

# Desensitization and Sensitization of Cells to Fluoropyrimidines with Different Antisenses Directed against Thymidylate Synthase Messenger RNA<sup>1</sup>

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## ABSTRACT

Previous studies have shown that the cytotoxicity of fluoropyrimidines is mediated, in large part, by inhibition of the enzyme thymidylate synthase (TS). The aim of this study was to determine whether the chemosensitivity of human cancer cells to fluoropyrimidines could be increased by decreasing TS expression with antisense oligodeoxyribonucleotides (ODNs). ODNs (18-mers) targeted at the AUG translational initiation site of TS mRNA inhibited translation in a sequence- and dose-dependent manner in a rabbit reticulocyte lysate *in vitro* translation system. Treatment of human colon cancer HT-29 cells with antisense ODNs decreased TS catalytic activity in the cells in a dose-dependent manner over a short period, but the longer-term effect of the TS antisense ODN treatment was actually to increase the amount of TS in the cells and to decrease their sensitivity to 5-fluoro-2'-deoxyuridine (FdUrd). However, when human nasopharyngeal cancer KB31 cells were transfected with a plasmid (pHaMAGRP) construct containing the TS antisense fragment (+1 to +422) under the control of a glucose-regulated promoter, the expression of both TS protein and TS catalytic activity was decreased by nearly 30% ( $P = 0.014$ ), and sensitivity of these cells to FdUrd was enhanced by ~8-fold ( $P = 0.021$ ). No changes in the levels of expression of TS protein or FdUrd-associated cytotoxicity were observed in control, vector-transfected cells. No change was observed in the sensitivity of transfected cells toward either cisplatin or Adriamycin. These results show that the level of

expression of TS in human malignant cells can be down-regulated with antisense TS RNA, and their sensitivity to fluoropyrimidines can, thereby, be increased.

## INTRODUCTION

TS<sup>3</sup> catalyzes the reductive methylation of dUMP to dTMP, an essential step in DNA biosynthesis (1). This reaction constitutes the sole intracellular *de novo* source of thymidylate for DNA replication and repair. TS is an essential enzyme in cells that are rapidly proliferating, and as a result, it represents an important target in cancer chemotherapy (2–4).

The TS gene is a member of the family of S-phase genes, which have increased expression at the G<sub>1</sub>-S boundary, once the cell is committed to initiate DNA replication (5). Previous studies have shown that the expression of TS is regulated at the transcriptional level, but there is now also evidence that the expression of TS is also regulated at the translational level. Specifically, TS itself negatively regulates its own synthesis by directly binding to two different regions on the TS mRNA (6–9). In addition, recent studies have identified the presence of a naturally occurring antisense RNA (*rTS*) to human TS mRNA (10, 11). Although the significance of this antisense TS RNA remains to be elucidated, the initial studies suggest that the expression of sense TS protein may be regulated by either antisense TS RNA or even, perhaps, by antisense TS protein.

There are several lines of evidence that support the concept that TS represents a biologically and clinically relevant target for the fluoropyrimidines (12). First, *in vitro* studies have shown a strong association between the level of intracellular expression of TS enzyme activity and 5-FU sensitivity (13, 14). Thus, neoplastic cell lines and tumors with increased TS enzyme activity are relatively more resistant to the cytotoxic effects of 5-FU. Second, clinical studies have shown a strong correlation between the level of TS enzyme inhibition within patient tumor samples after 5-FU treatment and eventual clinical response to 5-FU-based chemotherapy (15, 16).

The above findings suggest a rational approach for converting non-5-FU-responsive tumors into 5-FU responders by down-regulating intracellular TS expression with an antisense strategy, thereby allowing TS inhibitory compounds to be more effective at decreasing intracellular TS activity. Here, we investigated the effect of antisense ODNs on the TS activity in cells, expression of TS protein, and sensitivity of cells to fluoropyri-

Received 12/16/97; revised 6/3/98; accepted 6/9/98.

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<sup>1</sup> This work was supported by United States Public Health Service Grant CA60859.

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<sup>3</sup> The abbreviations used are: TS, thymidylate synthase; 5-FU, 5-fluorouracil; ODN, oligodeoxyribonucleotide; FdUrd, 5'-fluoro-2'-deoxyuridine; FdUMP, 5-fluoro-dUMP; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt; GRP, glucose-regulated promoter; RT-PCR, reverse transcription-PCR.

midines. Our results show that, although an exogenously administered antisense ODN actually increased TS in cells and, consequently, decreased the cytotoxicity of FdUrd, transfection of cells with a plasmid vector designed to express a larger TS antisense fragment caused a decrease in TS protein and enhanced the cells' chemosensitivity to FdUrd.

## MATERIALS AND METHODS

**Synthesis of TS mRNA Template.** The human TS cDNA was obtained from Daniel Santi (Department of Biochemistry and Biophysics, University of California, San Francisco, CA). A PCR amplification was performed to isolate a TS fragment that contained the entire coding region, corresponding to nucleotides 1–966. The primer sequences used for this PCR were: TS-1, 5'-d(TAATACGACTCACTATAGGGAGAC-CACCATGCCTGTGGCCGGCTCGGAG)-3' (sense with T7 promoter TAATACGACTCACTATA); and TS-2, 5'-d(CCT-TCGAGCTCCTTTGAAAGCACC)-3' (antisense). Each PCR sample contained 12.5 pmol of the primers, 2.5  $\mu$ l of 10 $\times$  Taq buffer [500 mM KCl-100 mM Tris-HCl (pH 8.3)], 200 mM dNTPs, 1.9 mM MgCl<sub>2</sub>, 10–100 ng of template DNA, and 0.67 units of Taq polymerase in a total volume of 25  $\mu$ l. The entire reaction mixture was overlaid with 50  $\mu$ l of mineral oil. The PCR was run for 35 cycles of 1 min at 96°C, 1 min at 65°C, and 2.5 min at 72°C, with a final extension step of 7 min at 72°C.

The *in vitro* transcription mixture contained 40 mM Tris-HCl (pH 7.5), 12 mM MgCl<sub>2</sub>, 10 mM DTT, 4 mM spermidine, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.2 mM GTP, 2 mM 7-methyl G(5')ppp(5')G cap, 3  $\mu$ l of PCR template, and 100 units of T7 RNA polymerase, in a total volume of 25  $\mu$ l. The mixture was incubated at 37°C for 2 h. The *in vitro* transcription mixture was then purified on a Chromo Spin-30 column (Clontech, Palo Alto, CA). The RNA was precipitated with 2.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate, and the precipitated RNA was then dissolved in 25  $\mu$ l of 10 mM Tris-HCl (pH 7.5) and stored at –70°C until further use.

**Preparation of Antisense TS ODNs.** TS antisense, random control, and sense control ODNs were synthesized using an Applied Biosystem model 391 PCR-MATE DNA synthesizer by the phosphoramidite method. The ODNs were unmodified with a phosphodiester backbone, and each ODN was designed to target the TS mRNA translation initiation start site, the middle part of the coding region, and the 3' end of the coding region, respectively. The sequences of the TS antisense ODNs were as follows: AS-1, 5'-d(CGAGCCGGCCACAGGCAT)-3' (+1 to +18 of the TS coding region); AS-2, 5'-d(GGGCCGGCGCG-GCAGCTC)-3' (+19 to +36 of the TS coding region); AS-3, 5'-d(CTGTGCGGCGGGCAA)-3' (+37 to +54 of the TS coding region); AS-4, 5'-d(GATGTGCGCAATCATGTA)-3', (+697 to +714 of the TS coding region); AS-5, 5'-d(AACAGC-CATTTCCATTTT)-3' (3'-end of TS coding region); C-1, 5'-d(GCATACGACATGGACTGG)-3' (random control); and C-2, 5'-d(TTGCCCCCGCCGCACAG)-3' (sense control for AS-3).

Each ODN was purified by ethanol precipitation. The pellets were then vacuum-dried and reconstituted in 100  $\mu$ l of 10 mM Tris-HCl (pH 7.5). The concentration of each ODN was determined by UV spectrophotometry at 260 nm.

**In Vitro Translation.** Translation reactions were performed using a rabbit reticulocyte lysate *in vitro* translation system (Promega, Madison, WI) per the manufacturer's guidelines. In brief, a 20- $\mu$ l reaction mixture containing 1  $\mu$ l (0.4  $\mu$ M) of TS mRNA template, 2  $\mu$ l of amino acid mixture (minus methionine), 6  $\mu$ Ci of [<sup>35</sup>S]methionine (Amersham, Arlington Heights, IL; specific activity, 1.29 Ci/mmol), and 10  $\mu$ l of rabbit reticulocyte lysate was prepared. To this reaction, varying concentrations of TS antisense ODN targeted to different regions of the TS mRNA, including the AUG translational start site (AS-1, AS-2, and AS-3), the middle part of the protein-coding region (AS-4), and the 3' end (AS-5) were then added. All *in vitro* translations reactions were performed at 37°C for 90 min. The reactions were terminated by placing the reaction sample in a dry ice-isopropyl alcohol slurry. Translation reaction products were analyzed by SDS-PAGE (15% acrylamide) according to the method of Laemmli (17). The gels were fixed for 30 min in a 10% acetic acid-25% isopropyl alcohol solution, followed by 20 min in Amplify reagent (Amersham). The dried gels were exposed to Kodak X-Omat AR film overnight at –70°C. The translation protein products were excised from the SDS-polyacrylamide gels and then placed into scintillation vials containing 750  $\mu$ l of an 80% hyamine hydroxide solution (ICN Radiochemicals, Irvine, CA). The samples were incubated for 4 h at 50°C and allowed to cool. Radioactivity was determined by liquid scintillation counting.

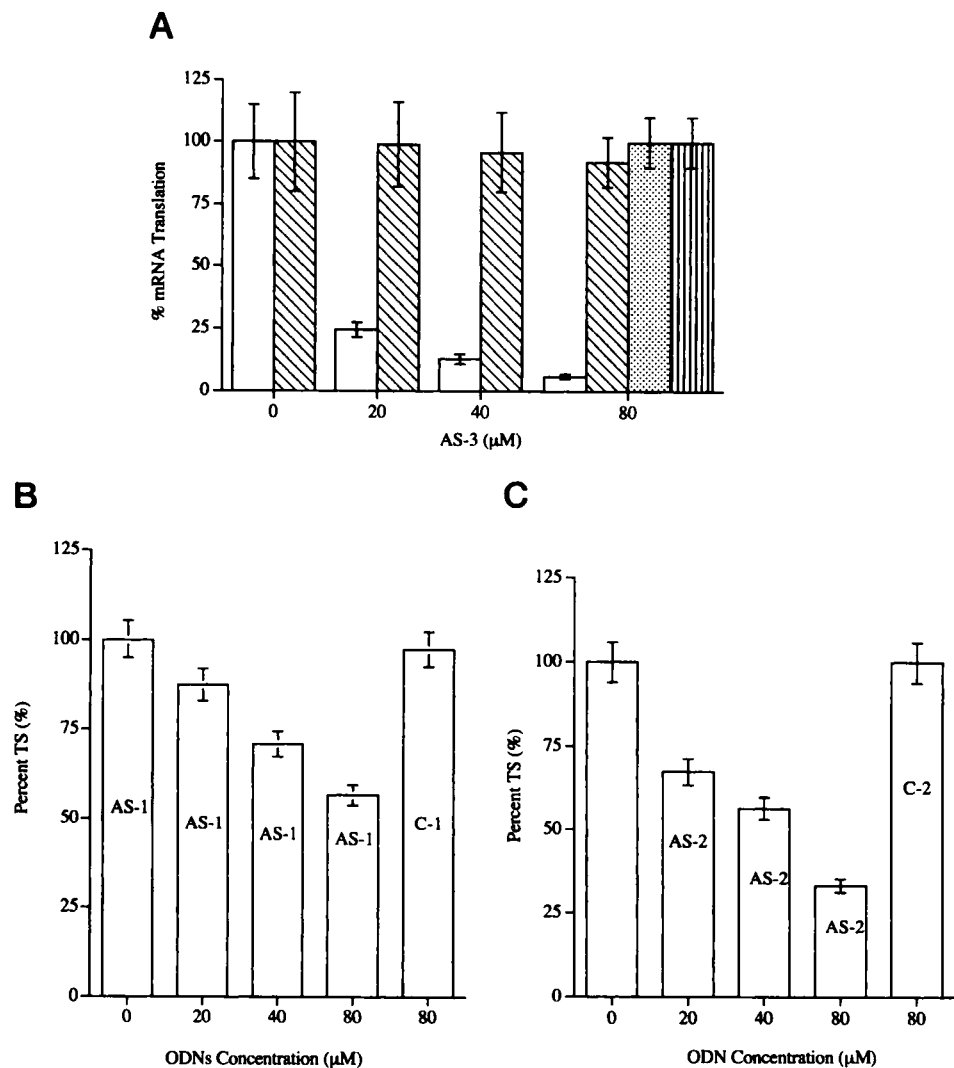
**Cell Culture.** The human colon cancer cell line HT-29 (American Type Culture Collection, Manassas, VA) was maintained in RPMI 1640 containing 10% dialyzed fetal bovine serum (Irvine Scientific, Santa Ana, CA). The human nasopharyngeal cancer cell line KB31 (obtained from Dr. Michael Gottesman, NIH, Bethesda, MD) was maintained in high-glucose DMEM containing 10% fetal bovine serum (Irvine Scientific), 5 mM glutamine, 50 units/ml penicillin, and 50 units/ml streptomycin. Both cell lines were maintained at 37°C with 5% CO<sub>2</sub> in a humidified incubator.

**Lipofectin Transfection.** To optimize the efficient delivery of ODNs into human cancer HT-29 cells, a Lipofectin transfection method was used per the manufacturer's protocol (Life Technologies, Inc.). In brief, 7.8  $\mu$ M Lipofectin were used with various concentrations of ODNs in 1 ml of serum-free medium. Cells were washed once with serum-free medium, and the DNA-Lipofectin mixture was then added to the cells for various amounts of time (0–36 h).

**Construction of TS Antisense Plasmid.** The plasmid pHaMAGRP is stress inducible and contains a GRP, as well as an MDR1 selection marker (18). A double-stranded DNA sequence corresponding to nucleotides +1 to +422 of the TS coding region was generated by PCR amplification. The primers used for this PCR contain a *Sal*I restriction site, as underlined below, at both ends. The sequences of the primers are as follows: 5'-d(CAGTGTGCGACATGCCTGTGGCCGGCTCGG-AG)-3' (sense); and 5'-d(CAGTGTGCGACATGCCTCCACTG-GAAGCCATAAAC)-3' (antisense).

The PCR amplification was performed as described above. The amplified DNA was resolved on a 1% agarose gel and purified using the Qiagen DNA purification kit. The pHaMAGRP and TS amplified DNAs were both digested with *Sal*I and purified with the Qiagen DNA purification kit. A ligation

**Fig. 1** Effect of antisense TS ODNs on translation of human TS mRNA in the rabbit reticulocyte lysate *in vitro* translation system. **A**, effect of antisense AS-3 on TS mRNA (□) and luciferase mRNA (▨) translation. C-1 (▤) ODN represents sense control ODN, and C-2 (▧) represents scramble control ODN. **B**, effect of antisense AS-1 on TS mRNA translation. C-1 represents sense control ODN. **C**, effect of antisense AS-2 on TS mRNA translation. C-2 represents scramble control ODN. The level of TS mRNA translation in the absence of antisense ODN was taken to be 100%.



reaction was then performed using T4 DNA ligase. After transformation, colonies containing the TS antisense sequence in the proper orientation were identified by restriction enzyme digestion and confirmed by direct sequencing.

**Transfection into the KB31 Cell Line.** The DNA plasmids pHMAGRP and pHMAGRP-TS were each transfected into KB31 cells by the CaPO<sub>4</sub>-DNA coprecipitation method (19). Cells were selected in the presence of 6 ng/ml colchicine (Sigma Chemical Co., St. Louis, MO). After 3 weeks, drug-resistant cells were pooled and grown to 80% confluence. The cells were subsequently maintained in medium containing 6 ng/ml colchicine.

**TS Enzymatic Activity Assay.** Two different assays were used to determine TS enzyme activity. (a) A radioligand-binding assay was used to determine TS enzyme activity by titration of TS active site in cell extracts with an excess of 5,10-CH<sub>2</sub>FH<sub>4</sub> and [<sup>3</sup>H]FdUMP, as described previously (20). Briefly, cells were trypsinized, pelleted by centrifugation, and resuspended in 0.1 M KH<sub>2</sub>PO<sub>4</sub> (pH 7.2). Cell lysis was accomplished by sonication using three 3-s bursts at 0°C. The extracts

were centrifuged at 10,000 × g for 30 min, and 100 μl of supernatants were incubated with 6 pmol of 5,10-CH<sub>2</sub>FH<sub>4</sub> and 6 pmol of [<sup>3</sup>H]FdUMP in a total volume of 125 μl. After 30 min of incubation at 37°C, 1 ml of ice-cold T-70-dextran and BSA-treated charcoal was added to the reaction mixture. After centrifugation, 0.8 ml of the supernatant was quantitated by liquid scintillation counting. (b) Activity of TS *in situ* was measured by the release of tritium from [5-<sup>3</sup>H]dUMP, as described previously (20). In brief, cells were treated with 0.5 μCi/ml [5-<sup>3</sup>H]deoxyuridine (10 μM) in a final volume of 0.5 ml of RPMI 1640 with 10% dialyzed fetal bovine serum medium. After incubation at 37°C for 60 min, 0.2 ml of medium was removed and added to 1 ml of ice-cold T-70-dextran and BSA-treated charcoal mixture to terminate the reaction. The suspensions were mixed and centrifuged for 30 min at 4400 × g. Radioactivity was determined by liquid scintillation counting after addition of the supernatant to 9 ml of scintillation mixture.

**Cytotoxicity Assay.** Cytotoxicity of FdUrd (Sigma) was determined by the tetrazolium-based cell proliferation XTT assay, according to the manufacturer's protocol (Promega). In

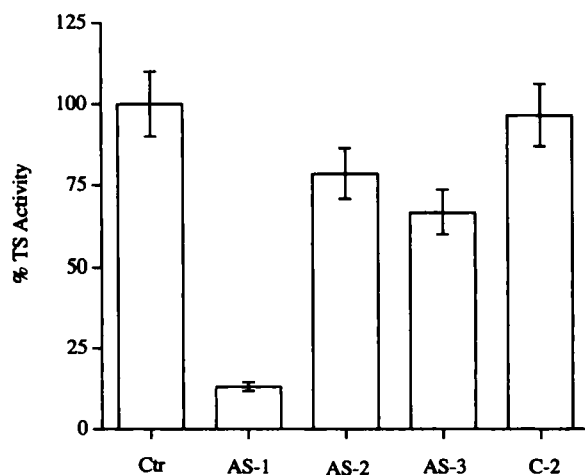


Fig. 2 Effect of antisense TS ODNs on the activity of TS in HT-29 cells. HT-29 cells were exposed to 50  $\mu$ M AS-1, AS-2, AS-3, and C-2 scrambled control ODN for 4 h. TS enzyme activity was measured by *in situ* TS catalytic activity assay. The level of TS enzyme activity in the absence of antisense TS ODN was taken to be 100%.

brief, a volume of 100  $\mu$ l of  $5 \times 10^4$  cells/ml was seeded overnight in 96-well plates. Cells were exposed to varying concentrations of FdUrd for 72 h. A separate set of experiments was performed, in which cells were exposed for 16 h to  $2 \times 10^{-4}$  mg/ml thapsigargin, which induces transcriptional activation of the glucose-regulated promoter (21). After removal of thapsigargin by washing with RPMI 1640, the cells were incubated with varying concentrations of FdUrd for 72 h. The XTT assay was then performed to determine analyze FdUrd cytotoxicity.

**PCR Quantitation of TS mRNA and TS Antisense RNA.** The method for quantitative PCR for TS expression level has been described previously in detail (22). Quantitation of the expression of  $\beta$ -actin gene was used as an internal standard. Each 5' primer contained the T7 promoter primer TAATACGACTCACTATA at the 5' end. The primers used were: TS60, 5'-d(GATGTGCGCAATCATGTACGTGAG)-3' (antisense, +697 to +720); TS61, 5'-d(T7-GGGAGAGGAGT-TGACCAATGCAAAGAGTG)-3' (sense, +469 to +492); BA67, 5'-d(T7-GGGAGAGCGGGAATCGTGCGTGACA-TT)-3' (sense, 2104–2127 of the  $\beta$ -actin genomic sequence, located on exon 3); and BA68, 5'-d(GATGGAGTTGAAG-GTAGTTTCGTG)-3' (antisense, 2409–2432 of the  $\beta$ -actin genomic sequence, located on exon 3).

To confirm the presence of TS antisense plasmid in stably transfected KB31 cells, PCR was used to amplify a region containing the TS antisense sequence and the GRP promoter sequence. The primers used to confirm the presence of pHa-MAGRP-TS vector were as follows: GRP antisense, 5'-d(TA-ATACGACTCACTATAGGGAGCTGTGACTACACTGAC)-3'; and TS sense, 5'-d(AGATCCAACACATCCTCCGC)-3'. The PCR was run for 30 cycles at 96°C for 15 s, 60°C for 30 s, and 72°C for 30 s with a final extension for 7 min at 72°C. All PCR products were transcribed *in vitro* with radiolabeled [ $\alpha$ - $^{32}$ P]CTP using T7 RNA polymerase and then resolved on a 6% polyacrylamide denaturing gel.

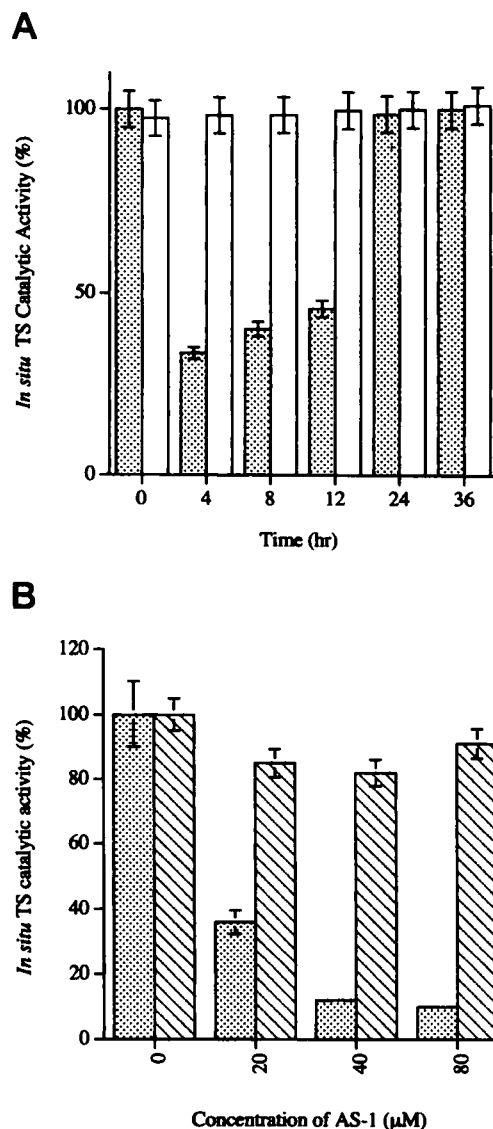
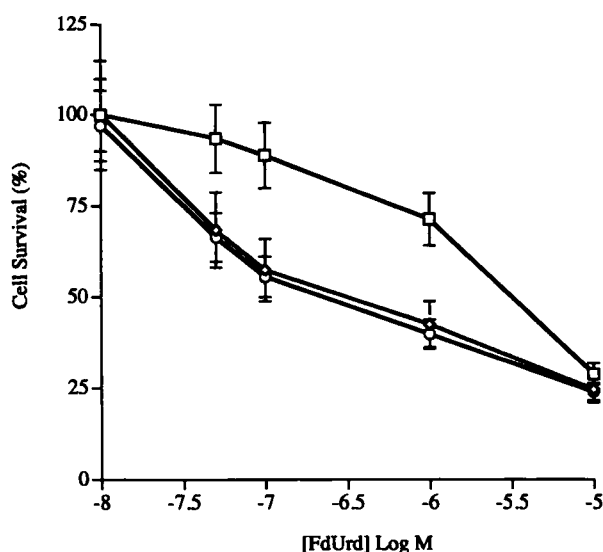


Fig. 3 Effect of antisense AS-1 on TS enzyme activity in HT-29 cells as a function of time. A, HT-29 cells were incubated with AS-1 ODN (50  $\mu$ M) for 0–36 h (■), and TS enzyme activity was determined by the *in situ* assay, as described in "Materials and Methods." C-2 ODN was used as a control (□). Cells were continuously exposed to AS-1 from 0 to 36 h. B, dose-dependent study of TS antisense effect on TS enzyme activity (*in situ* TS activity assay was performed at 4 h of incubation with AS-1; ■). C-1 was used as control ODN (□). The 100% reference point was the control without antisense ODN.

## RESULTS

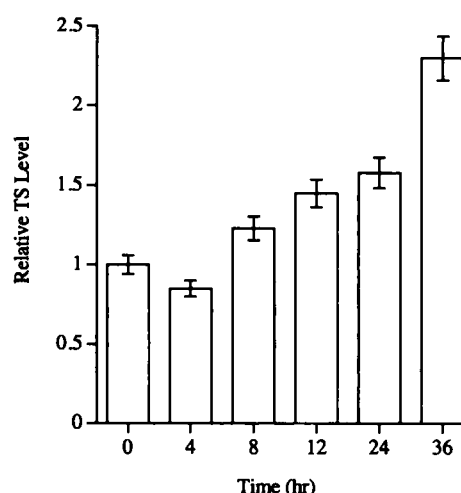
**Inhibition of *in Vitro* TS mRNA Translation by Antisense TS ODNs.** The effect of antisense ODNs on *in vitro* translation of human TS mRNA was first investigated using a rabbit reticulocyte lysate system. Among all of the antisense TS ODNs tested, the most potent inhibitor of TS mRNA translation was AS-3 targeted to nucleotides 37–54 of the TS coding region. This antisense ODN repressed TS mRNA translation in a dose-dependent manner, with an  $IC_{50}$  of  $\sim 10$   $\mu$ M (Fig. 1A). In contrast, the control sense (C-2) and scrambled ODNs (C-1) had



**Fig. 4** Effect of antisense AS-1 on cytotoxicity of FdUrd in HT-29 cells. HT-29 cells were incubated with 50  $\mu$ M antisense ODN AS-1 (□) or control ODN C-1 (◇) for 4 h. FdUrd was then added for an additional 8 h. The drugs were removed, and cells were grown for 48 h. Cell survival was measured by the XTT assay. ○, control non-ODN-treated cells.

absolutely no inhibitory effect on *TS* mRNA translation (Fig. 1A). To further demonstrate specificity, the effect of the most potent antisense ODN AS-3 on the translation of an unrelated mRNA transcript was tested, as seen in Fig. 1A; this ODN did not repress the translation of an unrelated luciferase mRNA (Fig. 1A). Antisense ODNs AS-1 and AS-2, targeted to nucleotide sequences 1–18 and 19–36 of the *TS* mRNA coding region, respectively, were significantly less effective as inhibitors of *TS* mRNA translation (Fig. 1, B and C, respectively). In addition, antisense ODNs (AS-4 and AS-5), targeted to the middle part of the *TS* coding region and the 3' end of *TS* mRNA, respectively, had no inhibitory effect on *TS* mRNA translation (data not shown).

**Inhibition of Expression in HT-29 cells by *TS* Antisense.** Having demonstrated sequence-specific inhibition of *TS* mRNA translation in the rabbit reticulocyte lysate system, we next investigated the effect of antisense ODNs on the expression of *TS* protein in human HT-29 colon cancer cells. Although AS-3 was identified to be the most potent repressor of *TS* mRNA translation using the rabbit reticulocyte lysate system, AS-1, targeted to nucleotides 1–18, was the most effective antisense ODN at down-regulating *TS* activity in HT-29 cells (Fig. 2). On the basis of this initial result, we used AS-1 for subsequent studies in intact cells. As shown in Fig. 3A, *TS* enzyme activity, as measured by the *in situ* *TS* activity assay, was significantly decreased to nearly 30% of control by 4 h with AS-1. However, the inhibitory effect diminished with longer exposure time, possibly resulting from degradation of the ODNs (23). AS-1 inhibited *TS* enzyme activity in a dose-dependent fashion, with an  $IC_{50}$  of  $\sim 10$   $\mu$ M (Fig. 3B). In contrast, sense control (C-2) and scrambled control (C-1) had no inhibitory effect on *TS in situ* enzyme activity.



**Fig. 5** Effect of antisense AS-1 on *TS* enzyme activity in HT-29 cells. HT-29 cells were incubated with 50  $\mu$ M AS-1 antisense ODNs for various times (0–36 h), and *TS* activities were determined by the [ $^3$ H]FdUMP binding assay, as outlined in "Materials and Methods." Relative *TS* enzyme activity value was then determined using the 0-h time point as the control value.

**Cytotoxicity Studies in HT-29 Cells.** We next investigated the effect of antisense AS-1 on the cytotoxicity of FdUrd in HT-29 cells. Because intracellular *TS* enzyme activity was maximally decreased after 4 h of incubation with 50  $\mu$ M AS-1 (Fig. 3B), HT-29 cells were exposed to this same concentration of antisense AS-1 for 4 h and then treated with FdUrd for an additional 8 h. Cell viability was measured by the XTT assay after 48 h. However, instead of increasing the cytotoxic potency of FdUrd, as was expected, AS-1 decreased the sensitivity of the cells to FdUrd by nearly 12-fold ( $IC_{50} = 3.16$   $\mu$ M; Fig. 4). The sense control C-1 ODN had no effect on FdUrd cytotoxicity ( $IC_{50} = 0.25$   $\mu$ M). To determine whether AS-1 actually had any effect on the intracellular levels of *TS*, *TS* content of cell extracts was quantitated by the [ $^3$ H]FdUMP ligand-binding assay (20). We found that, consistent with the decreased sensitivity of the cells to FdUrd, the *TS* level had actually increased by almost 2-fold after a 36-h exposure of the cells to 50  $\mu$ M AS-1 (Fig. 5).

**Transfection of KB31 Cells with a *TS* Antisense RNA Vector.** Because treating the cells with the antisense segment AS-1 gave the unexpectedly anomalous result of *TS* up-regulation and decreased sensitivity to FdUrd, we tested the strategy of transfecting cells with an expression vector containing a long antisense fragment. The idea behind this approach was that the use of a longer fragment might increase the probability of forming a complex with *TS* mRNA that would either promote degradation of the mRNA or inhibit its translation. In addition, generating the antisense fragment endogenously should also overcome the instability and relatively short half-life of ODNs in tissue culture medium (23). A *TS* antisense sequence corresponding to positions +1 to +422 of the coding region was inserted into plasmid pHaMAGP, which contains the stress-inducible GRP. We encountered difficulties in transfecting human HT-29 cells with this plasmid, so human nasopharyngeal

pHaMAGRP pHaMAGRP-TS



Fig. 6 Characterization of KB31 cells transfected with pHaMAGRP and pHaMAGRP-TS vector by RT-PCR. RNA was isolated from each cell line and then subjected to RT-PCR to determine the presence of the antisense *TS* RNA, as described in "Materials and Methods."

Table 1 Effect of introduction of *TS* antisense-expressing plasmid on the levels of *TS* mRNA and *TS* *in situ* enzyme activity in KB31 cells

	No transfection	pHaMAGRP	pHaMAGRP-TS
<i>TS</i> mRNA level (TS/ $\beta$ -actin)	11.2	14.3	10.6
<i>TS</i> protein level (pmol/10 <sup>6</sup> cells)	0.79	0.78	0.52
<i>TS</i> protein level after thapsigargin (pmol/10 <sup>6</sup> cells)	0.79	0.79	0.49
<i>TS</i> enzyme activity (% of control)	100	98.2	72.3 <sup>c</sup>

<sup>a</sup>  $P = 0.014$ .

<sup>b</sup>  $P = 0.017$ .

<sup>c</sup>  $P = 0.023$ .

KB31 cells were used instead. The PCR was used to confirm the successful transfection of pHaMAGRP-TS antisense construct into the cells. Primers were designed to amplify a region containing the *TS* antisense sequence and a portion of the GRP promoter sequence. As shown in Fig. 6, a PCR product corresponding to this segment of the vector was generated from extracts of transfected KB31 cells. *TS* enzyme activity, as measured by the *in situ* *TS* activity assay, was decreased by 30% in pHaMAGRP-TS-transfected KB31 cells ( $P = 0.032$ ; Table 1). Consistent with this observation, the content of *TS* protein was also almost 30% lower in the transfectants than in the parental cells, as determined by the [<sup>3</sup>H]FdUMP binding assay ( $P = 0.019$ ). In contrast, transfection of a pHaMAGRP plasmid

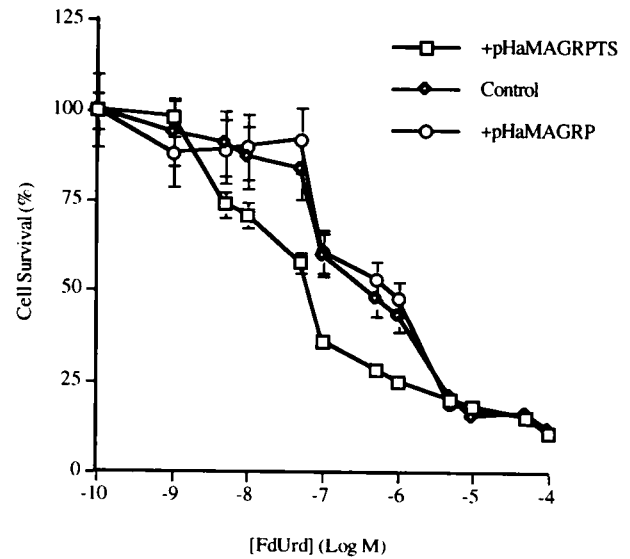


Fig. 7 Effect of antisense *TS* RNA on the cytotoxicity of FdUrd. Cells were exposed to various concentrations of FdUrd, and cell viability was determined by the XTT assay after 72 h.

not containing a *TS* insert had no inhibitory effect on *TS* enzyme activity. Despite the decrease in *TS* content and activity, no difference in *TS* mRNA levels between plasmid-transfected and parental KB31 cells could be detected by quantitative RT-PCR. Thapsigargin had been previously shown to enhance GRP transcriptional activity (21). However, treatment of the KB31 transfectants with thapsigargin did not significantly lower *TS* levels in the cells (Table 1).

**Cytotoxicity Studies in KB31 Cells Transfected with pHaMAGRP *TS*.** KB31 cells transfected with the *TS* antisense plasmid were more sensitive to treatment with FdUrd ( $IC_{50} = 50$  nM), compared with control cells ( $IC_{50} = 400$  nM;  $P = 0.025$ ; Fig. 7). Transfection of the plasmid decreased cell viability from 55 to 35% at 0.1  $\mu$ M FdUrd. However, there was no difference in the sensitivity of the transfected and untransfected KB31 cells to either Adriamycin or cisplatin (Fig. 8).

## DISCUSSION

Here, we examined the effects of antisense *TS* ODNs targeted at human *TS* mRNA on *TS* translation *in vitro*, the intracellular status of *TS*, and the sensitivity of cells to *TS*-directed agents. The observation that *TS* antisense ODNs can specifically repress *TS* mRNA translation in the rabbit reticulocyte lysate system suggested that, in principle, it should be possible to down-regulate *TS* in intact cells. However, the anomalous unexpected effects observed when cells were treated with *TS* antisense ODNs indicate the occurrence of more complex events than those in the *in vitro* translation system. Although the initial observation of decreased *TS* activity within 4 h in cells treated with AS-1 might, at first, appear to be consistent with the expected effect of the ODN, it probably is not due to decline of intracellular *TS* due to translational suppression because *TS* protein is known to have a reasonably long half-life in cells (5). Instead, the rapid inhibition of *TS* activity

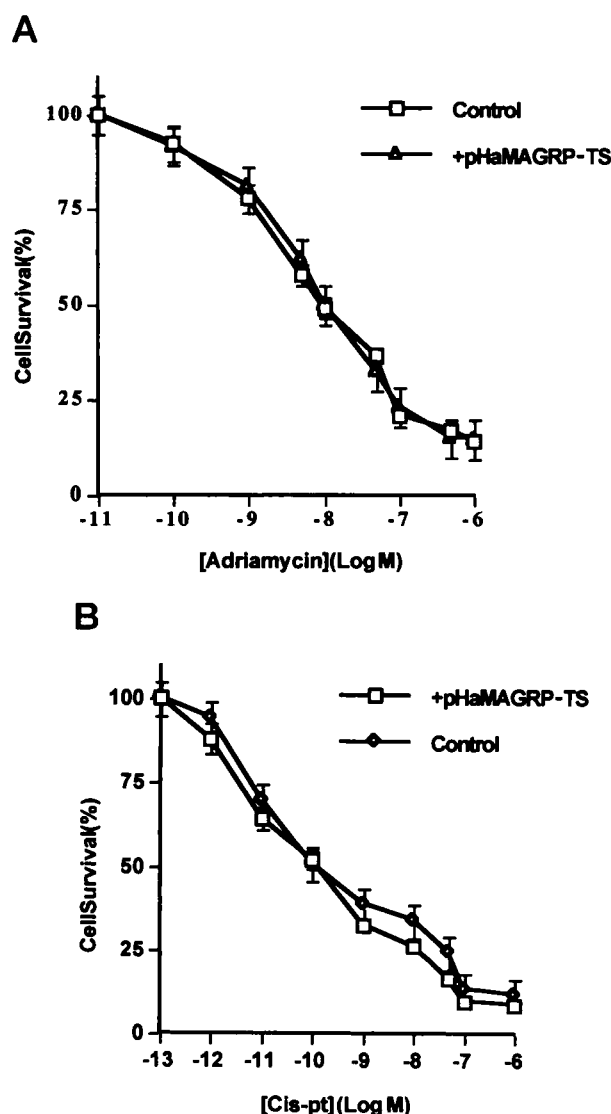


Fig. 8 Effect of antisense *TS* on the cytotoxicity of Adriamycin (A) and cisplatin (B). Cells were exposed to various drug concentrations, as indicated, and cell viability was determined by the XTT assay after 72 h.

suggests some sort of direct inhibitory activity by the ODN. *TS* is known to form complexes with a number of mRNAs, including those of p53, c-myc, and *TS* mRNA itself (8, 9). Thus, the polynucleotide binding site on *TS* appears to be fairly nonspecific, and it is possible that the ODN could bind to this site and, in doing so, could disrupt the correct folding of *TS*, thereby decreasing its activity. We have preliminary evidence that AS-1 does, indeed, lower the affinity for [<sup>3</sup>H]FdUMP to purified *Escherichia coli* *TS* protein *in vitro* (data not shown). A recent study found that methotrexate-resistant K562 cells, in which the expression of a naturally generated *TS* antisense segment (*rTS*) was elevated compared to parental K562 cells, had lower basal levels of *TS* activity and loss of growth-associated fluctuations of *TS* *in situ* activity (11). These data were considered as evidence for a link between *rTS* expression and regulation of *TS*

activity, and if so, it may be that the ODN is simulating some aspects of the regulatory mechanism of *rTS*. The rebound of *TS* activity after this initial inhibition is most likely due to degradation of the ODN, consistent with the known short half-life of phosphoester ODNs in culture (23). We first tried to overcome this instability by using phosphorothioate ODNs to down-regulate *TS* in cells, but these analogues exerted a nonspecific cytotoxic effect on cells, in which nonsense segments were as inhibitory as *TS*-specific ODNs (data not shown).

The surprising result of this experiment was that, despite short-term inhibition of *TS* activity, the longer-term effect of the *TS*-specific ODN was actually to increase *TS* levels in the cells, resulting in decreased rather than enhanced sensitivity to the *TS*-directed agent FdUrd. The precise mechanism of this effect is not yet clear, but we think it may be associated with the phenomenon of *TS* autoregulation (7). The observation that *TS* protein suppresses the translation of *TS* mRNA *in vitro* (7) led to the hypothesis that the aforementioned binding of *TS* mRNA to *TS* protein constitutes a mechanism that allows *TS* to regulate its own translation. Thus, if the ODN were to interfere with the binding of the mRNA to *TS*, more free *TS* mRNA would be available for translation and would eventually give rise to higher intracellular levels of *TS*. The ODN could compete with *TS* mRNA for the polynucleotide binding site on the enzyme or, alternately, the duplex that the antisense ODN presumably forms with *TS* mRNA may hinder its binding to *TS* protein.

To see if the effects of *TS* antisense in cells could be altered by endogenous expression and the use of a larger-sized ODN that would permit a number of different possibilities for interaction points, we inserted a segment of *TS* corresponding to bases +1 to +422 of the coding region into plasmid pHaMAGRP. We chose this particular plasmid because it contains the stress-inducible GRP, which is thought to be optimally active in tumor cells because tumors are "under growth stress" (18). This approach succeeded in lowering *TS* protein level in *TS* antisense vector-transfected cells, whereas that of control vector-transfected cells was unaffected (Table 1). The observation that *TS*-pHaMAGRP affected intracellular *TS* levels in a manner opposite to that of AS-1 might be reasonably explained by hypothesizing (a) that various AS ODNs have different degrees of capability for overcoming the translational autoregulation of *TS* and for inhibiting translation and (b) that the net effect of an AS fragment on *TS* levels is determined by a balance between a propensity for an increase in translation rate caused by the ODN and its efficacy at inhibiting translation. Thus, the lower amount of *TS* protein content after treatment with *TS*-pHaMAGRP may mean that this ODN is either less efficient than AS-1 at relieving the translational detainment of *TS* or more efficient than AS-1 at inhibiting the translational machinery, thereby compensating for the putative higher levels of free *TS* mRNA. The observation that total *TS* mRNA content of the cells was not noticeably altered by introduction of the *TS*-pHaMAGRP supports the hypothesis that the level of *TS* protein is regulated by effects on translation rather than at the transcriptional level by RNase H mediated hydrolysis of antisense-mRNA heteroduplexes.

Treatment of the cells with thapsigargin, a presumed enhancer of GRP promoter activity (21), resulted in little or no effect on *TS* protein level, indicating either that that GRP

promoter was only slightly induced by thapsigargin or that the inhibition of translation by the antisense was already at its maximal level.

The results presented here demonstrate the principle that, despite the complexity of TS regulation leading to anomalous effects with some antisense fragments, an antisense approach can decrease intracellular TS levels and increase the chemosensitivity of cells to TS inhibitors. Further development of this strategy may eventually make it possible to reduce TS content of highly TS-expressing tumors that are resistant to therapy with 5-FU or other TS-directed drugs. In this respect, it is encouraging that even the small decrease in TS produced by the present system significantly enhanced the sensitivity of the cells to FdUrd.

## REFERENCES

1. Danenberg, P. V. Thymidylate synthase: a target enzyme in cancer chemotherapy. *Biochem. Biophys. Acta*, 473: 73–79, 1977.
2. Moertel, C. G. Chemotherapy for colorectal cancer. *N. Engl. J. Med.*, 330: 1136–1143, 1994.
3. Grem, J. L. 5-Fluorouracil plus leucovorin in cancer therapy. In: V. T. De Vita, Jr., S. Hellman, and S. A. Rosenberg (eds.), *Principles and Practice of Oncology Update Series*, Vol. 2. Philadelphia: J. B. Lippincott Co., 1988.
4. Harrap, K. R., Jackman, A. L., Newell, D. R., Taylor, G. A., Hughes, L. R., and Calvert, A. H. Thymidylate synthase: a target for anticancer drug design. *Adv. Enzyme Regul.*, 29: 161–179, 1989.
5. Johnson, L. F. Post transcriptional regulation of thymidylate synthase gene expression. *J. Cell. Biochem.*, 54: 378–392, 1994.
6. Keyomarsi, K., Samet, J., Molnar, G., and Pardee, A. B. The thymidylate synthase inhibitor ICI D 1694 overcomes translational detainment of the enzyme. *J. Biol. Chem.*, 268: 15132–15149, 1993.
7. Chu, E., Koeller, D. M., Casey, J. L., Drake, J. C., Chabner, B. A., Elwood, P. C., Zinn, S., and Allegra, C. J. Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase. *Proc. Natl. Acad. Sci. USA*, 88: 8977–8981, 1991.
8. Chu, E., Voeller, D., Koeller, D. M., Drake, J., Takimoto, C. H., Maley, G. F., Maley, F., and Allegra, C. J. Identification of an RNA binding site for human thymidylate synthase. *Proc. Natl. Acad. Sci. USA*, 90: 517–521, 1993.
9. Chu, E., Cogliati, T., Copur, S. M., Borre, A., Voeller, D. M., Allegra, C. J., and Segal, S. Identification of *in vivo* target RNA sequences bound by thymidylate synthase. *Nucleic Acids Res.*, 24: 3222–3228, 1996.
10. Dolnick, B. J. Cloning and characterization of a naturally occurring antisense RNA to human thymidylate synthase mRNA. *Nucleic Acids Res.*, 21: 1747–1752, 1993.
11. Black, A. R., and Dolnick, B. J. Expression of rTS correlates with altered growth regulation of thymidylate synthase. *Cancer Res.*, 56: 700–705, 1996.
12. Berger, S. H., and Berger, F. G. Thymidylate synthase as a determinant of 5-fluoro-2'-deoxyuridine response in human colonic tumor cell lines. *Mol. Pharmacol.*, 34: 474–479, 1988.
13. Chu, E., and Allegra, C. J. Mechanisms of clinical resistance to 5-fluorouracil chemotherapy. *Cancer Treat. Res.*, 87: 175–195, 1995.
14. Van der Wilt, C. L., Pinedo, H. M., Smid, K., and Peters, G. J. Elevation of thymidylate synthase following 5-fluorouracil treatment is prevented by the addition of leucovorin in murine colon tumors. *Cancer Res.*, 52: 4922–4928, 1992.
15. Johnston, P. G., Lenz, H.-J., Leichman, C. G., Danenberg, K. D., Allegra, C. J., Danenberg, P. V., and Leichman, L. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res.*, 55: 1407–1412, 1995.
16. Johnston, P. G., Fisher, E. R., Rockette, H. E., Fisher, B., Wolmark, N., Drake, J. C., Chabner, B. A., and Allegra, C. J. The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. *J. Clin. Oncol.*, 12: 2640–2647, 1994.
17. Laemmli, U. K. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (Lond.)*, 227: 680, 1970.
18. Gazit, G., Kane, S. E., Nichols, P., and Lee, A. S. Use of the stress-inducible *grp78/BiP* promoter in targeting high level gene expression in fibrosarcoma *in vivo*. *Cancer Res.*, 55: 1660–1663, 1995.
19. Gorman, C., Padmanabhan, R., and Howard, B. H. High efficiency DNA-mediated transformation of primate cells. *Science (Washington DC)*, 221: 551, 1983.
20. Keyomarsi, K., and Moran, R. G. Quinazoline folate analogs inhibit the catalytic activity of thymidylate synthase but allow the binding of 5-fluorodeoxyuridylate. *J. Biol. Chem.*, 265: 19163–19169, 1990.
21. Li, W. W., Alexandre, S., Cao, C., and Lee, A. S. Transactivation of the *grp78* promoter by  $Ca^{2+}$  depletion: a comparative analysis with A23187 and the endoplasmic reticulum  $Ca^{2+}$ -ATPase inhibitor thapsigargin. *J. Biol. Chem.*, 268: 12003–12009, 1993.
22. Horikoshi, T., Danenberg, K. D., Stadlbauer, T. H. W., Volkenandt, M., Shea, L. C. C., Aigner, K., Gustavsson, B., Leichman, L., Frosing, R., Ray, M., Gibson, N. W., Spears, C. P., and Danenberg, P. V. Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using a polymerase chain reaction. *Cancer Res.*, 52: 108–116, 1992.
23. Corrias, M. V., and Tonini, G. P. An oligomer complementary to the 5' end region of *MDR1* gene decrease resistance to doxorubicin of human adenocarcinoma-resistant cells. *Anticancer Res.*, 12: 1431–1438, 1992.