment, HBT20-hTOP2MAM cells contained increased H-topo II protein (Figure 5, top panel). However, this increase in H-topo II protein was less by 24 h and no longer evident by 48 h (Figure 5, middle and bottom panels).

DNA–protein complex formation induced by etoposide

Formation of precipitable DNA–protein complexes following exposure of cells to etoposide was used to assess whether the H-topo II protein produced in the transfected cells was functional. As shown in Table 1, formation of cleavable DNA–protein complexes induced by 10 μM etoposide was significantly increased in HBT20-hTOP2MAM cells following 2 h of Dex treatment ($P < 0.05$). Similar to the mRNA and protein results above, after 24 h of Dex treatment no differences in DNA–protein complex formation could be detected compared with HBT20-MAM cells or HBT20-hTOP2MAM cells without Dex pre-treatment.

Effect of H-topo II transfection on sensitivity of cells to etoposide

Colony-forming assays were used to assess cell sensitivity and showed similar time course results (Figure 6). HBT20-hTOP2MAM cells pretreated for 2 h with Dex showed a significant increase in sensitivity to 10 μM etoposide compared with HBT20-Parent and HBT20-MAM cells, and with HBT20-hTOP2MAM without Dex treatment ($P < 0.05$). HBT20-Parent and HBT20-MAM cells with or without Dex treatment and HBT20-hTOP2MAM cells without Dex treatment were all more resistant to 10 μM etoposide. The increased sensitivity of HBT20-hTOP2MAM cells was not evident following 24 h of Dex treatment. The loss of sensitivity and decrease in mRNA expression and protein levels at 24 h could not be explained by a change in the response of the MMTV promoter to Dex. As shown in Figure 7, when HBT20-hTOP2MAM cells were transfected with the pMAMneo-CAT vector (MMTV-CAT), increased CAT activity was demonstrated for up to 48 h of Dex stimulation.

Cell cycle analysis measured by flow cytometry showed no significant difference between HBT20-MAM and HBT20-

Table 1 Formation of precipitable DNA–protein complexes as assessed by SDS/KCl assay

Values are expressed as a ratio of [^{3}H]thymidine/[^{14}C]leucine in etopside-treated cells divided by the ^{3}H/^{14}C ratio of untreated cells with or without Dex pretreatment. Values are means ± S.D. from three independent experiments. * $P < 0.05$ for Dex-treated HBT20-hTOP2MAM cells compared with untreated HBT20-hTOP2MAM cells and with HBT20-MAM cells treated or not with Dex (Students' t test).

		Complex formation			
		HBT20-MAM cells		HBT20-hTOP2MAM cells	
[Etoposide] (μM)	Duration of Dex treatment (h)	−Dex	+Dex	−Dex	+Dex
10	2	3.5 ± 0.3	3.6 ± 0.3	3.9 ± 0.1	5.3 ± 0.4*
10	24	3.9 ± 0.2	3.1 ± 0.4	3.8 ± 0.2	3.6 ± 0.2
50	2	7.3 ± 0.8	6.0 ± 1.4	8.2 ± 0.6	8.7 ± 1.3
50	24	6.6 ± 0.2	4.6 ± 0.5	5.5 ± 0.3	6.3 ± 0.3

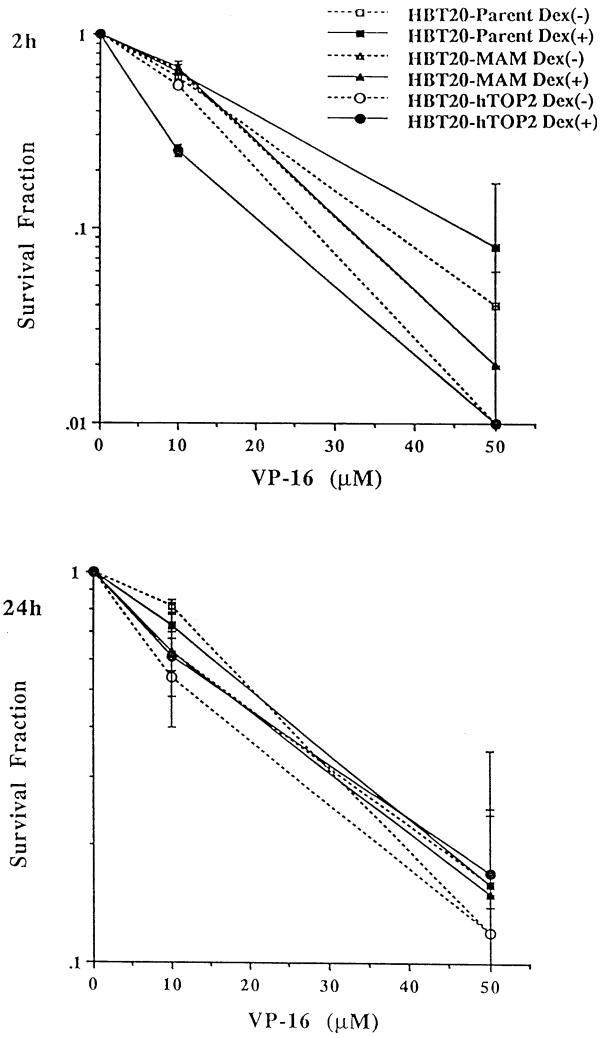

Figure 6 Survival of HBT20-Parent, HBT20-MAM and HBT20-hTOP2MAM cells measured using the colony-forming assay

Portions of 300 tumour cells were pretreated with or without Dex for 2 h (upper panel) or 24 h (lower panel) and then exposed to etoposide (VP-16) for 2 h. The cells were washed, refed and cultured for 12 days. The values are means ± S.D. from three independent experiments.

hTOP2MAM cells either with or without Dex treatment for 2–24 h (results not shown). Cell growth analysis also showed that the mean doubling times of the cell lines were not significantly

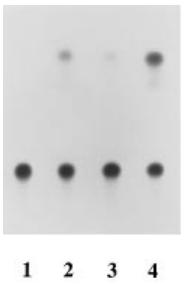

Figure 7 Effect of Dex on transfected pMAMneo-CAT in HBT20-hTOP2MAM cells

Cells were transfected with pMAMneo-CAT and then treated with or without Dex for 2 or 48 h. Cells were then harvested, and the CAT assay was performed as described in the Materials and methods section. Lane 1, 10 μM Dex for 48 h; lane 2, CAT construct without MMTV promoter with 10 μM Dex for 48 h; lane 3, pMAMneo-CAT (MMTV-CAT) without 10 μM Dex for 48 h; lane 4, MMTV-CAT with 10 μM Dex for 48 h.

different and were not affected by Dex treatment. Therefore a Dex-dependent growth arrest of HBT20-hTOP2MAM cells could not explain the variation in topoisomerase II transcription or cell sensitivity to etoposide.

Effect of H-topo II transfection on transcription rate of H-topo II, H-topo II mRNA stability and endogenous H-topo II promoter activity

To determine whether the decreased level of H-topo IIα mRNA in HBT20-hTOP2MAM cells after 24 h of Dex treatment was related to a decrease in the transcriptional activity of the gene, we compared the H-topo II transcription rates in nuclei isolated from Dex-treated and untreated tumour cells. As shown in Figure 8, the transcriptional rate of H-topo II was increased at 2 h but subsequently decreased following Dex treatment for 24 h. The H-topo II transcriptional rate in HBT20-MAM cells was not changed by Dex treatment (results not shown). We have previously shown that, when HBT-20 cells are transfected with D-topo II using the same vector, MMTV promoter activity and increased expression of D-topo II persisted at 24 h, compared with decreased endogenous H-topo II [13]. Although in our present system we cannot distinguish exogenous from endogenous H-topo II, based on the D-topo II data [13] and the finding that the endogenous MMTV promoter was still sensitive to Dex at 48 h (Figure 7), we concluded that the down-regulation of H-

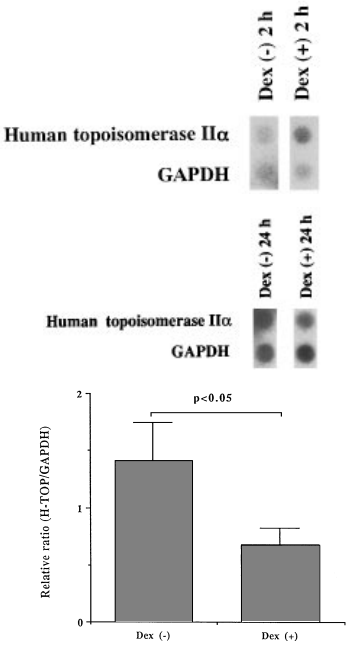

Figure 8 Effect of dexamethasone on H-topo IIα nuclear transcription

Nuclei were isolated from HTB20-hTOP2MAM cells incubated with or without Dex for 2 h or 24 h as indicated. Run-on transcription assays were performed as described in the Materials and methods section. The results from one representative experiment of three are shown. The bar graph shows a summary of the 24 h transcription assay. Relative transcriptional activity was calculated by normalization to GAPDH density. The values are means ± S.D. from three independent experiments.

topo II expression following Dex treatment involved decreased transcription of the endogenous H-topo II gene.

To further investigate the mechanism of Dex-induced endogenous H-topo II down-regulation at 24 h, HBT20-hTOP2MAM cells were transfected with various (H-topo II promoter)–CAT constructs (Figure 9) [18]. CAT expression from the construct comprising positions 0 (ATG site) to −295 (CAT295) was not affected by Dex treatment. Adding more upstream sequence (to −559 and −2400; CAT559 and CAT2400 respectively) led to a decrease in CAT activity following Dex treatment. CAT assays using the same constructs in HBT20-MAM cells showed no significant changes with CAT295, CAT559 and CAT2400 after 24 h of Dex treatment (Figure 9).

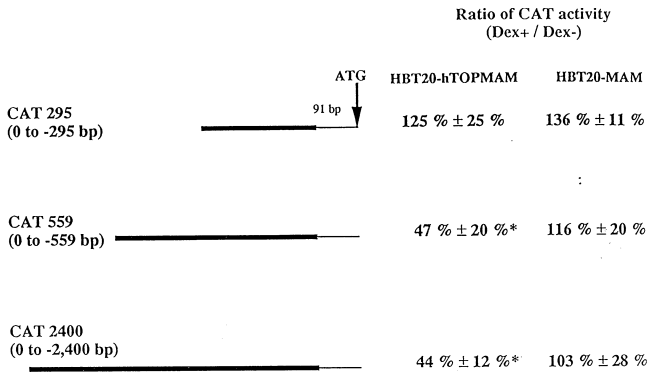

Figure 9 Diagrammatic representation of (H-topo II)–CAT chimaeric constructs, and CAT activities of the H-topo II gene promoter in HBT20-hTOP2MAM and HBT20-MAM cells

After 24 h of Dex treatment, CAT constructs were transfected into cells. At 48 h after transfection, cells were harvested and the CAT assay was performed. Following quantification for percentage acetylation of chloramphenicol, the relative ratio was calculated by dividing the percentage acetylation in the Dex-treated cells by that in the untreated cells. The values are means ± S.D. from three independent experiments. *$P < 0.05$ for significance of difference between HBT20-hTOP2MAM and HBT20-MAM cells.

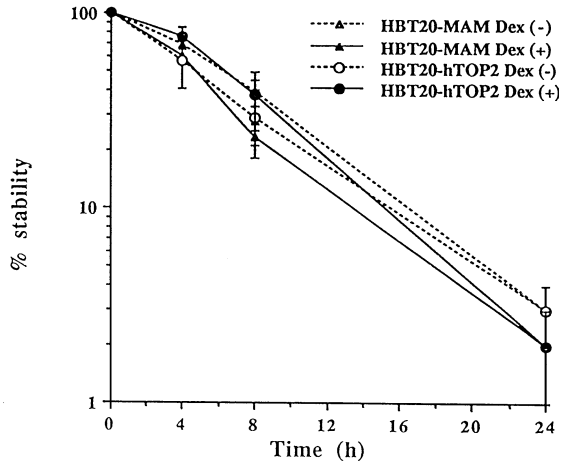

Figure 10 H-topo IIα mRNA half-life in Dex-treated HBT20-hTOP2MAM and HBT20-MAM cells

Tumour cells were treated with or without 10 μM Dex for 24 h. Actinomycin D (10 μM) was then added, and total RNA was extracted and analysed by Northern blot. Autoradiographs were scanned and the H-topo IIα mRNA level was expressed as a percentage of the level immediately before adding actinomycin D. The values are mean ± S.D. from three independent experiments.

Figure 10 H-topo IIα mRNA half-life in Dex-treated HBT20-hTOP2MAM and HBT20-MAM cells

Tumour cells were treated with or without 10 μM Dex for 24 h. Actinomycin D (10 μM) was then added, and total RNA was extracted and analysed by Northern blot. Autoradiographs were scanned and the H-topo IIα mRNA level was expressed as a percentage of the level immediately before adding actinomycin D. The values are mean ± S.D. from three independent experiments.

H-topo II mRNA stability in HBT20-hTOP2MAM cells was not altered after 24 h of Dex treatment (Figure 10).

DISCUSSION

The present study demonstrates that transfection of the H-topo II gene into intrinsically etoposide-resistant brain tumour cells and induction of the transfected promoter by Dex treatment resulted in increased expression of H-topo II mRNA, increased cellular H-topo II protein and an increase in the formation of etoposide-induced cleavable DNA–(H-topo II) complexes in these cells. An increase in cell sensitivity to etoposide was only observed at one drug concentration (10 μM) but not at the higher 50 μM concentration. The reason for this finding has not been determined. The MMTV promoter in our phTOP2MAMneo vector is relatively weak, yielding a small but significant increase in H-topo II expression. At low etoposide concentrations this small increase may give additional sites for drug action, leading to an increase in cytotoxicity. However, higher etoposide concentrations (e.g. 50 μM) may be sufficiently cytotoxic that small increases in H-topo II protein are of little consequence. These increases in H-topo II mRNA, H-topo II protein, etoposide-induced cleavable complex formation and etoposide sensitivity were transient. After 24 h of Dex stimulation, the transfected cells were indistinguishable from the non-Dex-treated cells and from the control transfected cells by all the above parameters.

We are unable to distinguish exogenous H-topo II from the endogenous H-topo II. Therefore it was difficult to determine

whether the decrease seen at 24 h was the result of a change in production of the exogenous or endogenous protein. A decrease in transfected MMTV promoter activity could result in a decrease in the amount of exogenously produced protein. Thus the total cellular protein (endogenous plus exogenous) measured by Western analysis would be decreased compared with that for the earlier time point. However, using a pMAMneo-CAT vector, we determined that the MMTV promoter was equally responsive to Dex at 48 h. Thus a loss of responsiveness to Dex of the transfected promoter could not explain the short-lived nature of the increase in H-topo II mRNA and protein.

An alternative hypothesis is that endogenous H-topo II was down-regulated following expression of the exogenous H-topo II gene. Such regulation could be via transcriptional or post-transcriptional mechanisms. However, it is unlikely that a post-transcriptional mechanism is involved, since the stability of H-topo II mRNA in the HBT20-hTOP2MAM cells was not altered following 24 h of Dex treatment. To address the question of regulation via a transcriptional mechanism, we transfected the HBT20-hTOP2MAM cells with the H-topo II promoter upstream of the CAT gene. We reasoned that, if the exogenous gene was down-regulating the endogenous gene via an interaction with the H-topo II promoter, then following Dex stimulation a decrease in CAT expression would occur in the H-topo-II-transfected cells. Indeed, following 24 h of Dex treatment, decreased CAT activity was demonstrated in the HBT20-hTOP2MAM cells but not in HBT20-MAM control cells transfected with the same CAT constructs (Figure 9). However, the susceptibility of the CAT constructs to Dex-induced down-regulation differed. CAT559 and CAT2400 were down-regulated following Dex treatment, while there was no effect on CAT295 activity. These results suggest that down-regulation of the promoter occurs in region -295 to -2400, and support our hypothesis that the decreased H-topo II mRNA seen at 24 h in the H-topo-II-transfected cells may be secondary to down-regulation of the endogenous H-topo II promoter by exogenous H-topo II. Whether it is the H-topo II protein itself that interacts with the promoter to down-regulate transcription is unclear at this time. This region contains inverted CCAAT boxes, which are implicated in cell cycle control of transcription of the human thymidine kinase gene [18,19] and also contains AP-2 and ATF sites [20]. Whether these sequences are important for mediating H-topo II gene regulation is under investigation.

Similar to our findings, other investigations have shown that nuclear extracts bind to the promoter region of H-topo II and down-regulate its expression (X. Bo, personal communication). It is therefore tempting to speculate that the H-topo II gene is tightly regulated and that a feedback mechanism exists in the cells to tightly control the intracellular levels of this gene product.

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In summary, we have demonstrated that transfection of the H-topo II gene sensitizes etoposide-resistant human brain tumour cells to the actions of the drug. The time frame of this sensitization is, however, limited. The increased exogenous H-topo IIα expression is quickly followed by a decrease in endogenous H-topo IIα expression. Our data indicate that this down-regulation is mediated via a transcriptional mechanism involving the H-topo IIα promoter.

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