Identification and Characterization of a Deletion Mutant of DNA Topoisomerase I mRNA in a Camptothecin-resistant Subline of Human Colon Carcinoma

Kae Yanase,1,2 Yoshikazu Sugimoto,1 Satomi Tsukahara,1 Tomoko Oh-hara,1 Toshiwo Andoh2 and Takashi Tsuruo1,3,4

1Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170-8455, 2Department of Bioengineering, Faculty of Engineering, Soka University, 1-236 Tangi-cho, Hachioji, Tokyo 192-8577 and 3Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657

In previous studies, we established two camptothecin (CPT)-resistant sublines, HT-29/CPT and St-4/CPT, from the human colon cancer cell line HT-29 and the human stomach cancer cell line St-4, respectively. Cellular contents of DNA topoisomerase I (topo I) in the resistant cells were eight-fold less than those in the corresponding parental lines. In this study, we have shown expression of two species of the TOP1 mRNA in HT-29/CPT. The longer mRNA (4.0 kb) is the wild-type TOP1 mRNA, and the shorter mRNA (3.3 kb) proved to have a deletion of 672 bp (nucleotides 58–729 or 59–730) that caused the in-frame deletion of amino acids 20–243 of human topo I. The deleted region is identical to exons 3–9 of the TOP1 gene. The expression level of the 3.3-kb mRNA was similar to that of the wild-type mRNA in HT-29/CPT. St-4/CPT expressed only the wild-type TOP1 mRNA in lesser amounts than did St-4. Mouse NIH3T3 cells transfected with the wild-type TOP1 cDNA showed higher sensitivity to CPT than the parental cells, whereas those transfected with the deleted TOP1 cDNA showed levels similar to those of the parental cells. Expression of the exogenous TOP1 mRNA was confirmed; however, expression of the truncated topo I was not detected in cells transfected with the deleted TOP1 cDNA. These results suggest that the expression of the deleted TOP1 mRNA led to the low expression of CPT-sensitive topo I in the resistant cells.

Key words: DNA topoisomerase I — Camptothecin — Drug resistance — Deletion mutant

Camptothecin (CPT) is an antitumor alkaloid isolated from Camptotheca acuminata, a tree native to southern China. Although this compound showed strong activity against experimental tumor models, it was found to be ineffective in clinical studies.1–3) Severe side effects included myelosuppression, vomiting, diarrhea, and hemorrhagic cystitis, and as a result clinical trials were discontinued.4,5) Many derivatives of CPT that have less toxicity and higher solubility in water have been synthesized and evaluated. CPT-11, a water-soluble analog of CPT, has broad-spectrum activity against murine tumors and human tumor xenografts, including multidrug-resistant tumors.6–8) A recent study showed that a half-molecule ABC transporter BCRP/MXR/ABCP is involved in CPT-11 resistance.9) Glucuronidation of SN-38, an active metabolite of CPT-11, by UDP-glucuronosyltransferase is another important determinant for CPT-11 sensitivity.10) Clinical studies demonstrated that CPT-11 is effective against various types of cancer.11–14) A survival advantage of CPT-11 treatment was found especially for colon cancer.14)

CPT inhibits both DNA and RNA synthesis15,16) and induces reversible DNA strand breaks in mammalian cells,17) due to the inhibition of DNA topoisomerase I (topo I).18,19) Eukaryotic DNA topoisomerases are involved in balancing torsional stress arising in DNA during transcriptional and replicational processes.20) Topo I resolves torsional problems by a mechanism that involves concerted breaking and rejoining of DNA along with strand passage. CPT binds to topo I in the enzyme-DNA complex and thereby inhibits the religation reaction of the enzyme.18,21) Crystal structures of human topo I have been reported, and a mode of CPT binding to the enzyme-DNA complex was proposed based on the structural information.22) One possible mechanism of CPT-induced cell killing is that the drug-stabilized cleavage complex interferes with replication forks, resulting in replication arrest and fork disassembly.23,24)

To examine the role of topo I in the mechanism of CPT action and to characterize the resistance mechanism to CPT-11.
CPT, we have established CPT-resistant tumor cell lines of different origins. In previous studies, we demonstrated the quantitative decrease in the cellular content of topo I in three CPT-resistant lines, HT-29/CPT, St-4/CPT and P388/CPT from the human colon cancer cell line HT-29, the human stomach cancer cell line St-4 and the mouse leukemia cell line P388, respectively. In this study, we show that two species of TOP1 mRNA, the wild-type (4.0 kb) and a deleted-type (3.3 kb), are expressed in HT-29/CPT. Possible involvement of the expression of the deleted TOP1 mRNA in CPT resistance was examined by means of DNA transfection experiments.

MATERIALS AND METHODS

Cell culture and assay of drug sensitivity Two CPT-resistant sublines HT-29/CPT and St-4/CPT were established from human colon cancer cell line HT-29 and human stomach cancer cell line St-4, respectively, by treating the tumor cells in vitro with increasing concentrations of CPT. Cells were grown in DMEM supplemented with 10% fetal bovine serum. The sensitivities of the cultured cell lines to drugs were evaluated in terms of the inhibition of cell growth after incubation at 37°C for 5 days with various concentrations of drugs, as described previously. The number of tumor cells was counted in a Coulter counter, and the IC50 values were determined.

Immunoblot analysis Cells were solubilized with 2% SDS, 5% 2-mercaptoethanol, and 50 mM Tris-HCl, pH 7.5. The cell lysates (1×10⁷ cells equivalent) were subjected to electrophoresis on 5–20% SDS-polyacrylamide gels. After electrophoresis, the protein was transferred onto a nitrocellulose membrane. For the detection of topo I, the blot was incubated with the anti-topo I monoclonal antibody T14C,25 washed, and then reacted with 125I-labeled anti-mouse IgG (Amersham). The membrane-bound peroxidase activity of the truncated enzyme, the wild-type and the deleted TOP1 mRNA were visualized using an ECL Plus kit (Amersham). The resulting bands were visualized using an ECL Plus kit (Amersham). The resulting bands were visualized using an ECL Plus kit (Amersham). The resulting bands were visualized using an ECL Plus kit (Amersham). The resulting bands were visualized using an ECL Plus kit (Amersham). The resulting bands were visualized using an ECL Plus kit (Amersham). The resulting bands were visualized using an ECL Plus kit (Amersham).

RNA blot analysis A TOP1 cDNA clone λhTOP1-D2 was kindly provided by Dr. L. F. Liu.28 Total cellular RNA was extracted after disruption of tumor cells in guanidine thiocyanate solution followed by CsCl density gradient centrifugation. Polyadenylated mRNA was obtained by chromatography on oligodeoxythymidylyl cellulose. The mRNA samples (5 µg/lane) were subjected to electrophoresis on a 1% formaldehyde-agarose gel and transferred to a nitrocellulose membrane. The TOP1 cDNA probe was radiolabeled with [α-³²P]dCTP (3000 Ci/mmol, Amersham), and hybridization was carried out as described previously.29 The membrane filter was washed and subjected to autoradiography. The equality of the mRNA loading in each lane of the blot was confirmed by control hybridization with a human β-actin cDNA probe, as described.30

Isolation of cDNA clones and construction of expression vectors The TOP1 clones corresponding to 4.0-kb and 3.3-kb TOP1 mRNAs were isolated from an HT-29/CPT cDNA library in λgt11 using the TOP1 cDNA clone λhTOP1-D2 as a probe. Nucleotide sequences of the wild-type (4.0 kb) and the deleted (3.3 kb) TOP1 cDNA were confirmed by an ABI PRISM 377 DNA Sequencing System (Perkin-Elmer, Foster City, CA). Next, the HA-tag was added upstream from the first ATG codon of the wild-type or the deleted TOP1 cDNA to differentiate between the endogenous and exogenous topo I in the transfected cells. For this purpose, the 5′-parts of the wild-type and the deleted TOP1 cDNAs were amplified by PCR using 5HA-S (5′-CGCGCTAGCATGTACCCCATACGACGTCCAGACTACGCTATGAGTGGGGACCCACTCTCA C-3′; HA-antigen sequence and nucleotides 1 to 22 from the first ATG of TOP1 cDNA) and 5HA-AS (5′-CATGATTCGTCCTCTTCGATCGCCCATCTTTG-3′; antisense of nucleotides 1104 to 1134 of TOP1 cDNA) as primers. The resulting PCR products were ligated with the 3′-part of TOP1 cDNA. The wild-type and the deleted TOP1 cDNAs with the HA-tag were cloned into a mammalian expression vector, pCALNLw, that has a strong CAG promoter to drive the expression of the cDNA.34 Nucleotide sequences of the constructs were confirmed.

Transfection Mouse fibroblast NIH3T3 cells (5×10⁵ cells/60-mm dish) were cotransfected with the expression construct containing the TOP1 cDNA (5 µg) and a neomycin-resistant plasmid pMCIneopolyA (0.25 µg; Stratagene, La Jolla, CA) using the SuperFect Transfection Reagent (Qiagen, Valencia, CA). The cells were replated 2 days after transfection and selected in medium containing 600 µg/ml of G418. G418-resistant colonies were isolated and used in further studies. Integration of the TOP1 cDNA in the transfectants was examined by genomic PCR using a sense-strand primer p1 (5′-TACGACGTCCAGACT-3′; the sequence in the HA-tag) and an antisense-strand primer p2 (5′-CAGAGGGCTCTATCCTCTTT-3′; antisense of nucleotides 1060 to 1075 of TOP1 cDNA). Expression of the exogenous TOP1 mRNA in the transfectants was examined by RT-PCR using a sense-strand primer p1 (5′-TACGACGTCCAGACT-3′; the sequence in the HA-tag) and an antisense-strand primer p3 (5′-AGAACTCTGCCTCTTG-3′; antisense of nucleotides 2306 to 2321 of TOP1 cDNA). As an internal standard of RT-PCR, β₂-microglobulin mRNA in the transfectants was amplified using a sense-strand primer (5′-ATATCCAAACCTACTGATG-3′) and an antisense-strand primer (5′-ATATCCAAACCTACTGATG-3′).

In vitro transcription/translation To examine the topo I activity of the truncated enzyme, the wild-type and the
deleted TOP1 cDNAs were cloned into a pGEM-4 vector (Promega, Madison, WI) with an SP6 promoter, and the respective topo I proteins were synthesized using in vitro transcription/translation systems (Promega). Topo I activity was assayed essentially as described by Ishii et al. in brief, the activity was measured by relaxing the supercoiled plasmid DNA at 37°C for 15 min in a 20 µl reaction mixture containing 0.2 µg of supercoiled pT2GN plasmid DNA, 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA, 0.5 mM dithiothreitol, 30 mg/ml BSA, 10% glycerol and various concentrations of in vitro translation products. The reaction was terminated by adding SDS to 1%. The extent of DNA relaxation was evaluated after electrophoresis on 0.8% agarose gels.

RESULTS

Expression of TOP1 mRNA in HT-29/CPT and St-4/CPT

Degrees of resistance of these CPT-resistant lines to inhibitors of topo I and II are summarized in Table I. HT-29/CPT and St-4/CPT expressed approximately one-eighth the amount of topo I as did the corresponding parental lines (Fig. 1A). To understand the molecular basis of this lowered expression in the CPT-resistant cells, RNA blot analysis was carried out using the human TOP1 cDNA probe λhTOP1-D2. As shown in Fig. 1B, two species of the TOP1 mRNA were found in HT-29/CPT. The longer mRNA (4.0 kb) is the wild-type TOP1 mRNA, and the shorter mRNA (3.3 kb) appears to possess a deletion. The amounts of the two mRNAs in HT-29/CPT were approximately one-half the amount of the wild-type mRNA expressed in the parental HT-29. On the other hand, St-4/CPT expressed only the wild-type TOP1 mRNA, and at a lower level than in St-4 (Fig. 1B).

Identification of the deleted region in the 3.3-kb mRNA

To identify the deleted region of the 3.3-kb mRNA expressed in HT-29/CPT, we carried out a series of gene amplification experiments using various primers with cDNA templates from HT-29 and HT-29/CPT. One set of primers, 2F (5′-TCCCAGATCGAAGCGGATTT-3′; nucleotides 28 to 47 from the first ATG of TOP1 cDNA 33)) and 5R (5′-GATTCGTCTCTTCAGCATGC-3′; antisense of nucleotides 1112 to 1131) successfully amplified the deleted cDNA fragment in HT-29/CPT (data not shown). The expected size of the PCR fragment was 1104 bp; however, the amplification gave rise to a 432-bp fragment in HT-29/CPT. The difference between the lengths of the PCR fragment and the expected fragment, 0.7 kb, was the same as the difference between the lengths of the two TOP1 mRNAs expressed in HT-29/CPT (Fig. 1B). The

Table I. Drug Resistance of HT-29/CPT and St-4/CPT

| Cell lines | Degree of resistance (x-fold) |
|------------|-----------------------------|
|            | CPT  | ADM  | VP-16 | m-AMSA |
| HT-29/CPT  | 6.9* | 0.3  | 1.4   | 0.2   |
| St-4/CPT   | 8.0  | 1.8  | 3.2   | 0.9   |

* Degree of resistance was calculated by dividing the IC₅₀ value of the resistant lines by that of the corresponding parental line.

Fig. 1. (A) Immunoblot analysis of whole cell lysates from CPT-sensitive and -resistant cell lines. Cell lysates from 1×10⁶ cells were prepared, subjected to SDS-PAGE in 5–20% polyacrylamide gel and subsequently transferred to a nitrocellulose filter. The blot was reacted with the anti-topo I monoclonal antibody T14C and 125I-labeled anti-mouse IgG. (B) RNA blot analysis of mRNA from CPT-sensitive and -resistant cell lines. Polyadenylated mRNA (5 µg) was electrophoresed in a formaldehyde-agarose gel, transferred to a nitrocellulose filter, and hybridized with 32P-labeled human TOP1 cDNA probe λhTOP1-D2.

Fig. 2. (A) Nucleotide and amino acid sequences at the break points of the exon 3–9-deleted TOP1 cDNA. (B) Schematic structures of wild-type and deleted TOP1 cDNAs. The diagonal region is identical to exons 3–9 of the TOP1 gene.
A 432-bp PCR fragment was subcloned into SmaI-digested pUC13 vector and sequenced. The nucleotide sequence of the PCR fragment was identical to the reported sequence of TOP1 cDNA but lacked 672 bp (nucleotides 58–729 or 59–730). This deletion should cause an in-frame deletion of amino acids 20–243 of human topo I (Fig. 2). Since the nucleotides at 58 and 730 are both G, we cannot identify which was deleted. However, it is likely that the region 59–730 was deleted because it is identical to exons 3–9 of the human TOP1 gene. Therefore, we refer to the short mRNA as exon 3–9-deleted TOP1 mRNA. In spite of the expression of the truncated mRNA with an in-frame deletion in HT-29/CPT, we could not detect the corresponding 70-kDa protein by western blots with anti-topo I monoclonal antibody T14C (Fig. 1A) or anti-topo I polyclonal antibody Scl-70 (Topogen, Columbus, OH) (data not shown).

Mammalian expression experiments The wild-type and the deleted TOP1 cDNAs were cloned into a pCALNLw vector and were termed pCAL-TW and pCAL-TD, respectively. Mouse fibroblast NIH3T3 cells were cotransfected with pCAL-derived construct and pMC1neopolyA. The transfectants were selected in medium containing 600 µg/ml of G418. G418-resistant colonies were isolated and used in further studies.

Integration of the TOP1 cDNA and expression of the exogenous TOP1 mRNA in NIH3T3 cells transfected with wild-type or deleted TOP1 cDNA. (A) Positions of PCR primers and expected sizes of the amplified products. (B) Detection of the exogenous TOP1 mRNA by RT-PCR using p1-p3 primers. β2-MG: Detection of β2-microglobulin mRNA as an internal control of RT-PCR. M, DNA size marker φX174/HaeIII. 3T3, parental NIH3T3 cells. n, mock transfectant clones. The numbers 1, 2, 3 and 4 represent clones n1, n2, n3 and n4, respectively. W, clones transfected with the wild-type TOP1 cDNA. The numbers 2, 4 and 5 represent clones W2, W4 and W5, respectively. C, control PCR product using wild-type TOP1 cDNA as a template. D, clones transfected with the deleted TOP1 cDNA. The numbers 1, 2, 3, 4 and 5 represent clones D1, D2, D3, D4 and D5, respectively. C, control PCR product using the deleted TOP1 cDNA as a template.

432-bp PCR fragment was subcloned into SmaI-digested pUC13 vector and sequenced. The nucleotide sequence of the PCR fragment was identical to the reported sequence of TOP1 cDNA but lacked 672 bp (nucleotides 58–729 or 59–730). This deletion should cause an in-frame deletion of amino acids 20–243 of human topo I (Fig. 2). Since the nucleotides at 58 and 730 are both G, we cannot identify which was deleted. However, it is likely that the region 59–730 was deleted because it is identical to exons 3–9 of the human TOP1 gene. Therefore, we refer to the short mRNA as exon 3–9-deleted TOP1 mRNA. In spite of the expression of the truncated mRNA with an in-frame deletion in HT-29/CPT, we could not detect the corresponding 70-kDa protein by western blots with anti-topo I monoclonal antibody T14C (Fig. 1A) or anti-topo I polyclonal antibody Scl-70 (Topogen, Columbus, OH) (data not shown).

Mammalian expression experiments The wild-type and the deleted TOP1 cDNAs were cloned into a pCALNLw vector and were termed pCAL-TW and pCAL-TD, respectively. Mouse fibroblast NIH3T3 cells were cotransfected with pCAL-derived construct and pMC1neopolyA. The transfectants were selected in medium containing 600 µg/ml of G418. G418-resistant colonies were isolated and used in further studies.

Integration of the TOP1 cDNA and expression of the exogenous TOP1 mRNA in the transfectant cells were examined in a series of PCR experiments. The position of PCR primers and the expected sizes of amplified products are summarized in Fig. 3A. In this series, only the exogenous TOP1 cDNA and mRNA were amplified since primer p1 codes the HA-tag. First, integration of the exogenous TOP1 cDNA with the HA-tag was examined by genomic PCR using p1-p2 primers. Transfectant clones with integration of the exogenous TOP1 cDNA (three clones, W2, W4 and W5, transfected with the wild-
Deletion Mutant of Topo I

type TOP1 cDNA and five clones, D1, D2, D3, D4 and D5, transfected with the deleted TOP1 cDNA) were used in further experiments (Fig. 3B). Integration of the exogenous full-length TOP1 cDNA in these clones were confirmed by another genomic PCR using primers p1 and p3 (data not shown). As shown in Fig. 3C, expression of the exogenous, wild-type TOP1 mRNA (2.3 kb) was detected in W2, W4 and W5 by RT-PCR using the p1 and p3 primers. Expression of the deleted TOP1 mRNA (1.6 kb) was detected in D1, D2 and D3, but not in D4 and D5.

Western blot analysis using anti-HA antibody revealed high-level expressions of the exogenous topo I in all three clones transfected with the wild-type TOP1 cDNA (Fig. 4). No exogenous protein with the expected size was found in clones transfected with the deleted TOP1 cDNA (Fig. 4).

CPT sensitivity of transfected clones The sensitivity to CPT of the transfectants was examined using a cell growth inhibition assay (Fig. 5). The mock transfectant clones n1, n2, n3 and n4 showed similar CPT sensitivity to the parental 3T3 cells (IC_{s0} values were 4.6–5.5 ng/ml) (Fig. 5A). The IC_{s0} values to CPT of W2, W4 and W5 were 2.1, 1.6 and 1.3 ng/ml, respectively, which were approximately 3-fold higher than that of the control cells (Fig. 5B). The IC_{s0} values to CPT of D1, D2, D3, D4 and D5 were 4.3 to 6.3 ng/ml, which showed no significant changes in drug resistance from that of the control cells (Fig. 5C). The IC_{s0} values of these clones are plotted in Fig. 6.

**In vitro transcription/translation of topo I** To examine the catalytic activity of the deleted topo I, in vitro translation experiments were carried out using pGEM-TW or pGEM-TD with the HA-tagged wild-type or deleted TOP1 cDNA, respectively. High-level expression of the wild-type topo I (100-kDa protein) was detected by western blot analysis. The deleted topo I protein (70 kDa) was also

---

Fig. 5. Sensitivity to CPT of clones transfected with wild-type or deleted TOP1 cDNA. To evaluate the sensitivity of the transfectants to CPT, cells were cultured in medium containing various concentrations of CPT. After 5 days, viable cells were counted, and the percentage of cell growth was calculated (means±SEM, n=4 per group). Closed circles, CPT sensitivity of the parental NIH3T3 cells. (A) CPT sensitivity of the mock transfectant clones. ● 3T3, □ n1, ◊ n2, △ n3, ○ n4. (B) CPT sensitivity of three clones transfected with the wild-type TOP1 cDNA. ● 3T3, □ W2, ◊ W4, △ W5. (C) CPT sensitivity of five clones transfected with the deleted TOP1 cDNA. ● 3T3, □ D1, ◊ D2, △ D3, ○ D4, △ D5.

---

Fig. 6. IC_{s0} values for CPT of clones transfected with the TOP1 cDNA. (A) Closed circle, IC_{s0} value of parental NIH3T3 cells; open circles, IC_{s0} values of mock transfectant clones. (B) Open squares, IC_{s0} values of clones transfected with the wild-type TOP1 cDNA. (C) Open triangles, IC_{s0} values of clones transfected with the deleted TOP1 cDNA.
DISCUSSION

In this study, we showed the expression of two species of TOP1 mRNA in HT-29/CPT cells. The longer mRNA (4.0 kb) was the wild-type TOP1 mRNA, and the shorter mRNA (3.3 kb) had a deletion that resulted in the internal deletion of 224 amino acids (residues 20–243) of human topo I. The cDNAs corresponding to both mRNAs were isolated and introduced into NIH3T3 cells. Cells transfected with the wild-type TOP1 cDNA showed higher sensitivity to CPT than did 3T3 cells. On the other hand, transfectant clones with the deleted TOP1 cDNA showed similar levels of drug resistance to 3T3 cells.

The amounts of the wild-type and mutant mRNAs in HT-29/CPT were the same and together were approximately one-half the amount of wild-type mRNA expressed in the parental HT-29 (Fig. 1B). This suggested that the deleted TOP1 mRNA in HT-29/CPT cells was transcribed from one of the alleles of the genome. To identify the possible deletion or rearrangement in one of the alleles of the TOP1 gene, Southern blot analysis was carried out using eight restriction endonucleases. However, we could not identify significant rearrangements or deletions of the TOP1 gene in HT-29/CPT (data not shown). Therefore, it is likely that certain mutations in the splicing donor/acceptor sites in one of the alleles in HT-29/CPT resulted in a 672-bp deletion of the TOP1 mRNA. Isolation and analysis of the genomic DNA of HT-29/CPT is ongoing to clarify this point.

In this study we showed the expression of truncated TOP1 mRNA as a mechanism of CPT resistance. Expression of shorter mRNA was reported by Matsumoto et al. as a mechanism of resistance to topo II inhibitor. They reported that an acquired 600-bp deletion in one topo II allele resulted in a reduced topo II protein level of a VP-16-resistant subline MDA-MB-231-VP7. Therefore expression of shorter mRNA may be an infrequent event, but it can happen in other systems.

Quantitative reduction of topo I in resistant cells has been reported in various systems and seems to be the most common mechanism of CPT resistance. Any mechanism which would result in lowered protein expression can be related to CPT resistance. Such mechanisms include low mRNA expression, genomic deletion, alternative splicing, protein instability, and so on. We previously reported the reduction of cellular topo I content in HT-29/CPT, St-4/CPT and P388/CPT. In this study, we found that the expression of TOP1 mRNA was also decreased in St-4/CPT from the parental St-4. These results suggest that the decrease in the cellular content of topo I in St-4/CPT was caused simply by the reduction in the cellular content of its mRNA. In contrast, the total amount of TOP1 mRNA in HT-29/CPT appeared similar to the mRNA expressed in the parental HT-29, suggesting that mechanisms related to the transcription efficiency and mRNA stability were not altered in HT-29/CPT. The amount of wild-type TOP1 mRNA expressed in HT-29/CPT cells was approximately one-half that in parental HT-29; however, the cellular content of topo I in HT-29/CPT was eight-fold less than that in the parental HT-29. Therefore, the expression level of wild-type TOP1 mRNA in HT-29/CPT does not account for the low amount of the protein. This result suggests that there exists a mechanism other than lowered mRNA expression for the low-level content of topo I in HT-29/CPT. One possible mechanism is an acceleration of topo I degradation in HT-29/CPT. The stability of topo I protein and proteasome function in HT-29/CPT is to be examined to clarify this point.

NIH3T3 cells transfected with the wild-type TOP1 cDNA showed higher sensitivity to CPT than the control.
cells (Figs. 5, 6). TOP1-transfected baby hamster kidney cells expressed two- to five-fold higher amounts of wild-type topo I and showed increased cell death in the presence of CPT.\(^4\) In another system, COS cells expressing \textit{Saccharomyces cerevisiae} topo I showed increased CPT sensitivity.\(^4\) These results clearly demonstrate that cellular contents of topo I directly correlate to the sensitivity of cells to CPT.

Mechanisms of CPT resistance other than the quantitative reduction of topo I have been reported in various experimental systems. We previously reported the expression of mutant topo I in a CPT-resistant human T lymphoblastic leukemia cell line, CPT-K5.\(^2\) An amino acid change from Asp to Gly at residue 533 is responsible for the CPT resistance of the enzyme.\(^3\) The expression of mutant (Gly-533) topo I confers a dominant form of CPT resistance in cells expressing wild-type topo I.\(^4\) Maliepaard \textit{et al.} recently reported that a half-molecule ABC transporter BCRP/MXR/ABCP is involved in CPT-11 resistance.\(^5\) Glucuronidation of SN-38, an active metabolite of CPT-11, by UDP-glucuronosyltransferase is another important determinant for CPT-11 sensitivity.\(^6\)

The exon 3–9-deleted topo I of HT-29/CPT lacks amino acid residues 20–243. The deleted region contains the putative nuclear localization signal (residues 141–210).\(^7\) Human topo I with the deletion of the 70-amino-acid region showed topo I catalytic activity \textit{in vitro}. Overexpression of topo I in \textit{S. cerevisiae} is lethal, whereas overexpression of the topo I with the deletion of the 70-amino-acid region did not result in cell death,\(^8\) suggesting that the truncated enzyme is inactive \textit{in vivo} because of a defect in its transport into the nucleus. Therefore, it is not surprising that the CPT sensitivity of the NIH3T3 cells was not affected by the transfection of the deleted \textit{TOP1} cDNA. It is possible to speculate that the exon 3–9-deleted topo I is unstable when it exists in the cytoplasm. In \textit{in vitro} translation experiments, expression of a 70-kDa protein was detected from the deleted \textit{TOP1} cDNA. But, as shown in Fig. 7A, the level of the 70-kDa protein was much lower than that of the 100-kDa protein from the wild-type \textit{TOP1} cDNA. The inefficient expression of the 70-kDa protein supports the notion that the 3–9-exon region of \textit{TOP1} gene is involved in the enzyme’s stability. This may be the reason why we could not detect the 70-kDa protein expression in HT-29/CPT or in cells transfected with the deleted \textit{TOP1} cDNA.

Whether or not the exon 3–9-deleted topo I encodes a functional enzyme was not clarified in this study. Vaccinia virus topo I consists of 314 amino acids and has a molecular weight of 32000, which is one-third of the molecular weight of the human enzyme.\(^9\) In addition, the vaccinia virus enzyme does not have the region that corresponds to exons 3–9 of the human enzyme. Therefore, it is possible that the exon 3–9-deleted topo I does, in fact, function. However, DNA relaxation activity of the 70-kDa protein synthesized \textit{in vitro} translation could not be detected, probably due to the low expression of the protein (Fig. 7B). Large-scale preparation and purification of the 70-kDa protein will be necessary to elucidate this point.

In this study, we identified the exon 3–9-deleted \textit{TOP1} mRNA in CPT-resistant cells. Cells transfected with the deleted \textit{TOP1} cDNA showed no significant changes in CPT sensitivity, probably because of the instability of the truncated topo I protein in the cells. These results suggest that expression of the deleted \textit{TOP1} mRNA leads to low expression of CPT-sensitive topo I in resistant cells. Possible interactions of the truncated topo I with wild-type enzyme and endogenous topo I-interacting proteins could be examined more precisely by means of a double transfection experiment with both the deleted and wild-type \textit{TOP1}.

**ACKNOWLEDGMENTS**

We would like to thank Dr. Leroy F. Liu for providing us the DNA topoisoenzyme I cDNA probe and Ms. C. Kakalec for editing the manuscript. This work was partly supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science, Sports and Culture, Japan, the Ministry of Health and Welfare, Japan, and the Vehicle Racing Commemorative Foundation, Japan.

(Received December 24, 1999/Revised February 25, 2000/Accepted February 28, 2000)

**REFERENCES**

1) Gottlieb, J. A., Guarino, A. M., Call, J. B., Oliverio, V. T. and Block, J. B. Preliminary pharmacologic and clinical evaluation of camptothecin sodium (NSC 100880). *Cancer Chemother. Rep.*, \textbf{54}, 461–470 (1970).
2) Gottlieb, J. A. and Luce, J. K. Treatment of malignant melanoma with camptothecin (NSC-100880). *Cancer Chemother. Rep.*, \textbf{56}, 103–105 (1972).
3) Moertel, C. G., Schutt, A. J., Reitemeier, R. J. and Hahn, R. G. Phase II study of camptothecin (NSC-100880) in the treatment of advanced gastrointestinal cancer. *Cancer Chemother. Rep.*, \textbf{56}, 95–101 (1972).
4) Muggia, F. M., Creaven, P. J., Hansen, H. H., Cohen, M. H. and Selawry, O. S. Phase I clinical trial of weekly and daily treatment with camptothecin (NSC-100880); correlation with preclinical studies. *Cancer Chemother. Rep.*, \textbf{56}, 515–521 (1972).
5) Schaeppi, U., Fleischman, R. W. and Cooney, D. A. Toxicity of camptothecin (NSC-100880). *Cancer Chemother.
Rep. Part 3, 5, 25–36 (1974).

6) Kunimoto, T., Nitta, K., Tanaka, T., Uehara, N., Baba, H., Takeuchi, M., Yokokura, T., Sawada, S., Miyasaka, T. and Mutai, M. Antitumor activity of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin, a novel water-soluble derivative of camptothecin, against murine tumors. Cancer Res., 47, 5944–5947 (1987).

7) Houghton, P. J., Cheshire, P. J., Hallman, J. C., Bissery, M. C., Mathieu-Boué, A. and Houghton, J. A. Therapeutic efficacy of the topoisomerase I inhibitor 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin against human tumor xenografts: lack of cross-resistance in vivo in tumors with acquired resistance to the topoisomerase I inhibitor 9-dimethylaminoethyl-10-hydroxycamptothecin. Cancer Res., 53, 2823–2829 (1993).

8) Tsuruo, T., Matsuizaki, T., Matsushita, M., Saito, H. and Yokokura, T. Antitumor effect of CPT-11, a new derivative of camptothecin, against pleiotropic drug-resistant tumors in vitro and in vivo. Cancer Chemother. Pharmacol., 21, 71–74 (1988).

9) Maliepaard, M., van Gastelen, M. A., de Jong, L. A., Pluim, D., van Waardenburg, R. C., Ruevekamp-Helmers, M. C., Flook, B. G. and Schellens, J. H. Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. Cancer Res., 59, 4559–4563 (1999).

10) Takahashi, T., Fujiwara, Y., Yamakido, M., Katoh, O., Watanabe, H. and Mackenzie, P. I. The role of glucuronidation in 7-ethyl-10-hydroxycamptothecin resistance in vitro. Jpn. J. Cancer Res., 88, 1211–1217 (1997).

11) Ohno, R., Okada, K., Masaoka, T., Kuramoto, A., Arima, T., Yoshida, Y., Ariyoshi, H., Ichimaru, M., Sakai, Y., Oguro, M., Ito, Y., Morishima, Y., Yokokura, S. and Ota, K. An early phase II study of CPT-11, a new derivative of camptothecin, for the treatment of leukemia and lymphoma. J. Clin. Oncol., 8, 1907–1912 (1990).

12) Fukuoka, M., Naitani, H., Suzuki, A., Motomiya, M., Hasegawa, K., Nishiwaki, Y., Kuriyama, T., Ariyoshi, Y., Negoro, S., Masuda, N., Nakajima, S. and Taguchi, T. A phase II study of CPT-11, a new derivative of camptothecin, for previously untreated non-small-cell lung cancer. J. Clin. Oncol., 10, 16–20 (1992).

13) Masuda, N., Fukuoka, M., Kusunoki, Y., Matsui, K., Takafuji, N., Kudoh, S., Negoro, S., Nishioaka, M., Nakagawa, K. and Takada, M. CPT-11, a new derivative of camptothecin for the treatment of refractory or relapsed small-cell lung cancer. J. Clin. Oncol., 10, 1225–1229 (1992).

14) Rothenberg, M. L. Efficacy and toxicity of irinotecan in patients with colorectal cancer. Semin. Oncol., 25, 39–46 (1998).

15) Bosmann, H. B. Camptothecin inhibits macromolecular synthesis in mammalian cells but not in isolated mitochondria or E. coli. Biochem. Biophys. Res. Commun., 41, 1412–1420 (1970).

16) Kessel, D. Effects of camptothecin on RNA synthesis in leukemia L1210 cells. Biochim. Biophys. Acta, 246, 225–232 (1971).

17) Horwitz, M. S. and Horwitz, S. B. Intracellular degradation of HeLa and adenovirus type 2 DNA induced by camptothecin. Biochem. Biophys. Res. Commun., 45, 723–727 (1971).

18) Hsiang, Y. H., Hertzberg, R., Hecht, S. and Liu, L. F. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J. Biol. Chem., 260, 14873–14879 (1985).

19) Bjornsti, M. A., Benedetti, P., Viglianti, G. A. and Wang, J. C. Expression of human DNA topoisomerase I in yeast cells lacking yeast DNA topoisomerase I: restoration of sensitivity of the cells to the antitumor drug camptothecin. Cancer Res., 49, 6318–6323 (1989).

20) Pommier, Y., Pourquier, P., Fan, Y. and Strumberg, D. Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. Biochim. Biophys. Acta, 1400, 83–106 (1998).

21) Hsiang, Y. H. and Liu, L. F. Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. Cancer Res., 48, 1722–1726 (1988).

22) Redinbo, M. R., Stewart, L., Kuhn, P., Champoux, J. J. and Hol, W. G. Crystal structures of human topoisomerase I in covalent and noncovalent complexes with DNA. Science, 279, 1504–1513 (1998).

23) Avemann, K., Knippers, R., Koller, T. and Sogo, J. M. Camptothecin, a specific inhibitor of type I DNA topoisomerase, induces DNA breakage at replication forks. Mol. Cell. Biol., 8, 3026–3034 (1988).

24) Hsiang, Y. H., Lihou, M. G. and Liu, L. F. Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complex as a mechanism of cell killing by camptothecin. Cancer Res., 49, 5077–5082 (1989).

25) Sugimoto, Y., Tsukahara, S., Oh-hara, T., Isoe, T. and Tsuruo, T. Decreased expression of DNA topoisomerase I in camptothecin-resistant tumor cell lines as determined by a monoclonal antibody. Cancer Res., 50, 6925–6930 (1990).

26) Sugimoto, Y. and Tsuruo, T. DNA-mediated transfer and cloning of human multidrug-resistant gene of Adriamycin-resistant leukemia K562. Cancer Res., 47, 2620–2625 (1987).

27) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680–685 (1970).

28) Juan, C. C., Hwang, J., Liu, A. A., Whang-Peng, J., Knutsen, T., Huebner, K., Croce, C. M., Zhang, H., Wang, J. C. and Liu, L. F. Human DNA topoisomerase I is encoded by a single-copy gene that maps to chromosome region 20q12-13.2. Proc. Natl. Acad. Sci. USA, 85, 8910–8913 (1988).

29) Sugimoto, Y., Asami, N. and Tsuruo, T. Expression of P-glycoprotein mRNA in human gastric tumors. Jpn. J. Cancer Res., 80, 993–999 (1989).

30) Sugimoto, Y., Tsukahara, S., Oh-hara, T., Liu, L. F. and
Tsuruo, T. Elevated expression of DNA topoisomerase II in camptothecin-resistant human tumor cell lines. *Cancer Res.*, **50**, 7962–7965 (1990).

31) Young, R. A. and Davis, R. W. Efficient isolation of genes using antibody probes. *Proc. Natl. Acad. Sci. USA*, **80**, 1194–1198 (1983).

32) Kieffer, B. I. Optimised cDNA size selection and cloning procedure for the construction of representative plasmid cDNA libraries. *Gene*, **109**, 115–119 (1991).

33) D’Arpa, P., Machlin, P. S., Ratrie, H., III, Rothfield, N. F., Cleveland, D. W. and Earnshaw, W. C. cDNA cloning of human DNA topoisomerase I: catalytic activity of a 67.7-kDa carboxyl-terminal fragment. *Proc. Natl. Acad. Sci. USA*, **85**, 2543–2547 (1988).

34) Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S. and Saito, I. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucleic Acids Res.*, **23**, 3816–3821 (1995).

35) Pelham, H. R. P. and Jackson, R. J. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.*, **67**, 247–256 (1976).

36) Krieg, P. and Melton, D. Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. *Nucleic Acids Res.*, **12**, 7057–7070 (1984).

37) Ishii, K., Hasegawa, T., Fujisawa, K. and Andoh, T. Rapid purification and characterization of DNA topoisomerase I from mouse mammary carcinoma FM3A cells. *J. Biol. Chem.*, **258**, 12728–12732 (1983).

38) Kunze, N., Yang, G., Dolberg, M., Sundarp, R., Knippers, R. and Richter, A. Structure of the human type I DNA topoisomerase gene. *J. Biol. Chem.*, **266**, 9610–9616 (1991).

39) Matsumoto, Y., Takano, H. and Fojo, T. Cellular adaptation to drug exposure: evolution of the drug-resistant phenotype. *Cancer Res.*, **57**, 5086–5092 (1997).

40) Madden, K. R. and Champoux, J. J. Overexpression of human topoisomerase I in baby hamster kidney cells: hypersensitivity of clonal isolates to camptothecin. *Cancer Res.*, **52**, 525–532 (1992).

41) Hann, C., Evans, D. L., Fertala, J., Benedetti, P., Bjornsti, M.-A. and Hall, D. J. Increased camptothecin toxicity in mammalian cells expressing *Saccharomyces cerevisiae* DNA topoisomerase I. *J. Biol. Chem.*, **273**, 8425–8433 (1998).

42) Andoh, T., Ishii, K., Suzuki, Y., Ikegami, Y., Kusunoki, Y., Takemoto, Y. and Okada, K. Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. *Proc. Natl. Acad. Sci. USA*, **84**, 5565–5569 (1987).

43) Tamura, H., Kohchi, C., Yamada, R., Ikeda, T., Koiwai, O., Patterson, E., Keene, J. D., Okada, K., Kjeldsen, E., Nishikawa, K. and Andoh, T. Molecular cloning of a cDNA of a camptothecin-resistant human DNA topoisomerase I and identification of mutation sites. *Nucleic Acids Res.*, **19**, 69–75 (1991).

44) Andoh, T., Tamura, H., Kohchi, C., Yamada, R., Ikeda, T., Koiwai, O., Yasui, Y., Nishizawa, M. and Okada, K. Mechanism of camptothecin resistance in mammalian cells: mutation in topoisomerase I gene and its implication in enzymatic function. In “Molecular Biology of DNA Topoisomerasers and Its Application to Chemotherapy,” ed. T. Andoh. H. Ikeda and M. Oguro, pp. 229–235 (1993). CRC Press, Boca Raton.

45) Yanase, K., Sugimoto, Y., Andoh, T. and Tsuruo, T. Retroviral expression of a mutant (Gly-533) human DNA topoisomerase I cDNA confers a dominant form of camptothecin. *Int. J. Cancer*, **81**, 134–140 (1999).

46) Alsnér, J., Svejstrup, J. Q., Kjeldsen, E., Sorensen, B. S. and Westgaard, Ø. Identification of an N-terminal domain of eukaryotic DNA topoisomerase I dispensable for catalytic activity but essential for *in vivo* function. *J. Biol. Chem.*, **267**, 12408–12411 (1992).

47) Shuman, S., Goldner, M. and Moss, B. Characterization of vaccinia virus DNA topoisomerase I in *Escherichia coli*. *J. Biol. Chem.*, **263**, 16401–16407 (1988).