1992

# SEQUENCE SPECIFICITY OF TENIPOSIDE-INDUCED DELETION AND INSERTION MUTATIONS AT THE APRT LOCUS OF CHINESE HAMSTER CELLS

Yi-Hong Han

Virginia Commonwealth University
School of Basic Health Sciences

This is to certify that the dissertation prepared by Yi-Hong Han entitled "Sequence Specificity of Teniposide-Induced Deletion and Insertion Mutations at the *aprt* locus of Chinese Hamster Cells" has been approved by her committee as satisfactory completion of the dissertation requirement for the degree of Doctor of Philosophy.

[redacted]

Lawrence F. Povirk, Director of Dissertation

[redacted]

David A. Gewirtz, School of Basic Health Sciences

[redacted]

Louis S. Harris, School of Basic Health Sciences

[redacted]

Phillip B. Hylemon, School of Basic Health Sciences

[redacted]

Paul S. Swerdlow, School of Medicine

[redacted]

Louis Harris, Department Chairman

[redacted]

S. Gaylen Bradley, Dean, School of Basic Health Sciences
Chairman, MCV Graduate Committee

16 March 1992

Date

# SEQUENCE SPECIFICITY OF TENIPOSIDE-INDUCED DELETION AND INSERTION MUTATIONS AT THE *APRT* LOCUS OF CHINESE HAMSTER CELLS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Medical College of Virginia, Virginia Commonwealth University

by

Yi-Hong Han
B.M. Beijing Medical University
August, 1984

Director: Lawrence F. Povirk, Ph.D.,
Department of Pharmacology and Toxicology

Medical College of Virginia
Virginia Commonwealth University
Richmond, Virginia
May, 1992

Dedicated to

*My parents*

ACKNOWLEDGEMENT

I would like to express my heartfelt appreciation to Dr. Larry Povirk for his support and guidance without which this work would not have been possible. I feel privileged to have been able to observe and experience first hand his famous Povirkian approach to problem solving. His insights and thoroughness have never failed to put me to shame.

I would like to thank Dr. David Gewirtz for his invaluable advice and assistance which he offered, from time to time, with generosity and patience.

Special thanks must go to Drs. Louis Harris, Phillip Hylemon and Paul Swerdlow for their contributions to this dissertation. The encouragement I received from them enabled me to survive the stress of my difficult times.

Appreciation are also due to those whom I worked with side by side at one time or another in the laboratory. Among them are Web Houlgrave, Bob Steighner, Finley Austin, Richard Bennett, Peng Wang, Todd Bunch and Gwen Bouer. Their help and friendship all contributed to making my past few years more fruitful and enjoyable.

The last but not the least person I would like to thank is my husband. His understanding and emotional support made the long journey seem not so long.

Table of Contents

## List of Tables

## List of Figures

## List of Abbreviations

| | |
|---|---|
| *aprt* | adenine phosphoribosyl transferase |
| ATP | adenosine triphosphate |
| bp | base pair |
| CHO | Chinese hamster ovary cell |
| Ci | Curie |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytosine triphosphate |
| ddATP | dideoxyadenosine triphosphate |
| ddCTP | dideoxycytosine triphosphate |
| ddGTP | dideoxyguanosine triphosphate |
| ddTTP | dideoxythymidine triphosphate |
| dGTP | deoxyguanosine triphosphate |
| $dH_2O$ | distilled water |
| dTTP | deoxythymidine triphosphate |
| DNA | deoxyribonucleic acid |
| *E.coli* | Escherichia coli |
| EDTA | ethylenediaminetetraacetic acid |
| *hgprt* | hypoxanthine guanine phosphoribosyl transferase |
| HPLC | high pressure liquid chromatography |
| kb | kilo base pairs |
| M.F. | mutation frequency |
| PCR | polymerase chain reaction |
| PMSF | phenylmethylsulfonyl fluoride |
| PRPP | 5-phosphoribosyl-1-pyrophosphate |
| RNA | ribonucleic acid |
| SDS | sodium dodecyl sulfate |
| *tk* | thymidine kinase |
| Topo II | topoisomerase II |

SEQUENCE SPECIFICITY OF TENIPOSIDE-INDUCED DELETION AND INSERTION MUTATIONS AT THE *APRT* LOCUS OF CHINESE HAMSTER CELLS

ABSTRACT

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Medical College of Virginia, Virginia Commonwealth University.

Yi-Hong Han

Medical College of Virginia--Virginia Commonwealth University, 1992.

Advisor: Dr. Lawrence F. Povirk

Previous studies suggested that teniposide is a strong clastogen, and that the DNA breakage effect of this drug is mediated by the nuclear enzyme topoisomerase II. Ripley et al found evidence for a correspondence between sites of acridine-induced frameshift mutations in bacteriophage T4 and sites of *in vitro* DNA cleavage by T4 topoisomerase II. To identify the sequence specificity of teniposide-induced deletion and insertion mutations in mammalian cells, the CHO-D422 cell line, which is hemizygous at the *aprt* locus, was employed in this study. Sixty-eight teniposide-induced and 42 spontaneous *aprt* mutants were analyzed at the DNA sequence level. Compared with the spectrum of spontaneous mutations in which two thirds of the mutations are base substitutions, two thirds of teniposide-induced mutations are deletions and insertions of different sizes. Significant site correspondence between teniposide-induced deletion/insertion mutations and *in vitro*

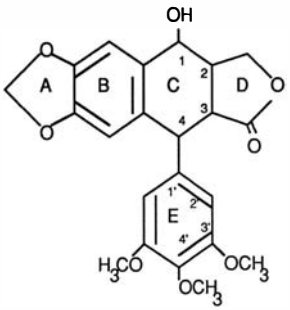
DNA double strand cleavages by purified mammalian topoisomerase II was also found in this study, which suggests that the majority of teniposide-induced deletions and insertions observed in this study were generated at the sites of topoisomerase II mediated DNA double strand breaks in the cells. However, considering the positions of the double cleavage sites in the mutation sequences, no consistent pattern was found which could suggest a unified mechanism of DNA double strand break repair. Three models are proposed to try to explain the possible events occurring in the cells following teniposide exposure which resulted in observed deletion and insertion mutations.

# INTRODUCTION

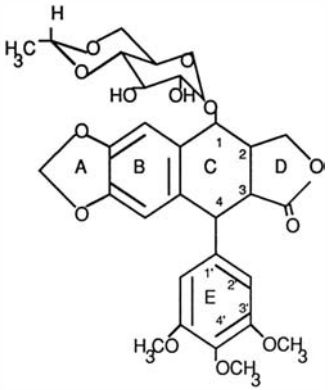
Teniposide (4'-dimethyl-epipodophyllotoxin-thenylidene beta-D-glucopyranoside; VM-26, Figure 1) is a semisynthetic epipodophyllotoxin derivative which actively used in clinical cancer chemotherapy since early 1970's.

Previous studies showed that teniposide produced a cytotoxic effect in mammalian cells mainly by causing extensive DNA single and double strand breaks (Loike et al., 1974), and the DNA fragmentation effect of teniposide was mediated by topoisomerase II, an essential nuclear enzyme which functions to resolve DNA topological problems during certain genetic activities such as replication and transcription (Long and Minocha, 1983). Teniposide is able to inhibit the enzyme's transient DNA breakage resealing activity, and this is presumably the main mechanism of action of the drug.

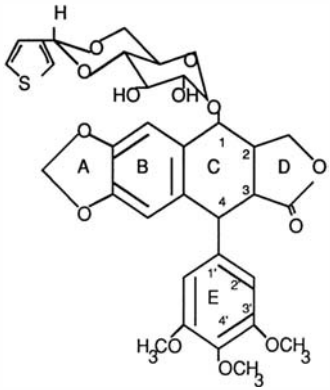
Therapy-related acute myeloid leukemias have been known to occur among long term cancer survivors who were treated with radiation and/or certain anticancer drugs such as alkylating agents. A unique type of acute monocytic leukemia has been identified in recent years and attributed to the use of epipodophyllotoxins and other topoisomerase II inhibitors




Podophyllotoxin




Etoposide




Teniposide

Fugure 1. Structural formulas of podophyllotoxin, etoposide and teniposide.

including acridines and anthracyclines, etc. (Ratain et al., 1987). The demand for effective anticancer drugs with greater therapeutic indexes urges us to provide detailed knowledge about the mutagenic properties of drugs like teniposide in order to direct future efforts in drug development.

Teniposide was found to be strongly mutagenic in mammalian cells as indicated by a significant increase in the mutation frequencies at the hypoxanthine-guanine phosphoribosyl transferase (*hgprt*) and the thymidine kinase (*tk*) loci following teniposide treatment (Singh and Gupta 1983; DeMarini et al., 1987). Sister chromatid exchanges were also induced by teniposide in Chinese hamster ovary (CHO) cells. However, the drug failed to exhibit significant mutagenic effect on a number of prokaryotic mutagenesis systems (Gupta, et al, 1987). These results suggest that teniposide is a strong clastogen and that the DNA breakage effect of the drug is probably due to the inhibition of mammalian topoisomerase II.

Due to the lack of good model systems, only a few studies were focused on looking for the DNA sequence specificity of mutations induced by topoisomerase II inhibitors, and none of the work was done in mammalian cells. Ripley et al (1988) reported that hotspot sites for acridine-induced frameshift mutations in bacteriophage T4 correspond exactly to the *in vitro* cleavage sites of the T4 topoisomerase II. These results not only suggested that inhibition of T4 topoisomerase II by acridine was responsible for the generation of the observed

mutations, but also showed that the *in vivo* topoisomerase II mediated DNA cleavages could be reproduced *in vitro* with the same sequence specificity. It would be especially sensible to find out whether such co-specificity is also present in mammalian cells.

The present study is aimed to reveal the DNA sequence specificity of teniposide-induced mutations in mammalian cells. A special CHO cell line D422, which is hemizygous at the *aprt* gene, was employed in this study. The *aprt* gene encodes the nonessential enzyme adenine phosphoribosyl transferase, the product of which, adenosine-5'-monophosphate (AMP) is used in DNA synthesis. Cells that carry mutationally inactivated *aprt* genes could be selected by 8-azaadenine, which is a toxic analog of adenine. Because of the hemizygous nature and the small size (2.6 kb) of the locus, the *aprt* mutants could be analyzed at the DNA sequence level. Possible co-specificity between teniposide-induced mutations and the *in vitro* topoisomerase II cleavage sites could provide clues for identification of possible mechanisms of teniposide mutagenesis as well as the mechanisms of DNA double strand break repair.

# LITERATURE REVIEW

## HISTORY

*Podophyllum emodi* Wall., which grows in the Himalayan region, and the American *Podophyllum peltatum L.* (may apple, mandrake) are old medicinal plants. They belong to the family of the *Berberidaceae* and were used by the natives of both continents as cathartics and anthelminthics during the last two centuries. After Kaplan discovered the therapeutic effect of podophyllin, an alcoholic extract of the *Podophyllum* rhizomes, in the treatment of condylomata acuminata in the 1940s (Kaplan, 1942), King and Sullivan (1946), Sullivan and Wechsler (1947) and others described arrest in metaphase produced by this plant extract. In the meantime, successes with condyloma therapy inspired intensive work with malignant tumors in several separate laboratories and podophyllin was shown to exhibit a significant cytotoxic effect (Cornman and Cornman, 1951). The chemical analysis of podophyllin revealed several compounds of which Podophyllotoxin is the main cytotoxic constituent. All these substances belong to the class of lignans, natural products containing the 2,3-dibenzylbutane skeleton. Although possessing antitumor

properties they were unacceptable for human use because toxicity prevented their administration in high enough dosage to give significant clinical activity (Issell and Crooke, 1979).

In the early 1950s, chemists in the pharmaceutical research department of Sandoz, Ltd. in Switzerland reasoned that *Podophyllum* lignans might be present in the plant as glycosides. It was hoped that, in analogy to cardiac glycosides, they would exhibit pharmacological properties superior to those of the aglycones. After systematic chemical modification of the podophyllotoxin molecule and testing in biological systems, two preparations, namely SP-G, the condensation product of the *Podophyllum* glucoside fraction with benzaldehyde, and SP-I, podophillinic acid ethyl hydrazide were demonstrated to be therapeutically useful and were commercialized in 1963. After two years of more combined chemical and biological search for still better antitumor agents, a compound, namely DEPBG (D, 4'-demethyl-; E, epi-; P, podophyllotoxin; B, benzylidene; G, B-D-glucoside) was found in SP-G which was a potent inhibitor of cell proliferation. To their surprise, DEPBG exhibited a different mode of action compared with that of previous *Podophyllum* compounds: instead of producing an arrest of mitosis in metaphase, it prevented proliferating cells from entering mitosis and thus induced a premitotic block (Stähelin and Cerletti, 1964). Subsequently, a large number of other aldehydes were condensed to this glucoside (Kuhn et al., 1969; Kuhn and Wartburg, 1969) and the

products were analyzed for their biological effects. Two of them, the condensation products with thiophene aldehyde (teniposide, VM-26, Vumon) and acetaldehyde (etoposide, VP-16, VePesid) were selected for clinical testing based on their high cytostatic potency in mouse leukemia L1210 and a number of animal tumor models (Stähelin 1969; 1970).

Clinical trials of teniposide and etoposide started in 1967 and 1971, respectively, and promising therapeutic activities in different types of cancer have been found. In 1978, teniposide and etoposide were licensed out to the United States company Bristol-Myers, which carried on the development of the two epipodophyllotoxins and introduced etoposide to the U.S. market in 1983.

The mechanisms of action of epipodophyllotoxins was understood only at the cellular level until Loike et al. reported fragmentation of DNA in Hela cells by teniposide and etoposide in 1974. Another breakthrough was made several years later by Long and Minocha (1983) who correlated the DNA fragmentation with the inhibition of the nuclear enzyme topoisomerase II and proposed that inhibition of topoisomerase II by teniposide and etoposide is responsible for in vivo DNA breakage and cytotoxicity. These important findings opened a new chapter of epipodophyllotoxin history.

## CURRENT ROLE IN CANCER CHEMOTHERAPY

Since the first introduction of teniposide in 1967 and

etoposide in 1971, these drugs have been increasingly used in cancer chemotherapy. Teniposide and etoposide share the same mode of action --- they are phase-specific cytotoxic drugs acting in the late S and early $G_2$ phase and inducing a premitotic block in the cell cycle (Krishan et al., 1975; Kalwinsky et al., 1983). This mode of action is quite different from that of their parent compound podophyllotoxin which is a spindle poison causing inhibition of mitosis by blocking microtubular assembly (Kelleher, 1977; Loike and Horwitz, 1976a). Teniposide and etoposide also have very similar experimental and clinical features (Rozencweig et al., 1977), except that teniposide is about seven to ten times more potent than etoposide as regards production of DNA damage and cytotoxicity (Ross et al., 1984), and etoposide appears to have a greater therapeutic index (Clark and Slevin, 1987).

Etoposide and teniposide had been extensively studied in clinical trials sponsored by the National Cancer Institute and demonstrated to be active antineoplastic agents. They not only have a wide range of anticancer activity, but also are relatively well tolerated (O'Dwyer et al., 1984; 1985). They are used in treatment of several malignancies either alone or in combination with other cancer chemotherapeutic agents. The activity of etoposide against different types of cancers has been more extensively explored and it is frequently used as a first-line drug in the treatment of small cell lung cancer, germ cell tumors, lymphomas, acute non-lymphocytic leukemia and more recently Kaposi's sarcoma associated with acquired

immune deficiency syndrome (Arnold, 1979; Issell and Crooke, 1979; O'Dwyer et al., 1985; Sinkule, 1984;). Teniposide has tended to be used in pediatric oncology, especially for neuroblastoma (Hayes et al., 1985; O'Dwyer et al., 1984), but is also active in acute lymphocytic leukemia, lymphomas, brain tumors and small cell lung cancer (O'Dwyer et al., 1984).

Neither teniposide nor etoposide has appreciable activity in breast, colorectal, or head and neck carcinomas and melanomas (Sinkule, 1984; Radice et al., 1979).

## CHEMISTRY

The registered name of the formulation of teniposide (VM-26, PTG, and NSC122819) in clinical use is Vumon (Bristol Myers), and its chemical name is 4'-demethyl-epipodophyllotoxin-9(4,6-O-thenylidene-β-D-glucopyranoside). The empirical formula of teniposide is $C_{32}H_{32}O_{13}S$, and the molecular weight is 656.7.

Teniposide and etoposide were synthesized starting from podophyllotoxin (Fig. 1) which is a component of the ethanolic extracts (podophyllin) of dried roots and rhizomes of species of the genus *Podophyllum*. The commonly used sources of podophyllin are the *Podophyllum peltatum L.* (May apple or American Mandrake) and the Indian species *Podophyllum emodi* Wallich (Holthuis, 1988).

Both teniposide and etoposide have a multiringed structure of the parent podophyllotoxin linked to a sugar

moiety, a glucopyranoside (Figure 1). They differ from podophyllotoxin at 3 positions: they have a glucoside moiety at the C-4 atom, their C-4 position is in the enantiomeric configuration of podophyllotoxin, and they have a hydroxy group at the C-4' position. Etoposide and teniposide only differ in substitution on the glucoside moiety of a methyl group (etoposide) for a thenylidene group (teniposide).

Studies on the structure-activity relationship suggested that the hydroxy group at the C-4' position was essential for the DNA breakage activity; epimerization of the C-4 position of the podophyllotoxin rings enhanced activity, whereas glucosylation of the hydroxy group at the 4-position diminished activity (Loike and Horwitz, 1976b; Long et al., 1984). Alteration or opening of the lactone moiety resulted in a partial or complete inactivation of etoposide, respectively (Jardine et al., 1982); The presence of the glucoside group greatly increased the ability of etoposide and teniposide to produce DNA breaks (Ayres and Lim, 1982; Loike and Horwitz, 1976b).

Teniposide is almost insoluble in water, and is soluble in organic solvents, such as alcohols, halogenated hydrocarbons, tetrahydrofurane and dimethyl sulfoxide (Holthuis, 1988). Because of the limited solubility and stability of etoposide and teniposide in aqueous solutions, a complex formulation is required for intravenous use of these drugs. Teniposide is supplied in 5 ml vials containing 50 mg of drug, 0.15 g of benzyl alcohol; 0.3 g of N,N-dinethyl

acetamidine; 2.5 g of polyethoxylated castor oil; maleic acid to Ph 5.1, and absolute alcohol to 5 ml. Etoposide is supplied in vials containing 100 mg of drug, 400 mg of Tween 80, 3.25 g of polyethylene glycol 300, 150 mg of benzyl alcohol, 10 mg of anhydrous citric acid and absolute alcohol to 5 ml (Rozencweig et al., 1977). The intact ampoules of teniposide and etoposide in such formula were found to be chemically stable for 4 years. The drugs are much less stable in physiological solutions such as 0.9% NaCl or 5% dextrose (Clark and Slevin, 1987). It was also found that the stability of these drugs varies with drug concentration and pH of the solution, but not with temperature differences or whether they are stored in dark or light environment. Lowered stability was observed with increased drug concentration (Clark and Slevin, 1987). Etoposide is most stable at pH 5 ($t^1/_2$=609h); at pH values less than 5 the sugar moiety is cleaved, whereas in basic solutions, etoposide epimerises yielding the cis-lactone which degrades further to give the cis-hydroxy acid derivative (Underberg et al., 1985).

## PHARMACOKINETICS

The pharmacokinetic properties of teniposide were first studied by Allen and Creaven in mid 1970s using tritium labeled teniposide. After a single infusion, teniposide showed a triexponential decay curve (Allen and Creaven, 1975; Creaven and Allen, 1975). When reverse-phase high performance liquid

chromatography was developed to improve specificity of the analysis, most studies showed a biexponential decay following intravenous infusion of teniposide (D'Incalci et al., 1985; Evans et al., 1982; Stewart et al., 1984). The elimination half-life of teniposide was 6 to 10 hours in those studies showing a biexponential decay and between 20 and greater than 48 hours in those exhibiting triexponential decay. The volume of distribution at steady state was 8 to 30 $L/m^2$. Total plasma clearance was 7 to 17 $ml/min/m^2$ and renal clearance was 1 to 3 $ml/min/m^2$ (Clark and Slevin, 1987). Biliary excretion accounted for 0 to 10% of the dose (Allen and Creaven, 1975; Creaven and Allen, 1975). Steady-state plasma concentrations of teniposide during a 72 hour infusion were linearly related to dose (Rodman et al., 1985). Several studies showed that only 5 to 20% of the administered dose was eliminated as unchanged drug, and the major metabolic products appeared to be the hydroxy acid, the picro-lactone isomer, and the aglycone glucuronide (Evans et al., 1982; Holthuis et al., 1986; Rossi et al., 1984).

The pharmacokinetics of etoposide was reported to follow biexponential decay after intravenous administration (Clark and Slevin, 1987). The terminal elimination half-life of etoposide was less than that of teniposide, and the plasma and renal clearances of etoposide were greater.

Uptake of etoposide and teniposide into mouse leukemia L1210 cells exhibited a membrane-controlled passive diffusion, and 20% of the drug was irreversibly retained in the cell

(Allen 1978a; 1978b; Allen and Shirley, 1977). The uptake and binding of teniposide was 7 to 10 times that of etoposide (Allen, 1978b), which may partly explain the difference in efficacy of equitoxic doses of the two drugs (Allen, 1978a; Stahelin, 1970). Teniposide was more highly protein bound than etoposide. More than 94% of etoposide and over 99% of teniposide was protein-bound in vitro over a wide range of drug concentrations (Allen et al., 1982). Such magnitude of protein binding is likely to influence their pharmacokinetics in vivo (Bailey-Wood et al., 1985; Gilette, 1973).

## TOXICITY

Short-term toxic effects of teniposide were described in the phase I and phase II clinical trials. At every schedule tested, the most pronounced toxicity has been myelosuppression. The severity and duration of myelosuppression increases with drug dose in a gradual fashion. For example, at 67 $mg/m^2$ weekly, the majority of patients did not manifest white blood cells counts < $2000/mm^3$ (Muggia et al., 1971), while at 165 $mg/m^2$ twice weekly in patients with leukemia, aplasia of the marrow for approximately two weeks was seen (Rivera et al., 1982). The period of aplasia was prolonged by increasing the dose further to 200 $mg/m^2$ (Rivera et al., 1982). However, the myelosuppression induced by teniposide appeared to be no more severe than that induced by most other antileukemic drugs

(Rivera et al., 1975).

Other toxic effects have been considerably less frequent. The incidence of type I hypersensitivity varies from 2% to 11% of patients, and no such reaction induced by this drug has been reported to be fatal (Weiss, 1982). Hypersensitivity reactions can occur within minutes of starting the infusion, with rapid onset of symptoms such as flushing, urticaria, wheezing, coughing, and vomiting (Hayes et al., 1985). Acute rises in blood pressure and, occasionally, hypotension were also manifested (Hayes et al., 1985; Muggia et al., 1971). All these reactions, except for hypotension, were usually self-limited and could be resolved with interruption of the infusion or by intravenous Benadryl and Solu-Cortef (Hayes et al., 1985; O'Dwyer et al., 1984).

The acute reactions usually occurred after repeated administration, but sometimes appeared on the first dose (Hayes et al., 1985), suggesting that this drug reaction was probably not mediated by antibody (Weiss, 1982), although one patient was described with an $IgG_1$ antibody to the drug (Habibi et al., 1982). It has been suggested that the solvent used for preparation of teniposide may be responsible for the allergic reactions, since other drugs which use dimethylacetamide or Cremophor EL as solvent have also been reported to induce allergic reactions (Budman et al., 1982; Dye and Watkins, 1980; Huttel et al., 1980). Etoposide dose not require this solvent for preparation and no acute anaphylactic reactions have been ascribed to this drug.

With regard to long-term complications, epipodophyllotoxins have been reported recently to be tumorigenic. This effect will be discussed in "therapy-related malignancy".

## MECHANISMS OF ACTION

Teniposide is one of the anticancer drugs which produce cytotoxic effects mainly by affecting DNA topoisomerases. Topoisomerases are essential nuclear enzymes that function to resolve DNA topological problems which arise during some critical cellular processes such as replication and transcription. Two major topoisomerases, types I and II, have been identified over the past two decades in both prokaryotic and eukaryotic cells (Wang, 1985; Gellert, 1981). These enzymes function by introducing transient protein-bridged DNA breaks to allow the passage of other DNA strand(s). Topoisomerase I breaks one strand, and topoisomerase II breaks both strands of duplex DNA. With the strand passing activity, both enzymes can relax supercoiled DNA, and topoisomerase II can also catenate/decatenate DNA circles.

Eukaryotic DNA topoisomerase II is a 170 kd homodimeric protein which is present in the nuclear matrix (Earnshaw et al., 1985). This enzyme appears to be the cellular target of a number of clinically important anticancer drugs from diverse chemical classes including the intercalating agents (acridines, anthracyclines, ellipticines, actinomycins) and

epipodophyllotoxins. These drugs interfere with the topoisomerase II by trapping a key covalent intermediate of the breakage-rejoining reaction, termed the "cleavable complex". Treatment of the cleavable complex with a strong protein denaturant such as SDS or alkali results in DNA single-strand and double-strand breaks, where the 5' end of each broken DNA strand is covalently linked to a topoisomerase subunit via a tyrosyl phosphate bond. In the case of a double-strand break, the 3'-hydroxyl end is recessed by four bases (four-base staggered double-strand break) (Tewey et al., 1984a). However, in the absence of protein denaturants, the cleavable complexes can be converted to noncleavable complexes upon removal of the drug from the reaction (Long et al., 1986). It has been widely accepted that accumulation of the drug-stabilized DNA-topoisomerase II cleavable complexes is lethal to proliferating cells and is responsible for the cytotoxic activity of these drugs.

The biological functions of eukaryotic DNA topoisomerase II are still not well characterized. Studies in the SV40 cell-free replication system have shown that topoisomerase II is essential for segregating completely replicated daughter molecules (Yang et al., 1987). Its strand-passing activity is also important for replication fork movement. The fact that the enzyme activity is dramatically increased when quiescent cells are stimulated for growth (Bodley et al., 1987) also indicates an important role for topoisomerase II in cell replication. Unlike DNA topoisomerase I, topoisomerase II is

uniformly distributed along the chromosomes, and is not enriched in actively transcribed regions (Earnshaw et al., 1985). On the other hand, strong topoisomerase II cleavage sites have been mapped to both 3' and 5' ends of the Drosophila heat-shock genes hsp70 (Rowe et al., 1986) and the SV40 enhancer region in SV40-infected monkey cells (Yong et al., 1985) which indicates that topoisomerase II may also be involved in the process of transcription. Other biological functions of eukaryotic topoisomerase II such as promotion of illegitimate recombination in vitro (Bae et al., 1988) and organization of chromosome structure (Earnshaw et al., 1985) have also been recognized.

Like many other anticancer drugs, teniposide and etoposide were entered into clinical trials based on their apparent tumorostatic effects. The mechanism of action at the molecular level began to be uncovered a few years later when Loike et al. (1974) found that treatment of cells with teniposide and etoposide resulted in DNA fragmentation. The lack of an effect of these drugs on purified DNA led to the hypothesis that a nuclear enzyme was responsible for the drug-induced cellular DNA degradation (Loike and Horwitz, 1976). Subsequent studies showed that topoisomerase II was the putative cellular target of teniposide and etoposide, and trapping of cleavable complexes was the primary mechanism of action of these drugs (Long and Minocha, 1983; Ross et al., 1984). The potency of different congeners of epipodophyllotoxins in stabilizing cleavable complexes was

found to correlate well with their cytotoxicity (Long, 1984). However, the mechanism by which the drug-stabilized cleavable complexes trigger cell death is still not well defined.

Several possibilities have been considered, which may not be mutually exclusive. First of all, collision of drug-stabilized cleavable complexes with replication forks may be the initial event resulting in DNA single- and double-strand breaks. It was found that double-strand-break-repair deficient mutants were hypersensitive to the cytotoxic effects of these drugs (D'Arpa and Liu, 1989). $G_2$ arrest has been demonstrated to occur following drug treatment (Drewinko and Bariogle, 1976). Analysis of chromosome damage in $G_2$-arrested HeLa cells indicated a direct correlation between the amount of heavily damaged chromatin and the presence of cells arrested in $G_2$ (Rao, 1980). $G_2$ arrest apparently provides time for repair of DNA damage prior to mitosis. However, when the level of DNA damage exceeds the repair capacity of the cell, certain cellular mechanisms, analogous to the SOS response in *E. coli*, may be triggered which lead to cell death. Moreover, some cell death may be attributed to the depletion of functional enzyme due to the trapping of the topoisomerase II in cleavable complexes by the drug. Topoisomerase II appears to be absolutely required in some cellular processes such as chromosome condensation and separation during mitosis (Uemura et al., 1987). Absence of topoisomerase II activity during these critical periods of the cell cycle might lead to cell death. As evidence for this hypothesis, exposure of a $G_2$

population of CHO cells to teniposide results in the production of a small number of cells having incompletely condensed or fused mitotic chromosomes in the form of micronuclei (D'Arpa and Liu, 1989).

Finally, the cellular level of topoisomerase II is one of the important factors in determining drug cytotoxicity. Both tumor cells and proliferating normal cells have higher levels of topoisomerase II. By using a number of normal and neoplastic cells, Hsiang et al. (1988) demonstrated that the level of DNA topoisomerase II was regulated by both serum growth factors and the cell density in normal cells. At lower serum concentrations and higher cell densities , the cellular level of topoisomerase II was reduced in these cells. Serum replenishment rapidly restored the high cellular level of topoisomerase II. However, these culture conditions had much less effect on topoisomerase II levels in tumor cells. Furthermore, while the cellular level of topoisomerase II was controlled in normal cells during the $G_0/G_1$ phase, the level of topoisomerase II in tumor cells remained relatively constant throughout the cell cycle. The high level of topoisomerase II in proliferating tumor cells and its altered regulation may partly explain the specificity of topoisomerase II inhibitors toward neoplastic tissues.

## THERAPY-RELATED MALIGNANCY

Therapy-related acute myeloid leukemia (t-AML) is a late

complication of extensive cytotoxic chemotherapy and/or radiotherapy for a wide range of primary disorders. It represents 10% to 15% of all AML, and is usually unclassifiable by the French-American-British (FAB) system. The characteristics that distinguish t-AML from de novo AML include a high incidence of antecedent myelodysplastic syndrome with clinical manifestations of pancytopenia, frequent cytogenetic abnormalities involving chromosomes 5 and 7, and poor prognosis due to low responsiveness to antileukemic therapy (Fourth International Workshop, 1984; Kantarjian and Keating, 1987). The median time from initial treatment to bone marrow dysfunction is 4-6 years. The development of this unique disorder has been attributed to the use of alkylating agents (e.g. L-phenylalanine mustard, chlorambucil or melphalan) and/or radiotherapy in the treatment of the primary malignancies (Albain et al., 1990; Le Beau et al., 1986; Lerner, 1978).

In recent years, several cases of t-AML have been reported in which neither the morphology nor the karyotype was typical for the commonly seen t-AML (Ratain et al., 1987; Pui et al., 1989). Patients with this new type of t-AML had several characteristics in common: epipodophyllotoxins, anthracyclines or actinomycin D were included in the chemotherapy in combination with either cis-platin or cyclophosphamide; this type of t-AML can be classified by FAB as types M4 (acute myelomonocytic leukemia) or M5 (acute monocytic leukemia) and have cytogenetic changes involving

chromosome 11q [t(9;11)(p22;q23) is frequently seen]; there was no preleukemic phase, and the interval between the treatment of the primary malignancy and the development of t-AML was short (about 2-3 years); patients with this type of t-AML responded well to antileukemic therapy (DeVore et al., 1989).

The mechanisms and molecular events underlying therapy-related leukemogenesis have not been studied in great depth. The drugs identified to cause this new type of t-AML are cytotoxic agents acting mainly through inhibition of topoisomerase II (Long and Stringfellow, 1988). Since these drugs are known potent clastogens and mutagens (Singh and Gupta, 1983; DeMarini et al., 1987), it is reasonable to speculate that the clastogenic effect of topoisomerase II inhibitors may confer a leukemia by inducing chromosomal aberrations, particularly at 11q23.

A recent in vitro cytogenetic study on the clastogenic effect of etoposide suggested that chromosome aberrations induced by etoposide are not random: chromosomes 1, 11 and 17 were most frequently involved, while chromosomes 4, 5 and X were seldom affected (Maraschin et al., 1990). It was also noticed that R-band-rich chromosomes were significantly more affected than G-band-rich chromosomes. This non-random distribution may be related to the fact that R bands are rich in genes that are actively transcribed and hence are more susceptible to topoisomerase II mediated damages. It was suggested that, since most aberrations detected were either

translocations or dicentrics, illegitimate recombinations between non-homologous chromosomes due to incorrect repair might be the molecular event resulting in leukemogenesis (Maraschin et al., 1990).

To further define the genetic loci and the critical regions on the chromosomes that are preferentially involved in leukemogenesis will be helpful in understanding the role certain genes may play in the processes of transformation. Detailed information on topoisomerase II inhibitor-induced DNA damage and repair mechanisms will also be important for elucidating the mutagenic properties of these drugs at the molecular level. Such knowledge will be of great value in the effort to separate the cytotoxic and mutagenic properties of these drugs in order to achieve safer chemotherapy in the future.

## MUTAGENESIS

Teniposide is one of the many cytotoxic anticancer drugs developed in recent years that produce their effects by interacting with cellular DNA and causing a variety of genetic alterations. Many of these drugs have been shown to be mutagenic and carcinogenic in a number of different experimental systems (Singh and Gupta, 1983). There have been more and more reports claiming the emergence of secondary malignancies among long-term cancer survivors who had been treated with these drugs (Kantarjian and Keating, 1987; Albain

et al., 1990), which raised the suspicion that some of these drugs may be carcinogenic. In view of their widespread clinical use, the mutagenic potential of these drugs has come to be of great concern and has produced considerable research interest.

To characterize their mutagenic properties, Singh and Gupta (1983) examined the mutagenic effects of thirteen anticancer drugs (namely, busulfan, chlorambucil, adriamycin, bleomycin, daunomycin, ellipticine, teniposide, etoposide, cis-platin, dacarbazine, lomustine, actinomycin D, and mitomycin C) at five independent genetic loci in Chinese hamster ovary (CHO) cells. The genetic loci are those conferring resistance to the nucleoside analogues 6-thioguanine ($Thg^r$) and 5,6-dichlororibofuranosylbenzimidazole ($Drb^R$), the cardiac glycoside ouabain ($Oua^R$), the protein synthesis inhibitor emetine ($Emt^r$) and the polyamine synthesis inhibitor methylglyoxal-bis(guanylhydrazone) ($Mbg^r$). Among these genetic loci, only the hypoxanthine-guanine phosphoribosyltransferase (*hgprt*) locus may be used to detect a wide range of genetic lesions, including those which may cause inactivation of the gene, since the product of this gene is a purine salvage pathway enzyme and its function is not essential for cell growth under normal conditions. In contrast to the *hgprt* locus, the other genetic loci appear to be essential for cell survival and growth (e.g., $Na^+$-$K^+$-ATPase in $Oua^R$ or 40S ribosomes in $Emt^r$ mutants). These loci, therefore, are expected to respond only to those agents which cause minor

DNA damages (e.g. base substitutions) that lead to resistance without inactivating the functions of these genes. The genotoxic activity of various anticancer drugs was also investigated in an independent assay system based on induction of sister chromatid exchange (SCE) (Gupta and Singh, 1983).

Results of these studies are summarized in Table 1. All thirteen anticancer drugs showed positive mutagenic responses in both assay systems. In comparison, teniposide was relatively strongly mutagenic at the *hgprt* locus, which suggested that this drug is producing predominantly the types of genetic lesions (e.g. deletions and frameshifts) that may cause inactivation of the affected function. Consistent with this result is the fact that teniposide was also a very potent inducer of SCE, which makes teniposide a possible potent clastogen.

The *hgprt* locus is X-linked -- most cell lines have only one functional *hgprt* gene. The functional hemizygosity of this locus makes it readily available for mutagenesis. Like the *hgprt* locus, the thymidine kinase (*tk*) locus is also one of the most frequently used target genes in mammalian cell mutagenesis assays. The *tk* locus is autosomal, and normal cell lines have two functional *tk* genes. However, several investigators have isolated *tk* heterozygous lines in rodent and human cells. The L5178Y/$TK^{+/-}$-3.7.2C mouse lymphoma assay measures mutations at the functionally heterozygous *tk* locus based on resistance to trifluorothymidine. Studies indicate that this assay may detect both intragenic mutations as well

Table 1. Summary of the Mutagenic Effects of Various Anticancer Drugs in Mutagenesis and SCE Assays

| Anticancer Drugs | Relative Mutagenic Responses of Different Genetic Markers[a] | | | | | SCE[b] assay |
|---|---|---|---|---|---|---|
| | $Emt^r$ | $Drb^R$ | $Oua^R$ | $Mbg^r$ | $Thg^r$ | |
| Chlorambucil | + | - | ++ | + | +++ | +++ |
| Busulfan | + | - | + | - | ++ | ++ |
| Lomustine | + | - | - | - | + | + |
| Dacarbazine | - | - | - | - | + | + |
| Adriamycin | - | - | ++ | - | + | + |
| Daunomycin | - | ++ | + | - | + | + |
| Bleomycin | - | - | + | + | + | + |
| Ellipticine | - | - | - | ++ | +++ | ++ |
| Teniposide | ++ | + | - | - | ++++ | ++++ |
| etoposide | + | + | - | - | +++ | ++++ |
| cis-Platin | - | - | - | - | +++ | ++++ |
| Actinomycin D | ++ | - | - | - | + | + |
| Mitomycin C | ++ | ++ | - | ++ | +++ | ++++ |

[a] Based on the mutagenesis significance ration of the observed mutagenic responses, the mutagenic effects of the chemicals are arbitrary classified from - (negative) to ++++ (very strong).

[b] Based on the fold increase in SCE as compared to the untreated cells, the potencies of the chemicals of SCE induction are arbitrarily classified from + (weak) to ++++ (very strong).

as chromosomal mutations (intergenic events resulting from chromosomal rearrangements and/or functional multilocus deletions) affecting expression of the *tk* locus. These two classes of genetic damage were found to produce colonies with different sizes that can be distinguished by an automatic colony counter (Moore et al., 1985b). Small-colony TK mutants appear to result from large scale alterations to chromosome 11, which carries the *tk* locus, while large-colony mutants appear to represent single-gene mutations (Hozier et al., 1985).

The mutagenic and clastogenic activities of teniposide were evaluated in L5178Y/TK$^{+/-}$-3.7.2C mouse lymphoma cells. At a dose which resulted in approximately 20% survival, teniposide induced as many as about 2,000 *tk* mutants/$10^6$ survivors, and the majority of the mutants formed small colonies (DeMarini et al., 1987). Compared with the above figure, teniposide induced much fewer mutants at the *hgprt* locus in CHO cells (Singh and Gupta, 1983). A reasonable explanation is available based on previous observations that both the *tk* and *hgprt* loci were equally mutable by gene mutagens and weak clastogens such as EMS and ICR170, whereas, agents acting primarily by clastogenic mechanisms, such as $\gamma$-rays, and m-AMSA were extremely potent mutagens at the *tk* locus of L5178Y cells but were much less mutagenic at the *hgprt* locus of CHO or V79 cells (DeMarini et al., 1987). The heterozygosity of the *tk* locus as opposed to the hemizygous nature of the *hgprt* locus appeared to be the major reason for

the differences in the mutagenic response of the two loci. Multilocus events induced by potent clastogens might be recoverable at a functionally heterozygous locus such as *tk*, where the loss of essential flanking gene function might be compensated for by functional genes on the homologous chromosome, while at a functionally hemizygous locus like *hgprt*, there is no homologous genetic material to compensate for the loss of essential flanking gene functions, so that these mutants could not be recovered. In cytogenetic analysis, teniposide was demonstrated to be a strong inducer of chromosome aberrations. It induced 244 aberrations/100 cells at 5 μg/ml (DeMarini et al., 1987). These results led to the conclusion that teniposide is probably a potent clastogen in mammalian mutagenic test systems.

The cytotoxic and mutagenic effects of teniposide and its congener etoposide in bacterial test systems has also been evaluated. In contrast to mammalian cells, treatment of either *S. typhimurium* or *E. coli* with a wide range concentrations of these drugs produced no appreciable increase in the mutation frequencies in several systems measuring either reverse or forward mutations (Gupta et al., 1987). Among these systems, two prokaryotic forward mutation assays in which the excision repair proficient *E. coli* K12 343/113 strain was used, should, in principle, detect all types of genetic lesions that cause inactivation of $gal^R$ or $trp^R$ gene products. However, treatment with up to 500 μg/ml of etoposide caused no increase in mutation frequency in these forward mutation detection systems

[i.e. ability to grow in presence of galactose ($gal^+$) or resistance to 5-methyltryptophan]. These results, in addition to the fact that neither etoposide nor teniposide showed significant cellular toxicity in the prokaryotic systems suggested that epipodophyllotoxins do not produce genotoxic lesions in bacterial cells (Gupta et al., 1987).

It has been widely accepted that the cytotoxic effects of teniposide and etoposide are brought about by interaction with mammalian DNA topoisomerase II, which breaks and rejoins double-stranded DNA in concert. The lack of toxicity of these drugs in prokaryotic as opposed to mammalian systems suggested that the enzymes from prokaryotic and mammalian cells might differ in their interaction with these drugs. The purified enzyme from mammalian sources is a homodimer of a polypeptide chain with molecular weight of 170 kd (Gellert, 1981; Wang, 1985). The corresponding type II topoisomerase in bacterial systems is DNA gyrase which is composed of two subunits with molecular weight of 90 kd and 100 kd, both of which are required for activity. The bacterial enzyme also differs from the mammalian enzyme in that while the catalytic activity of mammalian topoisomerase II is dependent upon ATP, the bacterial DNA gyrase can cause relaxation of superhelical DNA in the absence of ATP (Gellert, 1981; Wang, 1985). On the other hand, the behavior of the bacteriophage T4 topoisomerase II, a multisubunit protein, is more similar to mammalian type II topoisomerase and much less similar to *E. coli* DNA gyrase (Vosberg, 1985). Drugs that promote mammalian topoisomerase

II-mediated DNA cleavage also promote T4 topoisomerase II-mediated DNA cleavage (Ripley et al., 1988). Therefore, the differences between topoisomerase II from different biological systems in their interaction with teniposide and etoposide may account for the observed results in these systems.

A series of other drugs are also known to produce cytotoxic effects by disturbing DNA topoisomerase II. These drugs belong to different chemical classes, e.g., acridines, anthracyclines and ellipticines, represented by m-AMSA, 5-iminodaunorubicine, and 2-Me-9-OH-$E^+$, respectively. Unlike epipodophyllotoxins, these drugs interact directly with DNA molecules by intercalation. What they have in common with epipodophyllotoxins is that they are all able to stabilize the DNA-topoisomerase II complex. Good correlations have been found between the ability of a drug to cause cleavable complex accumulation and production of cytotoxicity (Long et al., 1986). For example, intercalators such as o-AMSA and ellipticine which are less cytotoxic than their congeners m-AMSA and 2-Me-9-OH-$E^+$ are also much less efficient in stimulating cleavable complex formation and producing DNA strand breaks in vitro (Nelson et al., 1984; Tewey et al., 1984). Whether the intercalative activity plays a role in their cytotoxic effect is still in dispute. DNA cleavage activity of intercalators and epipodophyllotoxins were observed both in cultured mammalian cells and in vitro system with purified mammalian or T4 topoisomerase II, which provided evidence for the involvement of topoisomerase II in the

cytotoxic effects of these drugs. On the other hand, DNA sequencing analysis revealed that topoisomerase II cleavage sites produced by drugs from different chemical classes are distinctly different (Tewey et al., 1984b) which suggested that although topoisomerase II may by the common target, the enzyme may interact differently with different drugs.

The search for the sequence specificities of DNA topoisomerase II cleavage sites both in the absence and presence of the drugs has been mostly limited to *in vitro* studies (Spitzner and Muller, 1988; Capranico et al., 1990) and the validity of the consensus sequences in predicting the cleavage sites in living cells has been questionable. Rather substantial differences have been found between the topoisomerase II cleavage sites *in vivo* and in naked DNA (Udvardy and Schedl, 1991). In one of the attempts to characterize the formation of mutations induced by topoisomerase II inhibitors, m-AMSA was used to induce mutations in bacteriophage $T_4$ and to promote $T_4$ topoisomerase-mediated DNA cleavage *in vitro* (Ripley et al., 1988). A co-specificity of the sites of the DNA damages in the two systems was found. The specific phosphodiester bonds cleaved *in vitro* are precisely those at which frameshifts are most strongly promoted by m-AMSA *in vivo*. This co-specificity enabled the author to postulate the molecular events (incorrect repair) leading from initial topoisomerase II-mediated DNA damage (a double strand break) to the resulting frameshift mutations (Ripley et al., 1988). This interesting result led us to the

present studies designed to determine whether such co-specificity is also present in mammalian systems.

## CHO/*aprt* SYSTEM

Genetic loci which encode nucleotide salvage pathway enzymes such as thymidine kinase (*tk*) and hypoxanthine guanine phosphoribosyltransferase (*hgprt*) are most extensively used as genetic targets in mutagenesis studies. These enzymes function to integrate free purine and pyrimidine bases into cellular metabolism and their activities are not essential for cell survival in normal growth medium. The nonessential nature of these enzymes made it possible to detect a wide range of genetic alterations by a single step forward selection for the mutants of these genes. The *aprt* gene, another such locus, encodes the salvage enzyme adenine phospho-ribosyltransferase (APRT) which catalyzes the synthesis of adenosine-5'-monophosphate (AMP) from adenine and 5-phosphoribosyl-1-pyrophosphate (PRPP). The *aprt* locus in Chinese hamster ovary (CHO) cells has been used mostly in studying sequence specificity of mutagenesis mainly because of the availability of hemizygous strains, and the small size (2.6 kb) of the structural gene which makes sequencing analysis much easier.

The CHO-D422 strain was isolated by Bradley and Letovanec (1982) through a direct selection method by plating wild type CHO cells in increasing concentrations of the purine analogue diaminopurine (DAP). Loss of APRT activity occurred in two

sequential steps. About half of total APRT activity was lost in the first step, and most or all of the remaining activity was lost in the second step. The D422 cell line retained 50 % of wild type CHO APRT activity was originally believed to be a heterozygote for *aprt* (Bradley and Letovanec, 1982). Subsequent studies, using Southern blot analysis, revealed that the CHO-D422 cell line carried a deletion of one of the *aprt* alleles, which rendered this cell line hemizygous at this locus (Nalbantoglu, 1983). This partial APRT deficient mutant cell line exhibited a stable phenotype and the rate of spontaneous mutation from partial to total APRT deficiency was on the order of $10^{-6}$ per cell per generation (Bradley and Letovanec, 1982). The mutation frequency could be enhanced by treatment with a mutagen and the *aprt*$^{-}$ mutants could be isolated by a simple one step selection for resistance to the drugs diaminopurine or 8-azaadenine (Jones and Sargent, 1974). In contrast, a number of heterozygous *aprt* lines obtained by sib-selection had spontaneous mutation frequencies two to three orders higher than that of the D422 strain. In addition, these sib-selected cell lines were not responsive to the mutagen EMS (Bradley and Letovanec, 1982). However, because of the hemizygous nature, a potential limitation of the CHO-D422/*aprt* system has been suggested to be the possible exclusion of mutational events that would inactivate essential genes within the region of hemizygosity (de Jong et al., 1988).

The hamster gene coding for APRT has been isolated by

using gene transfer and molecular cloning of transforming DNA (Lowy et al., 1980). The *aprt* gene is about 2.6 kb and is composed of 5 exons and 4 introns (Figure 2). The sequence of the entire gene has been determined by at least two independent laboratories (Nalbantoglu et al., 1986a; de Boer et al., 1989); the earlier sequence appears to contain several errors.

The spectrum of spontaneous mutations at the *aprt* locus has been documented. Although somewhat discrepant results are present, the most prominent characteristics in common have been the predominance of G:C to A:T transitions among base substitutions, which account for the majority of total mutations, the presence of short direct repeats at the termini of small deletions and the nonrandom distribution of the deletion mutations (Phear et al., 1989; Nalbantoglu et al., 1986b; de Jong et al., 1988). The latter characteristic has been attributed to the presence of certain DNA sequences such as dyad symmetry and short direct repeats in this region of the gene.

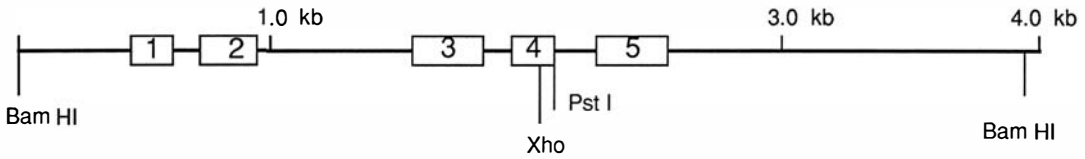



Figure 2. A schematic map of the *aprt* gene.

# MATERIALS AND METHODS

## TENIPOSIDE MUTAGENESIS

### Cell Culture:

The Chinese hamster ovary (CHO) cell subline D422 was a generous gift from Dr. B. W. Glickman. This cell line is hemizygous at the *aprt* gene and retains 50 % of wild type APRT enzyme activity. It was originally isolated by Bradley and Letovanec (1982). The *aprt* gene encodes the enzyme adenine phosphoribosyl transferase which catalyzes the synthesis of adenosine-5'-monophosphate (AMP) from adenine and 5-phosphoribosyl-1-pyrophosphate (PRPP). The cells were routinely maintained in α-Minimum Essential Medium (Gibco) supplemented with 5 % fetal bovine serum and 2.5 % horse serum (Gibco). Five mililiters of penicillin-streptomycin (Gibco) was added to each 500 ml medium to prevent contamination. A water-jacketed tissue culture incubator (Forma Scientific) kept the cells at 37°C, 5 % $CO_2$. The cells were routinely subcultured upon confluence. Following a brief wash with sterilized saline G (0.1 mM $CaCl_2 \cdot 2H_2O$, 5.4 mM KCl, 1 mM $KH_2PO_4$, 0.63 mM $MgSO_4 \cdot 7H_2O$, 137 mM NaCl, 1 mM $Na_2HPO_4 \cdot 7H_2O$, 11 % D-glucose), the cells were incubated with 3 ml of 0.25 % trypsin

(Gibco) in a 75 $mm^2$ tissue culture flask (Costar) for about 15 minutes to detach the cells from the wall of the flask. About 2 x $10^5$ cells were transferred into a new flask containing 20 ml of fresh medium.

Freeze storage of cell lines was done by spinning down a large number of cells at 500 rpm for 10 minutes and resuspending them in a small volume of fresh medium to make a final concentration of 3 - 5 million cells/ml. 0.9 ml of the cell suspension was combined with 0.1 ml sterilized dimethyl sulfoxide in a 1.5 ml sterilized vial and frozen down at -70 °C for a period of time before the cells were transferred into a liquid nitrogen tank.

Mutagenesis assay

Teniposide was a generous gift of Bristol Myers Inc. Teniposide powder was kept at -20 °C and was dissolved in dimethyl sulfoxide to 1 mM stock solution. Aliquots of the stock solution were kept at -70 °C for up to two weeks.

Each mutagenesis experiment started from five 1,000 cell inocula to minimize preexisting *aprt*$^-$ cells in the cultures. After 5 - 6 days of multiplication, 5 x $10^5$ cells were transferred from each inoculum to a 75 $mm^2$ flask containing 20 ml of fresh medium and allowed to settle and enter exponential growth for one day. Four of the five cultures were then mutagenized with different doses of teniposide (0.02 - 0.16 $\mu$g/ml) for 16 hours. The control culture was treated with the same volume of dimethyl sulfoxide.

Several experiments were done with calcium-depleted cells. Prior to the drug treatment, the cells were incubated in 5 mM EGTA for 4 hours to deplete calcium from the cultures. Various amounts of teniposide were added to the cultures and the drug treatment lasted only for one hour.

At the end of drug treatment, the medium was aspirated and replaced by fresh medium. The cells were allowed to grow for one day before they were trypsinized.

To determine cell survival, 2 x 700 cells were taken from each culture and plated in two 50 $mm^2$ tissue culture dishes (Costar) containing 15 ml medium. After 7 days of incubation, colonies were formed from viable cells. These colonies were fixed in 4 % formaldehyde (Fisher Scientific) for 5 minutes and stained by 1 % crystal violet (Sigma). The number of colonies with 100 or more cells was counted. Cell survival for each culture was calculated by dividing the average number of colonies from each treated culture by the average number of colonies from the control culture.

From each trypsinized culture, 2 x $10^6$ cells were transferred to a new flask with fresh medium. At the highest drug doses, there were often less than 2 x $10^6$ cells remaining attached to the flask following treatment, in which case all attached cells were transferred. The cells were allowed to grow for 6 days in order to express the *aprt*$^-$ phenotype. During the six day expression period, the cells were subcultured once at 2 x $10^6$ cells per flask.

To determine mutation frequency, a total of three million

cells from each culture were plated in six 56 $cm^2$ dishes in selective medium containing 0.4 mM 8-azaadenine (Sigma) and supplemented with 5 % dialyzed fetal bovine serum and 2.5 % dialyzed horse serum (Gibco). During the following 7 days of incubation, the mutated cells which were *aprt* deficient could survive in the selective medium and form colonies. In the meantime, 2 x 700 cells were also taken from each culture at the end of expression period and plated in nonselective medium with dialyzed sera. Colonies formed from these cells were divided by the number of cells plated (which was 700 cells) to give the plating efficiency of each culture. The total number of colonies formed in selective medium out of three million cells, corrected by plating efficiency, were used to determine mutation frequencies:

$$\frac{\text{Number of mutant colonies}}{3 \times 10^6 \times \text{Plating efficiency}}$$

From each drug treated culture in which the mutation frequency at least 8 fold greater than the background mutation frequency from the same experiment, two colonies were isolated. Colonies were also isolated from the control cultures. These cell lines were grown out in selective medium to a large number for DNA extraction.

## AMPLIFICATION OF THE *APRT* GENE

### Extraction of Genomic DNA

From each mutant strain, about $10^8$ cells were grown,

trypsinized and washed twice with cold phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM $KH_2PO_4$). The cells were then lysed in 1 ml digestion buffer [10 mM Tris-HCl, pH 7.5, 100 mM EDTA, 0.5 % sodium dodecyl sulfate (SDS), 25 μg/ml proteinase K (Sigma)] at 37°C overnight. 5 M NaCl was added into the lysate to make a final concentration of 0.1 M. An equal volume of phenol/chloroform (1/1) was mixed with the lysate and the mixture was centrifuged at 1,500 rpm for 10 minutes. The aqueous layer was transferred into a new tube and subjected to another phenol/chloroform extraction. The aqueous layer of the second extraction was then mixed with two volumes of 95 % ethanol, and the threaded DNA was spooled out, dried, and dissolved in one volume of Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

The extract was then digested with 50 μg/ml RNase (Sigma) for 4 - 5 hours at 37 °C. The digest was extracted twice with phenol/chloroform and precipitated as described above and dissolved in 200-500 μl TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The concentration of the DNA solution was determined with a Beckman spectrophotometer by measuring ultraviolet light absorbance at 260 nm. The final concentration was adjusted to 0.5 mg/ml.

## Preparation of PCR Primers

In order to analyze the mutations which caused inactivation of the *aprt* gene at the sequence level, the *aprt* gene was amplified by the polymerase chain reaction (PCR)

technique (Saiki, et al., 1985). Twenty-mer-oligonucleotides which are complimentary to the sequences flanking each exon of the *aprt* gene were synthesized and used as the primers in the polymerase chain reaction (Table 2). Synthesis of the oligonucleotides was done at the nucleic acid core facilities at the Medical College of Virginia by phosphoramidite method. Oligonuleotides were purified by ion-pairing HPLC on a 3.8 x 250 mm C-18 column (Rainin ultrosphere ODS). HPLC grade buffers were filtered and degassed prior to use. Buffer A consisted of 5 % acetonitrile, 0.1 M triethylammonium acetate, pH 7.0. Buffer B consisted of 50 % acetonitrile, 0.1 M triethylammonium acetate, pH 7.0. A linear gradient of 10-30 % buffer B was applied to the column over 60 minutes of elution time and the major ultraviolet-absorbing peak was collected. Purified oligonucleotides were dried and dissolved in distilled water. Concentrations of the oligonucleotide solutions were determined by measuring the UV absorbance on Beckman spectrophotometer and adjusted to 0.5 mg/ml.

Polymerase Chain Reaction:

Exons of the *aprt* gene that are located in close vicinity of each other were amplified together by the polymerase chain reaction. Exons 1 and 2 were amplified in one segment and exons 3, 4 and 5 were amplified in another segment. PCR was carried out in reaction volume of 100 $\mu$l containing 1 $\mu$g of genomic DNA, 0.25 $\mu$g of each primer flanking the exon to be amplified and 0.2 mM of each

Table 2. Oligonucleotide Primers Used in Polymerase Chain Reactions and Sequencing Reactions.

| Name | Sequence | Description | Position |
|---|---|---|---|
| 1U: | TGTTCCCGGACTGGTATGAC | upper strand exon 1 | -64--45 |
| 1L: | AACAAGGAGAGGCTGGTGGC | lower strand exon 1 | 203-184 |
| 2U: | GCCACCAGCCTCTCCTTGTT | upper strand exon 2 | 184-203 |
| 2L: | GGTTGAAGAAAGAAGGGATGG | lower strand exon 2 | 421-401 |
| 3U: | CTTACACCTCAGCCCTAACA | upper strand exon 3 | 1251-1270 |
| 3L: | GGAAGTAGAGTGAGAGTCTA | lower strand exon 3 | 1572-1553 |
| 4U: | TAGACTCTCACTCTACTTCC | upper strand exon 4 | 1553-1572 |
| 4L: | CTCTTGCTTAGACAGCACCC | lower strand exon 4 | 1731-1712 |
| 5U: | GGGTGCTGACTAAGCAAGAG | upper strand exon 5 | 1712-1731 |
| 5L: | CTGGTGGCTCACAAAGGTCA | lower strand exon 5 | 1998-1979 |

deoxynucleoside triphosphate (dNTP, Pharmacia). The reaction buffer consisted of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$ and 50 μg/ml bovine serum albumin (BSA, Sigma). The above mixture was heated to 95 °C for 5 minutes prior to addition of 2 units of Taq polymerase (Perkin Elmer-Cetus). The reaction mixture was overlaid by 50 μl light mineral oil (Sigma) to prevent evaporation.

Thirty cycles of PCR were run on a Perkin Elmer-Cetus thermal cycler. Each reaction cycle was composed of 1 minute denaturing time at 94 °C, 15 seconds (for exons 3, 4, 5) or 1.5 minutes (for exons 1, 2) annealing time at 55 °C and 2 minutes at 72 °C for polymerization. The final extension period was 7 minutes at 72 °C.

Single-stranded DNA was obtained by conducting an "asymmetric PCR" in which 10 μl of unpurified double-stranded PCR product was used as a template and 0.5 μg of one of the two primers was added. Twenty cycles of reaction were run with the same parameters used in regular PCR. Products of asymmetric PCR were separated from remaining nucleotides and primers by precipitation with 1 volume of 4 M ammonium acetate and 2 volumes of 2-propanol. 0.5 μg supercoiled plasmid DNA was added to facilitate pellet formation. Following 10 minutes of centrifugation in a microcentrifuge, the pellet was washed with 70 % ethanol and recentrifuged. The dried pellet was dissolved in 25 μl $dH_2O$, and used in direct sequencing reactions.

Agarose Gel Electrophoresis:

PCR products were detected by 1.5 % agarose gel electrophoresis. 1.5 g agarose gel powder was melted in 90 ml distilled water on a hot plate. 10 ml 10 x TBE buffer (10 x TBE: 890 mM boric acid, 890 mM Tris base, 20 mM EDTA) and 5 μl (10 mg/ml) ethidium bromide was added in the gel solution. The gel solution was solidified in a gel container. A 9 μl DNA sample was mixed with 1 μl loading solution (50 mM EDTA, 40% sucrose, 1 % bromophenol blue) and loaded on the gel. The DNA samples were electrophoresed in 1 x TBE buffer at 100 volts for 1 to 2 hours. A Polaroid film picture was taken under ultraviolet light to visualize the DNA bands.

DIRECT SEQUENCING OF PCR PRODUCTS

Sanger's Sequencing Reaction

Coding regions of the *aprt* gene were sequenced exon by exon using the dideoxy chain-termination method (Sanger) until the mutation was found. Single-stranded PCR products were used in the sequencing reaction. The annealing step was carried out by combining 7 μl (5 pmol) template DNA, 1 μl (5 pmol) of the oligonucleotide primer which anneals to the single-stranded template and 2 μl 5 x reaction buffer (5 x reaction buffer: 250 mM Tris-HCl, pH 8.8, 35 mM $MgCl_2$). The reaction was heated to 70 °C for 3 minutes and incubated at 42 °C for 10 minutes. The polymerization reaction was triggered by adding 2 units of Taq polymerase, 1 μl $\alpha$-$^{32}$P-dATP (800 Ci/mmole, 10 μCi/μl, New

England Nuclear) and 4.5 μl $dH_2O$. The above mixture was vortexed and used immediately for termination reactions in which 4 μl of the above reaction was transferred to each tube containing 4 μl of one of the termination mixes (each termination mix contained 20 μM of each dNTP and 60 μM ddGTP in G-mix, 800 μM ddATP in A-mix, 800 μM ddCTP in C-mix, and 400 μM ddTTP in T-mix). The tubes were incubated at 70 °C for 5 minutes before and after 1 μl "chase" (2 mM of each dNTP) was added into each tube. The reaction was stopped by addition of 4 μl formamide-dye stop solution (95 % deionized formamide, 2 mM EDTA, 0.5 % bromophenol blue, 0.5 % xylene cyanol FF).

Polyacrylamide-urea Gel Electrophoresis

Samples of DNA sequencing reactions were electrophoresed on a vertical polyacrylamide-urea denaturing gel which contained 7 % polyacrylamide/bisacrylamide (20:1) gel in 1 x gel buffer (130 mM Tris base, 45 mM boric acid, 2 mM EDTA). Immediately prior to pouring the gel, 0.6 ml freshly made 0.1 % ammonium persulfate and 40 μl temed were added into 90 ml gel solution. The samples were denatured at 95 °C for 5 minutes before being loaded on the sequencing gel. The gel (35 x 43 x 0.04 cm) was run at 80 watts for various lengths of time depending on the size of the DNA fragment.

SOUTHERN BLOT ANALYSIS

Preparation of the *aprt* Probe

The 3.8 kb Bam HI insert of the plasmid pHaprt (Lowy et al., 1980) contains the sequences coding for the enzyme APRT. The plasmid pHaprt was a gift of R. Axel and was amplified by other members of this laboratory. pHaprt plasmid (250 μg) was first digested with 50 units of the restriction endonucleases Xmn I (New England Biolabs) at 37 °C overnight and was then subjected to a second digestion with 125 units Bam HI (New England Biolabs) at 37 °C overnight. The effectiveness of digestion was monitored by agarose gel electrophoresis. DNA fragments were separated on agarose gel. The 3.8 kb band was isolated and the DNA was electroeluted in dialysis tubing (Fisher Scientific) at 50 volts overnight. TE buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA) was recirculated between two chambers of the electrophoresis apparatus during electroelution. The electric current was reversed for one minute at the end of the elution to detach DNA molecules from the wall of dialysis tubing. The DNA was concentrated and purified on a QIAGEN tip 20 according to the manufacturer's instructions. Following elution by the high salt solution, the DNA was then ethanol precipitated by additions of 2 volumes of 100 % ethanol followed by 1 hour of cooling at -70 °C. The DNA was then spun down in a microcentrifuge for 15 minutes. The pellet was washed with 200 μl 70 % ethanol, dried and dissolved in 60 μl $dH_2O$. The concentration of the DNA solution measured on the spectrophotometer was 0.37 μg/μl.

The 3.8 kb aprt fragment was labeled with a Bethesda Research Laboratories Life Technologies, Inc. (BRL) nick

translation system. In 50 μl reaction volume, 0.5 μg aprt fragment was combined with 5 μl solution $A_2$ (0.2 mM dATP, dGTP and dTTP, 500 mM Tris-HCl, pH 7.8, 50 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 100 μg/ml nuclease-free BSA), 90 μCi $\alpha$-$^{32}$P dCTP (10 mCi/ml, 3000 Ci/mmloe), 5 μl solution C (0.4 U/μl DNA Polymerase I, 40 pg/μl DNase I, 50 mM Tris-HCl, pH 7.5, 5 mM Mg-acetate, 1 mM 2-mercaptoethanol, 0.1 mM PMSF, 50 % glycerol, 100 μg/ml nuclease-free BSA). The reaction mixture was incubated at 15 °C for 1.5 hours and 5 μl solution D (0.3 M EDTA, pH 8.0) was added to stop the reaction. The $^{32}$P labeled probe was purified on QIAGEN tip 20 followed by ethanol precipitation and dissolved in 200 μl TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Radioactivity of the probe (~2 x $10^8$ cpm/μg) was measured on a Beckman liquid scintillation counter.

Digestion of Genomic DNA

For complete digestion, 10 μg genomic DNA was treated with 200 units restriction endonucleases Pst I or Bam HI (both from New England Biolabs) in the buffers provided by the manufacturer at 37 °C overnight. Ethanol precipitation was carried out by combining the samples with one tenth volume of 3 M sodium acetate and two volumes of 100 % ethanol. Following 1 hour of cooling at -70 °C, the DNA was spun down in microcentrifuge and the pellets were washed and dried. DNA was dissolved in 40 μl $dH_2O$ and 5 μl loading solution (40 % sucrose, 20 mM EDTA, 0.1 % bromophenoblue). The samples were

then loaded on a 360 ml 1 % agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) containing 0.5 μg/ml ethidium bromide. Also loaded on the agarose gel were 1 ng of the unlabeled 3.8 kb Bam HI fragment of pHaprt and a 1 kb size marker which is a mixture of DNA fragments of sizes between 750 base pairs and 12 kb. Electrophoresis was carried out in 1 x TAE buffer at 40 volts for 24 hours. A Polaroid picture of the gel was taken under ultraviolet light with a ruler placed on the side of the gel so that the distances to which the markers migrate could be determined on the picture and in turn, the sizes of the DNA fragments on the blot could be estimated.

Southern Transfer and Hybridization

The agarose gel containing fractionated DNA samples was soaked in 200 ml 0.25 N HCl for 10 minutes (acid depurination) followed by soaking in 200 ml denaturation solution (1.5 M NaCl, 0.5 M NaOH) twice for 15 minutes and in 200 ml neutralization solution (1.5 M NaCl, 1 M Tris-HCl, pH 7.4) twice for 15 minutes.

The buffer used for blotting was 10 X SSC (1 X SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The blotting stack was assembled as follows: one sheet of S&S GB003 filter paper saturated with blotting buffer was placed on a glass plate with both sides hanging down and dipping into blotting buffer in the container; three pieces of GB003 filter paper cut to a size slightly larger than the gel and saturated with blotting

buffer; the gel; Nytran membrane cut to the size of the gel and saturated with blotting buffer (air bubbles under the gel and between gel and Nytran membrane were removed); three pieces of GB003 filter paper saturated with blotting buffer; and a 1.5 inch stack of paper towels. On top of the stack, a light weight was added to keep all layers compressed. Transfer was continued at room temperature overnight. The Nytran membrane was then soaked in 100 ml 5 x SSC for 5 minutes to remove gel particles and baked at 75 °C for 1 hour to immobilize DNA fragments on the membrane.

The completely dried Nytran membrane was soaked in 20 ml prehybridization buffer [6 x SSC, 10 x Denhardt' reagent (0.2 % Ficoll, 0.2 % polyvinylpyrrolidone, 0.2 % BSA), 0.5 % sodium dodecyl sulfate (SDS), 100 μg low molecular weight DNA] at 42 °C for 2 hours.

Hybridization was carried out in a capped plastic container in 30 ml hybridization buffer (50 % deionized formamide, pH 7.4, 6 x SSC, 0.5 % SDS, 50 μg low molecular weight DNA, 8 % dextran sulfate 500 and $10^6$ cpm/ml probe) at 42 °C overnight.

The Nytran membrane was subjected to low and high-stringency washes: twice for 15 minutes each in 100 ml 6 x SSC, 0.2 % SDS at room temperature; twice for 15 minutes each in 100 ml 1 x SSC, 0.6 % SDS at 37 °C; and once in 100 ml 0.1 x SSC, 1 % SDS at 65 °C for 1 hour. The membrane was blotted dry and wrapped in plastic wrap prior to autoradiography which was at -70 °C with an intensifier screen.

## *IN VITRO* DNA CLEAVAGE REACTION

### Nuclear Enzyme Extraction

CHO-D422 cells were grown in α-Minimum Essential Medium supplemented with 10 % fetal bovine serum and 5 % horse serum. Cells were maintained in exponential growth in spinner flasks at 37 °C. A total of 5 x $10^8$ cells in 1 liter volume were sedimented and washed three times with cold buffer A (0.15 M NaCl, 10 mM $KH_2PO_4$, pH 7.5). Cells were suspended in 8.75 ml buffer G [5 mM $KH_2PO_4$, 2 mM $MgCl_2$, 4 mM dithiothreital (DTT), 0.1 mM $Na_2EDTA$, pH 7.0], and incubated on ice for about 10 minutes until 90 % of the cells were stained with trypan blue while remaining intact. Cells were checked every 5 minutes under a microscope. The cell suspension was immediately centrifuged at 400 x g for 5 minutes. The supernatant was aspirated, and the pellet was resuspended in 8 ml buffer H (5 mM $KH_2PO_4$, 2 mM $MgCl_2$, 4 mM DTT, 0.1 mM $Na_2EDTA$, 0.25 M sucrose, pH 7.0). The cell suspension was layered over 3 ml of buffer I (5 mM $KH_2PO_4$, 2 mM $MgCl_2$, 4 mM DTT, 0.1 mM $Na_2EDTA$, 0.3 M sucrose, pH 7.0), and centrifuged at 2,000 x g for 20 minutes. The cell pellet was resuspended in 0.5 ml buffer J (5 mM $KH_2PO_4$, 4 mM DTT, 1 mM $Na_2EDTA$), and incubated on ice for 15 minutes. 0.5 ml of buffer L (40 mM Tris-HCl, 0.7 M NaCl, 4 mM DTT, 60 % glycerol, pH 7.5) was added, and vortexed. The cell suspension was incubated on ice for 30 minutes and ultracentrifuged (Beckman Ti 70.1 rotor) at 100,000 x g for 1 hour at 4 °C in an Oak Ridge tube. The supernatant which

contains nuclear enzyme topoisomerase II was aliquoted, and stored at -70 °C. The following proteinase inhibitors were added to buffers G, H, I, J and L immediately before use: 1 mM benzamidine, 10 μg/ml soybean trypsin inhibitor, 50 μl/ml leupeptin, and 1 mM PMSF.

Test of Nuclear Enzyme Activity

Supercoiled DNA was used as substrate to test DNA cleavage activity of the nuclear extract. The cleavage reaction was carried out in 100 μl reaction buffer (10 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP) with 0.4 μg supercoiled DNA, 10 μM teniposide and 0.5 μl - 4 μl nuclear extract. The reaction was incubated at 37 °C for 30 minutes and terminated by addition of SDS and proteinase K (1 % and 0.1 mg/ml) and incubation at room temperature for 30 minutes. The reaction mixture was electrophoresed on an agarose gel to separate DNA in different forms (supercoiled, circular, linear) which was an indication of cleavage.

Preparation of DNA Substrates

To prepare end labeled PCR products of exons 2, 3, 4 and 5 of the *aprt* gene, PCR primers flanking these exons were 5'-$^{32}$P-end-labeled by the forward reaction. In 36 μl reaction volume, 1.5 μg each primer was mixed with 25 μl (83 pmol) γ-$^{32}$P-ATP (300 Ci/mmole, DuPont), 3.6 μl forward reaction buffer (0.5 M Tris-Cl, pH 7.5, 0.1 M $MgCl_2$, 50 mM DTT, 0.5

mg/ml BSA), 1 μl 100 mM spermidine, 20 units T4 polynucleotide kinase (Promega) and incubated at 37 °C for 1 hour. The reaction was terminated by adding 1 μl 0.5 M EDTA and heating to 70 °C for 10 minutes. Labeled oligonucleotide primers were ethanol-precipitated and dissolved in 30 μl of $dH_2O$.

A total of 8 labeled primers were used separately to generate 8 PCR products of exons 2, 3, 4 and 5. Four of them had $^{32}P$ label at 5'-ends of upper strands and the other 4 products had $^{32}P$ label at 5'-ends of lower strands. PCR was carried out in 100 μl PCR buffer described earlier with 0.2 mM each dNTP, 0.1 μg pHaprt plasmid (template), 15 μl labeled primer and 0.25 μg unlabeled primer of the opposite strand. The mixture was heated to 95 °C for 5 minutes before 2 units of Taq polymerase was added and overlaid with 50 μl mineral oil to prevent evaporation. Twenty cycles of reaction were carried out on Perkins Elmer Cetus thermal cycler, each at 94 °C for 1 minute, 50 °C for 1.5 minutes, 72 °C for 1 minute and final extension period at 72 °C for 7 minutes.

PCR products were purified by precipitation with 100 μl 4 M ammonium acetate and 200 μl 2-propanol. Following 10 minutes of incubation at room temperature, the DNA was centrifuged in a microcentrifuge for 10 minutes and the pellet was washed, dried and dissolved in 17 μl $H_2O$. To ensure that the substrates were full length molecules, the products were reacted with 1 μl Klenow fragment and 0.5 μl 2 mM dNTP mix at 30 °C for 15 minutes. The reaction was stopped by addition of 1 μl 0.5 M EDTA.

End labeled PCR products were purified on an 8 % polyacrylamide/bisacrylamide (30:1) nondenaturing gel (30 x 40 x 0.08 cm) prepared the same as was done for denaturing gel. The samples were electrophoresed at 30 watts for 4.5 hours. An X-ray film was exposed to the gel for 5 minutes and was used to direct isolation of the DNA bands of interest. Isolated DNA fragments were electroeluted in dialysis tubes in TE buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 50 volts overnight in a horizontal agarose gel box. The buffer was recirculated during elution. Electric current was reversed for 1 minute before the DNA in the dialysis tubes was concentrated on QIAGEN tip 20 according to manufacturer's instructions. Following ethanol precipitation, the end labeled PCR products were dissolved in $dH_2O$ to make final concentration approximately 0.01 $\mu g/\mu l$ which was estimated by comparison with samples of known concentration on an agarose gel.

Preparation of Purine Markers

Each end labeled PCR product (0.02 $\mu g$) was heated to 80 °C in 100 $\mu l$ 0.1 M KCl, 10 mM Sodium Citrate, pH 4.0 for 7.5 minutes. Following addition of 20 $\mu l$ 1 N NaOH the samples were heated to 90 °C for 30 minutes . Twenty micro liters of 1 N HCl and 5 $\mu g$ tRNA were added and the samples were ethanol precipitated and dissolved in 10 $\mu l$ $H_2O$ and 10 $\mu l$ formamide-dye solution (50 % deionized formamide, 2 mM EDTA, 0.5 % bromophenol blue, 0.5 % xylene cyanol FF).

*In Vitro* DNA Cleavage Reaction and Gel Electrophoresis

The end labeled DNA substrates (~0.02 μg) were cleaved *in vitro* in the presence of 2 μl topoisomerase II and 10 μM teniposide. Topoisomerase II used in this experiment was a generous gift from Dr. Y. Pommier. It was extracted from mouse leukemia cell line L1210 and was purified on HPLC. Cleavage reactions were carried out in 20 μl reaction buffer (0.01 M Tris-HCl, pH 7.5, 0.05 M KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP and 15 μg/ml BSA) at 37 °C for 10 minutes. Reactions were stopped by addition of SDS, EDTA and proteinase K with final concentrations of 1 %, 10 mM and 250 μg/ml, respectively, and incubation at 42 °C for 1 hour. Cleaved fragments were ethanol precipitated and dissolved in various volumes of formamide-dye solution (95 % deionized formamide, 2 mM EDTA, 0.5 % bromophenol blue, 0.5 % xylene cyanol FF) depending on the yield of precipitation.

The samples were loaded on a 7 % polyacrylamide-urea denaturing gel and electrophoresed at 80 watts.

# RESULTS

## Teniposide Mutagenesis

Independent cultures of CHO-D422 cells were mutagenized by teniposide at different concentrations for 16 hours and the cells were allowed to grow for 6 days before undergoing *aprt*$^-$ mutant selection. During the 6 days, the APRT enzyme remaining in the cells was diluted by degradation as well as cell division so that the mutant phenotype could be expressed. The numbers of 8-azaadenine resistant mutant colonies in each culture were counted at the end of the selection period and the mutation frequencies were calculated. Concurrent with these procedures, a small fraction of the cells was taken from each culture after the cells were treated with teniposide and plated in normal medium for determination of cell survival.

The results of teniposide mutagenesis studies are presented in Table 3. An exponential drop in cell survival was observed with increasing drug concentrations as was expected. The frequency of teniposide-induced mutations increased in a dose-dependent fashion at lower drug doses. However, starting from the doses that caused around 30 % cell survival, the mutation frequency quickly reached a plateau which is about

Table 3. Average Cell Survival and Mutation Frequency at the *aprt* Locus*

| Dose (μg/ml) | Percent Survival ( $\bar{x}$ ± s.e.) | | Mutation Frequency ( $\bar{x}$ ± s.e.) | |
|---|---|---|---|---|
| 0 | 100 | (n=22) | $1.4 \times 10^{-6} \pm 0.18$ | (n=45) |
| 0.02 | 72 ± 6.6 | (n=9) | $5.1 \times 10^{-6} \pm 1.56$ | (n=9) |
| 0.04 | 50 ± 4.1 | (n=23) | $8.1 \times 10^{-6} \pm 1.44$ | (n=20) |
| 0.06 | 37 ± 3.1 | (n=45) | $9.8 \times 10^{-6} \pm 1.15$ | (n=48) |
| 0.08 | 27 ± 3.6 | (n=33) | $10.2 \times 10^{-6} \pm 1.99$ | (n=32) |
| 0.16 | 20 ± 6.2 | (n=13) | $10.8 \times 10^{-6} \pm 4.29$ | (n=9) |

* The experiments were done under the standard mutagenesis protocol in which the cells were treated with teniposide for 16 hours.

10 fold greater than the background (spontaneous) mutation frequency.

In the hope of further maximizing the drug-induced mutation frequency, several experiments were done under different variations of mutagenesis protocol. First of all, since it was reported that calcium depletion was an effective measure to protect cells from drug cytotoxicity (Bertrand et al., 1990), the cells were incubated with EGTA for 4 hours followed by 1 hour drug treatment. However, the result indicated that calcium depletion did not improve mutability of the cells at increasing doses of teniposide (Table 4).

The second attempt was to allow mutagenized cells to multiply in suspension culture using spinner flasks (Breimer et al., 1986), so that proliferation of the mutant cells would not be inhibited due to cell confluence as would happen if the cells were grown in tissue culture flasks. The problem was that while the drug-induced mutations were only slightly increased, the background mutation frequencies were doubled or tripled. This is in contrast to results of Breimer et al. (1986) who reported a much lower spontaneous mutation frequency for cells growing in suspension.

Finally, the cells were synchronized in the presence of excess thymidine, so that the cells could be treated in either $G_1$ or $G_2$ phase of the cell cycle. However, no significant difference between the two groups was observed: mutation frequencies were approximately the same as for unsynchronized cells.

Table 4. Average Cell Survival and Mutation Frequency at the *aprt* Locus Under the Condition of Calcium Depletion

| Dose (μg/ml) | Percent Survival ( $\bar{x}$ ± s.e.) | Mutation Frequency ( $\bar{x}$ ± s.e.) |
|---|---|---|
| 0 | 100 ± - (n=5) | $0.5 \times 10^{-6}$ ± 0.14 (n=5) |
| 0.04 | 98 ± 2.0 (n=3) | $6.0 \times 10^{-6}$ ± 1.52 (n=3) |
| 0.08 | 100 ± 0.0 (n=4) | $3.2 \times 10^{-6}$ ± 0.88 (n=4) |
| 0.16 | 94 ± 4.3 (n=5) | $5.8 \times 10^{-6}$ ± 1.06 (n=4) |
| 0.32 | 60 ± 4.5 (n=5) | $3.5 \times 10^{-6}$ ± 1.49 (n=5) |
| 0.64 | 40 ± 8.9 (n=4) | $5.0 \times 10^{-6}$ ± 1.59 (n=4) |

* The experiments were done under the condition of calcium depletion in which the cells were treated with teniposide for one hour in the presence of EGTA.

In order to verify that the mutagenesis protocol we adopted was valid, mutation frequencies at the *aprt* locus and at the *hgprt* locus were compared. *hgprt*$^-$ mutants were selected with 6-thioguanine (Gupta and Singh, 1982). The mutation frequency at the *hgprt* locus was 5 to 10-fold greater than the *aprt* mutation frequency (Table 5) and was comparable to values obtained for the same locus by other investigators in CHO cells (Singh and Gupta, 1983). Thus, the nature of the *aprt* locus rather than the treatment protocol was probably responsible for the relatively low recovery of the teniposide-induced mutants.

## Sequence Analysis of the Mutations

*aprt*$^-$ mutants were collected for DNA sequence analysis. Two mutant colonies (sisters) were picked from each independently mutagenized culture for which the mutation frequency of the culture was at least 8-fold greater than the background mutation frequency from the same experiment. Mutants formed spontaneously were also isolated. To ensure that no colonies derived from a same mutant cell were included, when the mutation was found to be identical between sister mutant cell lines, one of them would be eliminated from the data pool.

Cellular DNA from each mutant strain was extracted and the mutated *aprt* gene was amplified by PCR in two segments. Each segment contains exons that are located in close vicinity

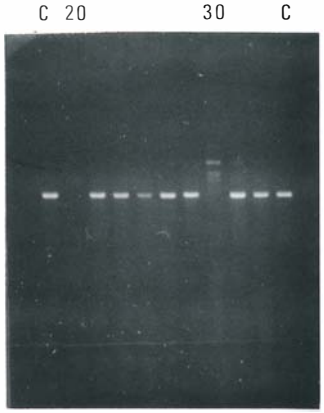
Table 5. Comparison of the Mutation Frequencies at the *aprt and hgprt* Locus*

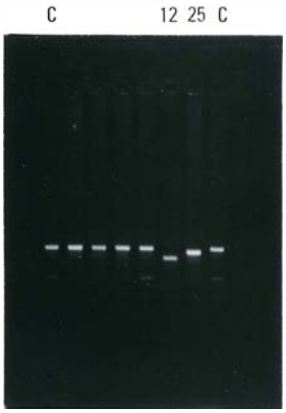
| Dose (μg/ml) | % Survival ($\bar{x}$ ± s.e.) | *aprt* M.F. x $10^{-6}$ ($\bar{x}$ ± s.e.) | *hgprt* M.F. x $10^{-6}$ ($\bar{x}$ ± s.e.) |
|---|---|---|---|
| 0 (n=2) | 100 | 0.2 ± 0.2 | 5.2 ± 4 |
| 0.04 (n=3) | 74 ± 19 | 5.7 ± 3.1 | 30.7 ± 7 |
| 0.06 (n=2) | 72 ± 8 | 8.8 ± 7.2 | 58.0 ± 11 |
| 0.08 (n=3) | 46 ± 10 | 7.9 ± 0.8 | 62.9 ± 27 |

* The experiments were done under the standard mutagenesis protocol in which the cells were treated with teniposide for 16 hours. *hgprt* mutants were selected in medium containing the toxic purine analog 6-thioguanine.

to each other. Exons 1 and 2 were amplified in one segment and exons 3, 4 and 5 were amplified in another segment. PCR products bearing small deletions or insertions could usually be detected by agarose gel electrophoresis (Figure 3). Three of the drug-induced mutants were shown to be insertions of about 190 to 330 base pairs. These bigger insertions are difficult to analyze at the DNA sequence level, because insertions of such sizes could not be properly amplified to yield a homogeneous PCR product (mutant 30 in Figure 3) even though a longer extension time was allowed in each reaction cycle. Since a single-stranded PCR product was used in the sequencing reaction, when there was no clean regular PCR product to serve as template, no usable single-stranded PCR product could be generated to serve as a template in the sequencing reaction. As a result, the sizes of these insertions were determined by comparing the PCR products with size markers on agarose gels. Another seven mutants were found to have missing PCR products, which indicated possible large deletions or rearrangements. These mutants were also excluded from sequence analysis but were subjected to Southern blot analysis in order to determine the nature and sizes of the alterations. For most of the spontaneous and drug-induced mutants, the PCR products of the *aprt* gene were able to be amplified further in an asymmetric manner where one of the two DNA strands were preferentially synthesized. The single-stranded products obtained from the asymmetric PCR were then sequenced by Sanger's sequencing reaction to reveal the

Figure 3. PCR products of exons 3, 4 and 5 of the *aprt* gene shown on a 1.5 % agarose gel. Lanes C are wild type PCR products (747 base pairs). Lane 20 shows that the PCR product of mutant 20t is missing. Lane 30 shows that mutant 30t carries a insertion of about 290 base pairs which was estimated by comparing the migration distance of the mutant DNA fragment with that of the wild type DNA fragment on a semi-log plot of 1 kb size markers. Lanes 12 and 25 show that mutants 12t and 25t carry deletions of 46 and 21 base pairs, respectively, which was determined by DNA sequencing.


C 20 30 C


C 12 25 C

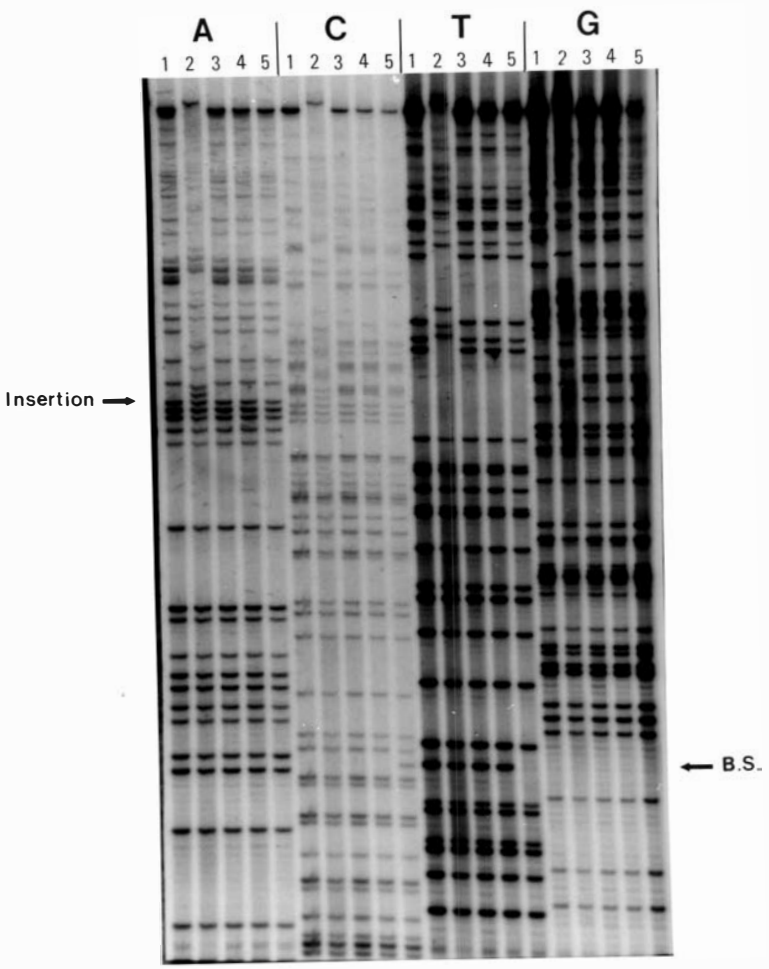
mutations (Figure 4).

The achievement of successful sequencing was built on many attempts and failures. First of all, denatured double stranded PCR product was used in the sequencing reaction; however, no sequence banding pattern was shown on the sequencing gel, which suggested that the PCR product quickly annealed and the sequencing primer could not anneal to the double-stranded template. When single-stranded template was used in the reaction, too many bands were shown on the sequencing gel, suggesting that the presence of PCR primers probably caused unwanted sequencing reactions to occur. Different purification procedures were tried to eliminate the PCR primers as well as nucleotides in the asymmetric PCR product. Among them, centricon ultrafiltration columns were used but single-stranded DNA molecules were found to bind to the filter membrane; hydroxyapatite purification of single-stranded asymmetric PCR product was also tried unsuccessfully. Finally, single-stranded PCR product was purified by co-precipitation with 0.5 $\mu$g supercoiled DNA in 1 volume of 4 M ammonium acetate and 2 volumes of 2-propanol.

It was also found that higher quality sequencing data were obtained if an internal primer (see Table 2), not used in the initial PCR, was used either for generation of the asymmetric product or for the sequencing reaction.

Sixty eight teniposide-induced and 42 spontaneous mutants were analyzed at the DNA sequence level of the *aprt* gene. Thirteen of the spontaneous mutant sequences were generously

Figure 4. DNA sequence alterations shown on the polyacrylamide denaturing gel. Lanes 1 through 5 represent 5 mutants analyzed. Letters A, C, T and G tell the nucleotide the bands represent. Lanes 2 show an insertion mutation the mutant carries; the arrow at the left shows the beginning of the sequence shift due to the insertion. Lanes 5 show a base substitution mutation the mutant carries; the arrow at the right indicates the T → C base substitution (B.S.).


A
C
T
G
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
Insertion
B.S.

provided by Dr. F. Austin from the same laboratory. The types of spontaneous and drug-induced mutations are categorized in Tables 6 and 7 and summarized in Tables 8 and 9, respectively.

Among all types of spontaneous mutations, 64 % are base substitutions. While the frequencies of transitions versus transversions are well balanced, G·C → A·T accounts for 71 % of the transitions and 37 % of total base substitutions. Figure 5 shows that most of the substitutions targeted amino acids which were previously recognized, but several substitutions took place at sites where mutations have never been detected before.

Twenty-one percent of the spontaneous mutations are small deletions ranging from 1 to 29 base pairs in size. Most of these small deletions are framed by short direct repeats; one copy of the repeated sequence is included in the deleted fragment and the other one is retained in the novojunction. Three duplications were also observed. A cluster of 5 deletion/duplication mutations is located in a region of exon 5 where a secondary structure could be formed (Figure 6, the second stem-loop structure).

Three of the 42 spontaneous mutations were found to have normal sequences of all 5 exons. The altered structures may reside in the regulatory components outside coding regions of the gene. Such cases were also encountered by other investigators (Phear et al., 1989) and were difficult to explain.

The spectrum of teniposide-induced mutations is quite

Table 6. Categorized spontaneous mutations. Lower case letters in deletion and insertion mutations represent the deleted or inserted DNA sequences. Short direct repeats appearing at the ends of deletion mutations are underlined. In the mutant sequence, one copy of the repeat is retained; thus, the actual endpoints of the deletion or insertion are indeterminate within the repeat, and are arbitrarily shown in the left most possible position.

| Strain | Type of Mutation | Mutation Sequence | Location (bp of gene) | Amino Acids Replacement |
|---|---|---|---|---|
| 58s | G·C → A·T | TGGTG G AGCTG | 1841 | E → Q 158 |
| 59s | G·C → A·T | TGGAG G TAAGA | 1661 | SJ |
| 61s | G·C → A·T | ACTTC C CCATC | 58 | P → S 20 |
| 67s | G·C → A·T | TCCTG G CCACT | 1651 | A → T 131 |
| 85s | G·C → A·T | TTTAG G TGAGA | 81 | SJ |
| 88s | G·C → A·T | CACTG G AGGTA | 1658 | G → E 133 |
| 100s | G·C → A·T | AACTA G AAATC | 1591 | E → K 111 |
| 118s | G·C → A·T | CATCC G GAAGC | 1366 | R → Q 87 |
| 35.6 | G·C → A·T | CAGGG G ATTCA | 1309 | G → E 68 |
| 37.6 | G·C → A·T | CCTGG G CTGTG | 1351 | G → D 82 |
| 63s | A·T → G·C | TAGAC T CCAGG | 1302 | S → P 66 |
| 64s | A·T → G·C | CAGGG A TATCT | 211 | D → G 28 |
| 103s | A·T → G·C | GAAGG A CCCCG | 232 | D → G 35 |
| 110s | A·T → G·C | AGATG A TCTCC | 1643 | D → G 128 |
| 65s | A·T → C·G | GCGCA T CCGCA | 38 | I → S 13 |
| 94S | A·T → C·G | TTCCC A GGGAT | 207 | SJ |
| 95s | A·T → C·G | GAGTG A CTGGA | 1912 | Stop → C |
| 115s | A·T → C·G | GCTGG T GGCGC | 26 | V → G 9 |
| 34.6 | A·T → C·G | CTGTT T AGGTG | 78 | F → L 26 |
| 41.5 | A·T → C·G | GCTGG T GGCGC | 26 | V → G 9 |
| 42.1 | A·T → C·G | GCCTG T GAGCT | 1789 | C → W 140 |
| 87s | G·C → C·G | TGGTG G AGCTG | 1841 | E → Q 158 |
| 116s | G·C → C·G | CCTGG G CTGTG | 1351 | G → A 82 |
| 40.2 | G·C → C·G | CAGGG G ATTCT | 1309 | G → A 68 |
| 57s | G·C → T·A | GCAGG C GAGTG | 317 | SJ |

| | | | | | |
|---|---|---|---|---|---|
| 117s | G·C → T·A | CCTGG G CTGTG | 1351 | G → V | 82 |
| 89s | A·T → T·A | GCGCA T CCGCA | 38 | I → N | 13 |

| | | | |
|---|---|---|---|
| 86s | 1 bp deletion | 7<br>GGCGGaATCTG | |
| 41.3 | 1 bp deletion | 44<br>CGCAGtTTCCC | |
| 114s | 5 bp deletion | 33<br>CGCAGcgcatCCGCAG | |
| 66s | 9 bp deletion | 299<br>GCAAGatcgactacATCGCAGG | |
| 107s | 11 bp deletion | 1775<br>AACCAtgtgcgctgccTGTGAGCT | |
| 78s | 16 bp deletion | 1783<br>GCGCTgcctgtgagctgctggGCCAG | |
| 62s | 18 bp deletion | 1817 1837<br>TGAGGtggtggag...gagccTGGTGGAG | |
| 42.2 | 18 bp deletion | 198<br>TCTCCttgtt...tatctCGCCC | |
| 42.4 | 29 and 1 bp deletion | 316 346<br>GCAGGcgagt...ggtccCCCaCT | |

| | | | |
|---|---|---|---|
| 41.1 | 1 bp duplication | 1821<br>GGTgGAGTGT | |
| 33.6 | 4 bp duplication | 1642<br>AGATGatctCCTGG | |
| 104s | 37 bp duplication | 1778 1817<br>CATGTgcgct...gctgaGGTGG | |

| | |
|---|---|
| 60s | No change detected |
| 39.6 | No change detected |
| 42.5 | No change detected |

Table 7. Categorized teniposide-induced mutations. Lower case letters in deletion and insertion mutations represent the deleted and inserted sequences. Short direct repeats appearing at the ends of deletions and insertions are underlined. In the mutant sequence, one copy of the repeat is retained; thus, the actual endpoints of the deletion or insertion are indeterminate within the repeat, and are arbitrarily shown in the leftmost possible position.

| Strain | Type of Mutation | Mutant Sequence | Location (bp of gene) | Amino Acids Replacement |
|---|---|---|---|---|
| 4t | A·T → C·G | TTCCC A GGGAT | 207 | SJ |
| 10t | A·T → C·G | CAGGA A CCATG | 1772 | T → P 135 |
| 53t | A·T → C·G | GAGTG A CTGGA | 1912 | Stop → C |
| 74t | A·T → C·G | AGATG A TCTCC | 1643 | D → A 128 |
| 80t | A·T → C·G | TCACC T TAAGT | 277 | L → R 50 |
| 81t | A·T → C·G | TTAGG T GAGAT | 82 | SJ |
| 16t | G·C → C·G | GTGAG C CTGGT | 1834 | S → R 155 |
| 18t | G·C → C·G | GCAGG C GAGTG | 317 | SJ |
| 26t | G·C → C·G | AAGTG G TTGTT | 1630 | V → L 124 |
| 27t | G·C → C·G | TCCTG G CCAGT | 267 | A → P 47 |
| 72t | G·C → C·G | CCTCC C TAGCT | 1329 | L → V 75 |
| 79t | G·C → C·G | GGCGA G TGGCC | 320 | SJ |
| 14t | G·C → T·A | TCCCC G ACTTC | 52 | D → Y 18 |
| 21t | G·C → T·A | TGGAG G TAAGA | 1661 | SJ |
| 31t | G·C → T·A | TGGTG G AGCTG | 1841 | E → Stop 158 |
| 72t | G·C → T·A | CTCAG G AGCTG | 1338 | E → Stop 78 |
| 48t | A·T → T·A | CTTCC T GTCTG | 1573 | SJ |
| 32t | A·T → G·C | TGATC T GCTGG | 1794 | L → P 142 |
| 36t | A·T → G·C | GGTGG A GCTGA | 1842 | E → G 158 |
| 42t | A·T → G·C | GCCCC T CCTGA | 223 | L → P 32 |
| 45t | A·T → G·C | CGAGT A TGGCA | 1420 | Y → C 105 |
| 54t | A·T → G·C | ACTCC A GGGGA | 1305 | R → G 67 |
| 75t | A·T → G·C | GAAGC T AGGAT | 1872 | L → P 168 |
| 92t | G·C → A·T | CTGAG G TGGTG | 1817 | V → M 150 |
| 68t | G·C → A·T | TTAAG G GCAGA | 1859 | G → S 164 |

| | | |
|---|---|---|
| 38t | 1 bp deletion | 232<br>AAGGAcCCCGC |
| 33t | 2 bp deletion | 1878<br>GGATCagTACCA |
| 101t | 3 bp deletion | 1644<br>ATGATctcCTGGC |
| 22t | 4 bp deletion | 294<br>TGGCGgcaaGATCG |
| 83t | 4 bp deletion | 273<br>CAGTCacctTAAGT |
| 3t | 6 bp deletion | 1801<br>TGGGCcagctaCAGGC |
| 76t | 6 bp deletion | 1877<br>AGGATcagtacCATTC |
| 52t | 7 bp deletion | 1585<br>GGCTGaactagaAATCC |
| 24t | 8 bp deletion | 1294<br>ACAGGcctagactCCAGG |
| 55t | 9 bp deletion | 1875<br>CTAGGatcagtaccATTCTTC |
| 33t | 10 bp deletion | 1863<br>GCAGAgagaagctagGATCAGT |
| 73t | 10 bp deletion | 269<br>TGGCCagtcaccttaAGTCcA |
| 96t | 12 bp deletion | 277<br>CACCTtaagtccacgcaTGGCG |
| 71t | 14 bp deletion | 1584<br>AGGCTgaactagaaatccaGAAAGAC |
| 13t | 15 bp deletion | 1387<br>CCAGGccccacagtgtcagccTCCT |
| 29t | 16 bp deletion | 1650<br>TCCTGgccactggaggtaagaGCCACT |
| 49t | 18 bp deletion | 1339 1358<br>CAGGAgctgg...tgtgtGCTCA |
| 25t | 21 bp deletion | 17 39<br>GAGTtgcagc...cgcaTCCGCA |

| | | |
|---|---|---|
| 56t | 36 bp deletion | 1874 1907<br>GCTAGgatca...tgagtGACTGGA |
| 12t | 46 bp deletion | 72 119<br>GCGTGctg...ttcCTGGGCTC |
| 44t | About 22 bp deletion in exon 5 | |

---

| | | |
|---|---|---|
| 11t | 1 bp duplication | 1655<br>GCCACtGGAG |
| 15t | 1 bp duplication | 1655<br>GCCACtGGAG |
| 39t | 2 bp duplication | 1778<br>CATGTgcGCTGCCT |
| 91t | 2 bp duplication | 1411<br>TATGCtcTCGAG |
| 5t | 4 bp duplication | 1595<br>GAAATccagAAAGA |
| 23t | 4 bp duplication | 1360<br>GTGCTcatcCGGAA |
| 28t | 4 bp duplication | 1848<br>CTGACctcaCTTAA |
| 34t | 4 bp duplication | 1352<br>TGGGCtgtgTGCTC |
| 37t | 10 bp duplication | 269<br>GCCagtcaccttaAGTCCA |
| 1t | 41 bp duplication | 216 258<br>TATCTcgcccctcctg...atcCGCCTCCTG |
| 90t | 85 bp complex duplication in exon4 | |

---

| | | |
|---|---|---|
| 35t | 3 bp insertion | 1857<br>CTTAAgttGGGCA |
| 33t | 6 bp insertion | 1900<br>TCCTGcaatgaCAATATGA |
| 40t | About 190 bp insertion in exon 3, 4, 5 | |
| 30t | About 290 bp insertion in exon 5 | |
| 98t | About 330 bp insertion in exon 5 | |

Table 8. Spectrum of Spontaneous Mutations at the *aprt* Locus

| Type of Mutation | Number | % |
|---|---|---|
| Transitions: | | |
| G·C → A·T | 10 | 23.8 |
| A·T → G·C | 4 | 9.5 |
| Transversions: | | |
| A·T → C·G | 7 | 16.7 |
| G·C → C·G | 3 | 7.1 |
| G·C → T·A | 2 | 4.8 |
| A·T → T·A | 1 | 2.4 |
| Small Deletions: | 9 | 21.4 |
| Insertions: | 3 | 7.1 |
| No Change: | 3 | 7.1 |
| Total: | 42 | 100 |

Table 9. Spectrum of Teniposide-Induced Mutations at the *aprt* Locus

| Type of Mutation | Number | % |
|---|---|---|
| Transitions: | | |
| A·T → G·C | 6 | 8.8 |
| G·C → A·T | 2 | 2.9 |
| Transversions: | | |
| A·T → C·G | 6 | 8.8 |
| G·C → C·G | 6 | 8.8 |
| G·C → T·A | 4 | 5.9 |
| A·T → T·A | 1 | 1.5 |
| Small Deletions: | 20 | 29.4 |
| Insertions: | 16 | 23.5 |
| Large Deletions or Rearrangements: | 7 | 10.3 |
| Total: | 68 | 100 |

Figure 5. Distributions of amino acid replacements resulting from base substitution mutations at the *aprt* gene. The amino acid displacements resulting from spontaneous mutations labeled above the APRT sequence were reported previously by other investigators (de Boer and Glickman, 1991). The amino acid replacements resulting from spontaneous (lower case letters) and teniposide-induced mutations labeled below the APRT sequence were results from this study.

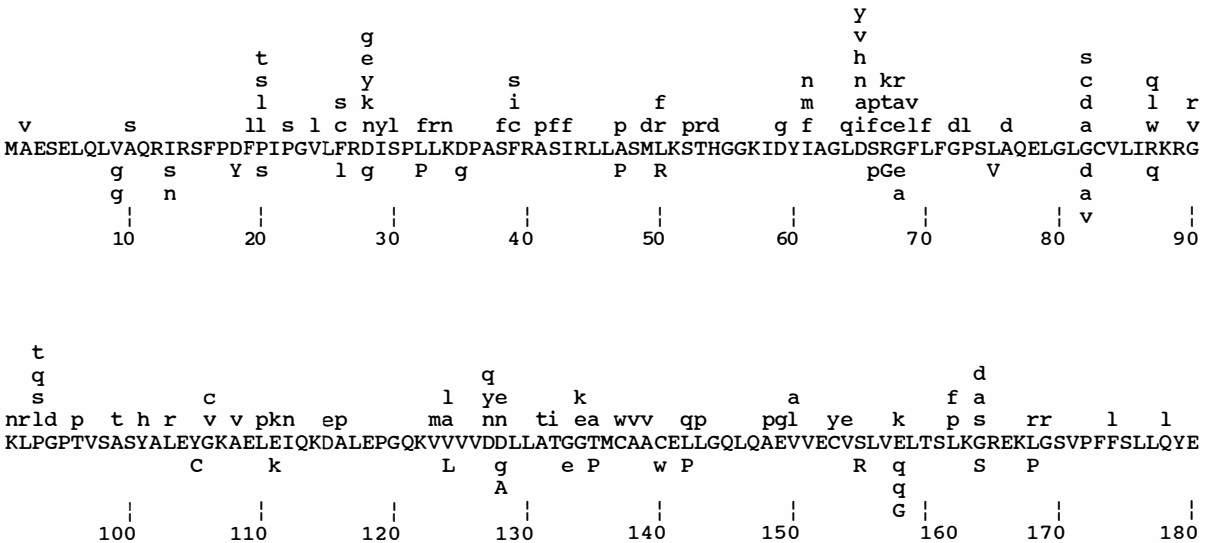
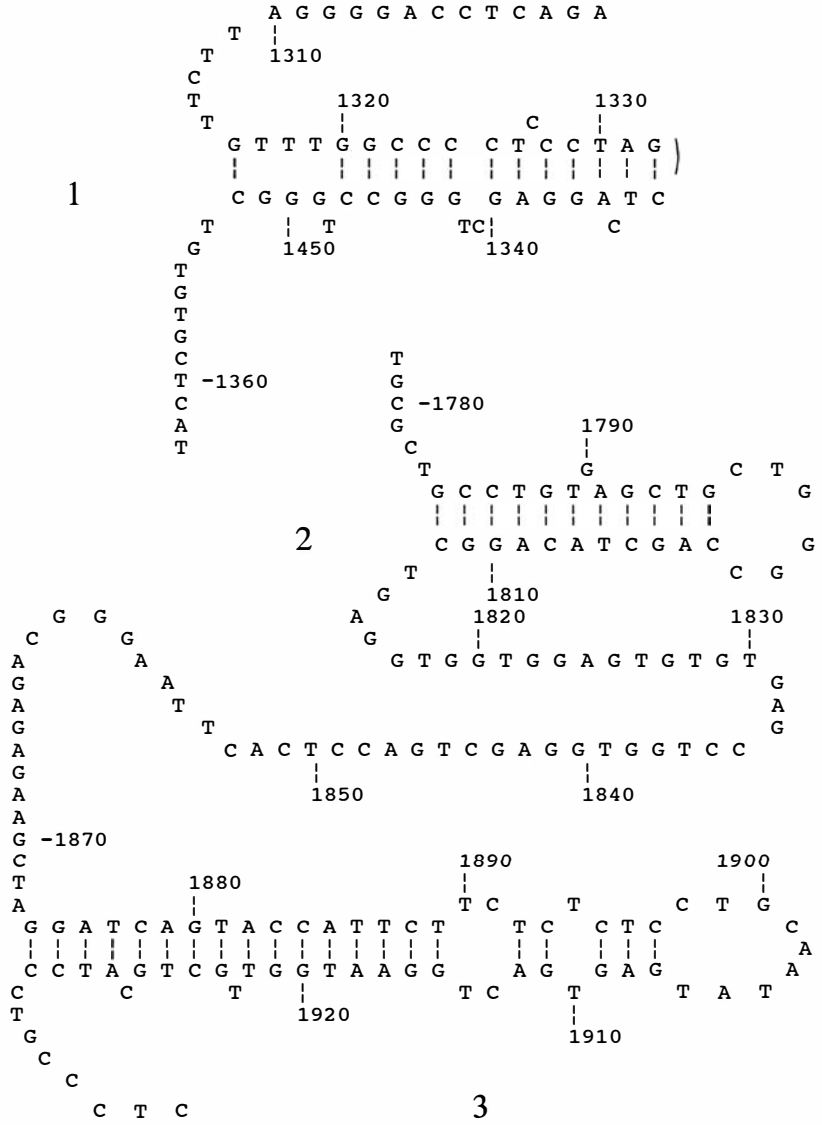

MAESELQLVAQRIRSFPDFPIPGVLFRDISPLLKDPASFRASIRLLASMLKSTHGGKIDYIAGLDSRGFLFGPSLAQELGLGCVLIRKRG
KLPGPTVSASYALEYGKAELEIQKDALEPGQKVVVVDDLLATGGTMCAACELLGQLQAEVVECVSLVELTSLKGREKLGSVPFFSLLQYE
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180

Figure 6. Regions of dyad symmetry at the *aprt* locus where secondary structures could be formed. The upper hair pin structure is in exon 3 and the lower ones are in exon 5.


A G G G G A C C T C A G A
1310
1320
1330
G T T T G G C C C C T C C C T A G
C G G G C C G G G G A G G A T C
1450
1340
1360
1780
1790
G C C T G T A G C T G
C G G A C A T C G A C
1810
1820
1830
G T G G T G G A G T G T G T
C A C T C C A G T C G A G G T G G T C C
1850
1840
1870
1880
1890
1900
1920
1910
1
2
3

different from that of spontaneous mutations. Sixty-three per cent of the mutations are deletions, insertions and rearrangements of different sizes. Teniposide-induced small deletions are similar in nature to the deletions formed spontaneously. The sizes of these small deletions are spread evenly between 1 and around 20 base pairs with the exception of two deletions of 36 and 46 base pairs. The presence of short direct repeats at the ends of the deletions is also characteristic of this group. There is a cluster of 4 deletions at exon 5 where the sequence is rich in short direct repeats and long stretch of dyad symmetry (Figure 6). These deletions (mutants 33t, 55t, 56t, 76t) begin between base pairs 1865 to 1879, which is around the base of a putative stem-loop structure.

Over two thirds of the teniposide-induced insertions are duplications and most of them are 4 base pairs or less. The insertions are either as small as 3 and 6 base pairs or as large as about 190 to 330 base pairs. The sizes of the three large insertions were estimated on agarose gel since no sequence data are available.

The composition of teniposide-induced base substitutions is somewhat different from that of spontaneous substitutions. Transversions were over two times more frequent than transitions and G·C → A·T transitions were much less prevalent. However, similar to the spontaneous ones, teniposide-induced base substitutions were rather evenly distributed throughout the coding sequences and splice

junctions of the *aprt* gene (Figure 5).

## Southern Blot Analysis of Large Scale Mutations

Among 68 teniposide-induced mutations, 7 of them have at least one exon which failed to be amplified by PCR. These mutations were examined by combined PCR and Southern blot analysis. The observed abnormalities are listed in Table 10.

Restriction endonucleases Bam HI and Pst I were used to digest mutant DNA (please refer to Figure 2 for restriction sites). After DNA fractionation by agarose gel electrophoresis and Southern transfer, the DNA was hybridized with a homogeneously $^{32}P$ labeled 3.8 kb Bam HI fragment of the plasmid pHaprt. Wild type DNA digested with Bam HI showed a 3.8 kb band which contains the entire coding sequences of the *aprt* gene (Figure 7). Pst I cuts the *aprt* gene into 2 segments. A 3.8 kb band contains exons 1, 2 and 3 and a 2.0 kb band contains exons 4 and 5 (Figure 8). Changes in banding patterns provided clues for determining the nature and size of a mutation. Large deletions or rearrangements were indicated in these mutations (Table 10).

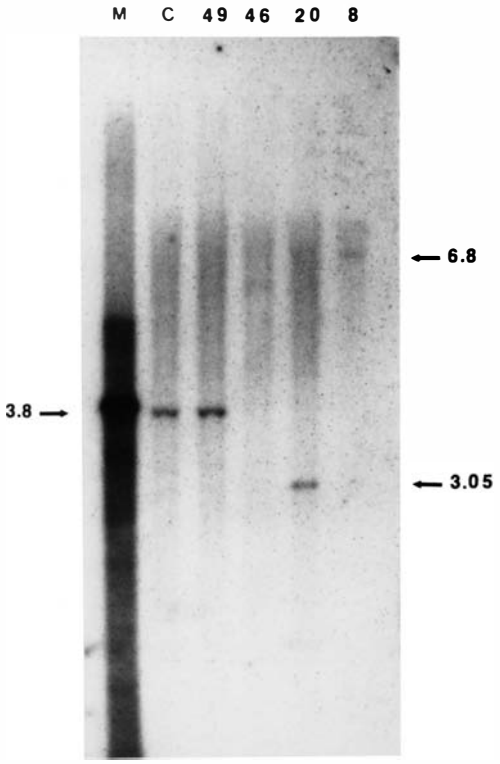
Mutants 6t and 8t seem to have similar alterations. Both have faint or missing PCR products for exons 1 and 2 which localized the alterations in this region of the gene. Displacements of 3.8 kb Pst I bands by smaller bands indicated large deletions. The fact that the 3.8 kb Bam HI bands are displaced by larger bands suggested that the large deletions

Table 10. PCR and Southern Blot Analysis of Large Deletions and Rearrangements

| Strain | PCR Analysis | Southern Blot Analysis | | Probable Lesion |
|---|---|---|---|---|
| | | Bam HI Site | Pst I Site | |
| 6t | Weak exon 1<br>no exon 2 | - 3.8 kb band<br>+ 3.85 kb band | - 3.8 kb band<br>+ 1.4 kb band | Deletion/rearrangement involving exons 1 and 2 |
| 8t | Weak exons 1 and 2 | - 3.8 kb band<br>+ 6.8 kb band | - 3.8 kb band<br>+ 1.5 kb band | Deletion/rearrangement involving exons 1 and 2 |
| 20t | No exons 3, 4 and 5 | - 3.8 kb band<br>+ 3.05 kb band | - both bands<br>+ 5.2 kb band | About 750 bp deletion at exons 3, 4 and 5 |
| 46t | No exons 3, 4 weak exons 1, 2 and 5 | - 3.8 kb band | - both bands | Deletion of most or all of the gene |
| 51t | No exon 4 | - 3.8 kb band<br>+ 2.8 kb band | + 2.3 kb band | Deletion/rearrangement involving exon 4 |
| 70t | No exons 3, 4 weak exons 1, 2 and 5 | - 3.8 kb band | - 3.8 kb band<br>+ 3.35 kb band | Deletion/rearrangement involving most or all of the gene |
| 77t | No exon 5 | - 3.8 kb band<br>+ 4.1 kb band | - 2.2 kb band<br>+ 6.0 kb band | Deletion/rearrangement involving exon 5 |

Symbols - and + represent loss and gain, respectively.

Figure 7. Southern blots of DNAs digested with the restriction endonuclease Bam HI. Arrows indicate bands of different sizes in kb. Lane C: wild type genomic DNA. Lane M: 3.8 kb *aprt* fragment marker. Due to the differences in the amount of DNA loaded on the agarose gel, the marker fragments migrated slower than genomic DNAs of the same size.


M
C
49
46
20
8
6.8
3.8
3.05

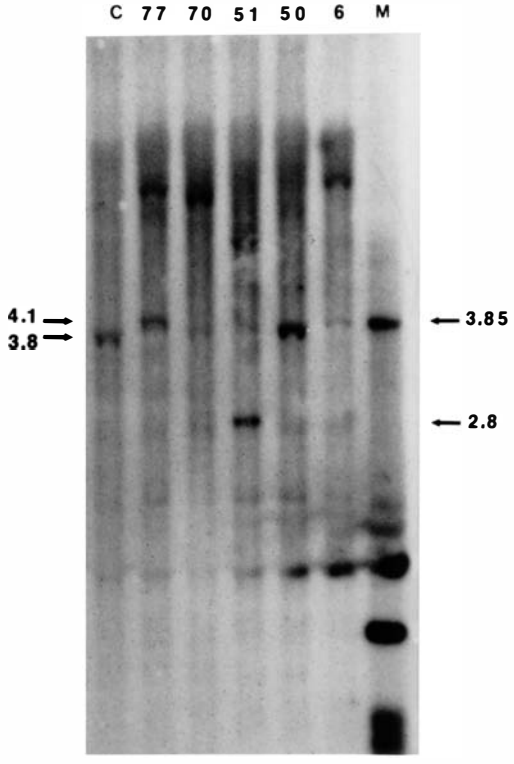
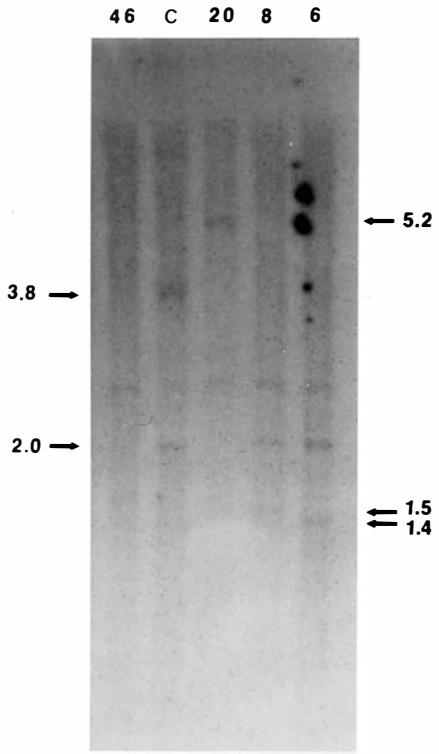
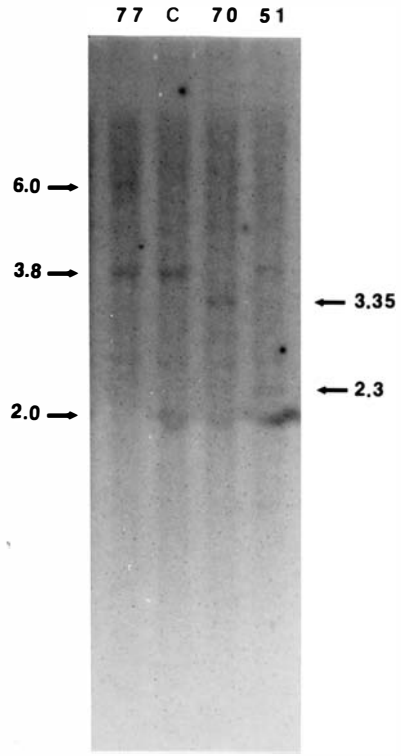

C
77
70
51
50
6
M
4.1
3.8
3.85
2.8

Figure 8. Southern blots of DNAs digested with the restriction endonuclease Pst I. Arrows indicate bands of different sizes in kb. Lane C: wild type DNA. The other lanes with different numbers represent the teniposide-induced mutants of the same number.


46 C 20 8 6
5.2
3.8
2.0
1.5
1.4


77 C 70 51
6.0
3.8
3.35
2.3
2.0

might have eliminated the upstream Bam HI site. The PCR and Southern blot results of mutant 20 are consistent and clearly indicate a deletion at exons 3, 4 and 5 of about 750 base pairs including the internal Pst I site. Mutant 46t has almost everything missing except for some faint PCR products of exons 1, 2 and 5. This could have resulted from deletion of most or all of the gene. However, since some of the PCR products were visible, although barely detected, it could also be interpreted as a bad preparation of cellular DNA which prevented detection of the mutation. Mutant 51t has a missing PCR product when exons 3, 4 and 5 were amplified together. But when they were amplified separately, only exon 4 was missing. Displacement of 3.8 kb Bam HI fragment by a 2.8 kb fragment made the mutation seem like a 1 kb deletion at exon 4. However, the size of the entire fragment of exons 3, 4 and 5 is only about 700 base pairs. In addition, the extra 2.3 kb band on the Pst I blot is also difficult to explain in terms of a simple deletion. A rearrangement of the gene structure involving exon 4, for example, a very large insertion in this exon, is more likely to be the cause. Mutant 70t has an ambiguity similar to that of mutant 46t. It could either be a deletion of most or all of the gene or bad preparation of the cellular DNA. The alteration of mutant 77t is apparently located toward the end of the gene. It is likely to be a large deletion or rearrangement involving exon 5 and the downstream Bam HI and Pst I restriction sites.

Although most of these mutations were not analyzed in

detail, it is certain that such large scale alterations only occurred among teniposide-induced mutations.

*In vitro* DNA Cleavage

Drug-induced mutations are probably the result of incorrect repair following DNA damages. To try to understand the sequence specificity of drug-induced DNA lesions and the cellular repair mechanisms, it was hoped that with the same specificity, DNA strand breaks could be generated *in vitro* in the presence of topoisomerase II and teniposide.

PCR products of exons 2, 3, 4 and 5 were 5'-end labeled with $^{32}P$ separately in upper and lower strands so that a total of 8 substrates were prepared. Since few deletion/insertion mutations were found at exon 1, this fragment was not prepared for *in vitro* cleavage reaction.

We first attempted to use a crude nuclear extract prepared from the CHO-D422 cell line to catalyze *in vitro* cleavage reaction. Although DNA cleavage was achieved at exon 4 as is shown in Figure 9, at other exons, significant cleavage was either not clearly observed or accompanied by a high nonspecific background cleavage. Purified topoisomerase II of mouse L1210 cells, which was provided by Dr. Y. Pommier, exhibited higher activity and specificity in the DNA cleavage reaction. Pictures shown in Figures 10, 11, 12 and 13 were results of in vitro cleavage reaction catalyzed by purified mouse topoisomerase II.

Figure 9. *In vitro* DNA cleavage mediated by crude nuclear extract in the lower strand of exon 4 of the *aprt* gene. Lanes 1, 2, 3 and 4: cleavage reactions in the presence of 10 $\mu$M teniposide and increasing amount of extract (0.5 - 3 $\mu$l). Lane T: reaction with 10 $\mu$M teniposide only. Lanes 5 and 6: reactions with extract only (1 and 2 $\mu$l). Lane C: reaction with substrate only (without teniposide and extract).

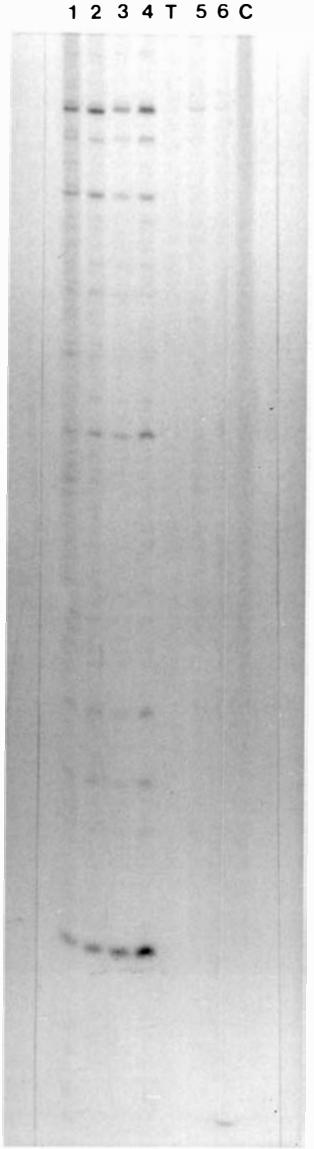
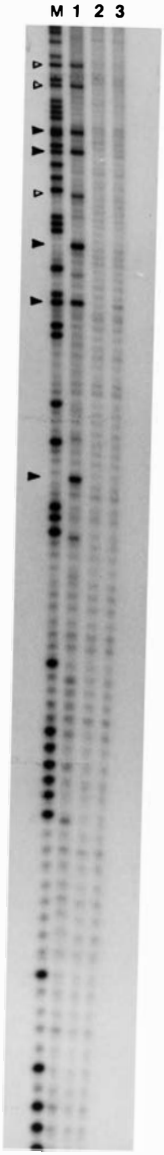

1 2 3 4 T 5 6 C

Figure 10. *In vitro* DNA cleavage in the upper (left) and lower (right) strands of exon 2 of the *aprt* gene. Lane M: purine markers. Lane 1: cleavage reaction with 10 $\mu$M teniposide and 2 $\mu$l purified topoisomerase II. Lane 2: reaction with 10 $\mu$M teniposide only. Lane 3: reaction with 2 $\mu$l topoisomerase II only. Solid and open triangles indicate strong and weak cleavage sites that were arbitrarily defined.


M 1 2 3

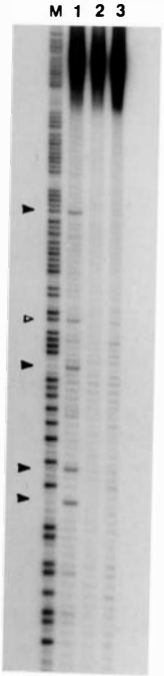

M 1 2 3

Figure 11. *In vitro* DNA cleavage in the upper (left) and lower (right) strands of exon 3 of the *aprt* gene. Lane M: purine markers. Lane 1: cleavage reaction with 10 μM teniposide and 2 μl purified topoisomerase II. Lane 2: reaction with 10 μM teniposide only. Lane 3: reaction with 2 μl topoisomerase II only. Solid and open triangles indicate strong and weak cleavage sites that were arbitrarily defined.

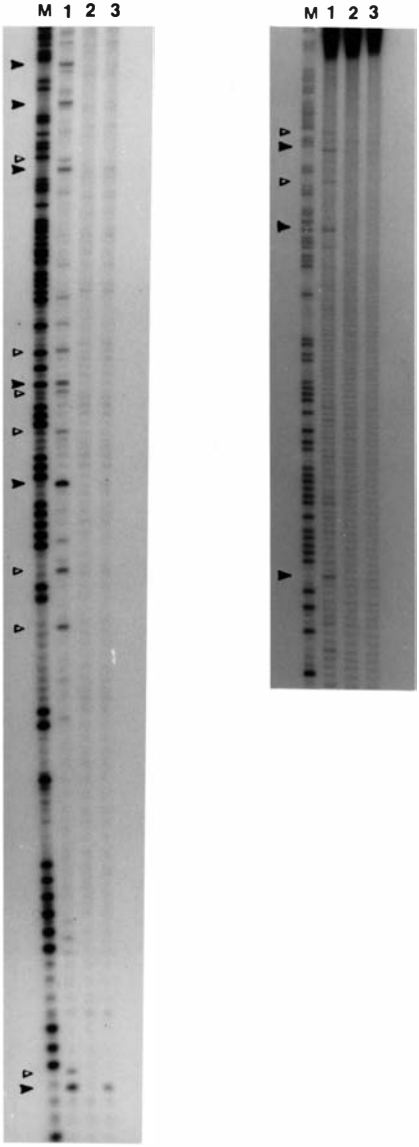
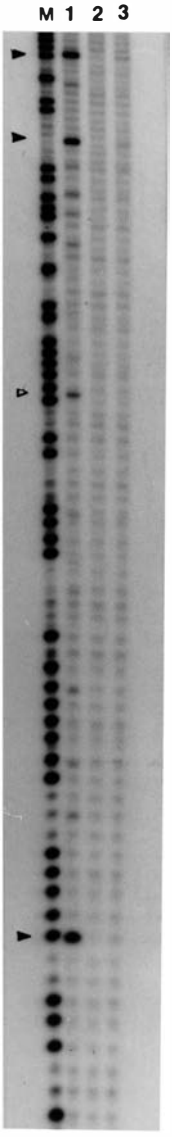

M 1 2 3
M 1 2 3

Figure 12. *In vitro* DNA cleavage in the upper (left) and lower (right) strand of exon 4 of the *aprt* gene. Lane M: purine markers. Lane 1: cleavage reaction with 10 $\mu$M teniposide and 2 $\mu$l purified topoisomerase II. Lane 2: reaction with 10 $\mu$M teniposide only. Lane 3: reaction with 2 $\mu$l topoisomerase II only. Solid and open triangles indicate strong and weak cleavage sites that were arbitrarily defined.


M 1 2 3

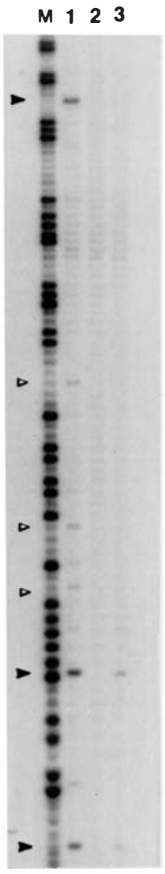
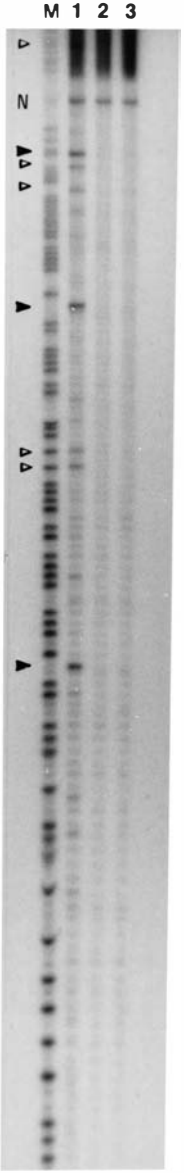
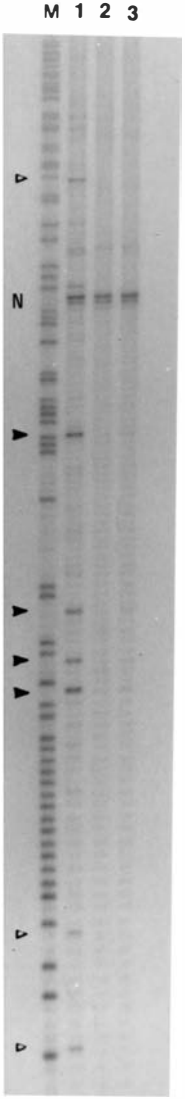

M 1 2 3

Figure 13. *In vitro* DNA cleavage in the upper (left) and lower (right) strands of exon 5 of the *aprt* gene. Lane M: purine markers. Lane 1: cleavage reaction with 10 $\mu$M teniposide and 2 $\mu$l purified topoisomerase II. Lane 2: reaction with 10 $\mu$M teniposide only. Lane 3: reaction with 2 $\mu$l topoisomerase II only. Solid and open triangles indicate strong and weak cleavage sites that were arbitrarily defined. The letter N indicates the nonspecific cleavage.


M 1 2 3
N


M 1 2 3
N

Each labeled substrate was incubated with 2 μl topoisomerase II and 10 μM teniposide which stabilized the DNA-topoisomerase II complexes. Subsequent treatment with SDS resulted in DNA strand breaks and the broken fragments were visualized by polyacrylamide-urea gel electrophoresis (Figures 10, 11, 12 and 13). Purine markers were run along with the sample DNAs so that the breakage sites could be located. Since a DNA fragment cut by topoisomerase II has a hydroxyl group at the 3' end, while the corresponding purine marker has a negatively charged phosphate at the 3' end, the topoisomerase II cleaved fragment migrates slower than the marker fragment of the same length on the sequencing gel. The shorter the fragment, the bigger the difference is. For example, a 70 base pair topoisomerase II cleaved fragment appearing on the gel is retarded from the corresponding marker fragment by a distance equivalent one half of a base, and a 30 base pair fragment is retarded by a distance of a complete base. This difference was verified by treating the purine marker with the 3' phosphatase activity of T4 polynucleotide kinase to remove its 3' end phosphate; however, since this removal seldom went to completion, it was not routinely employed.

Based on the intensity of the bands, the breakage points were arbitrarily classified into strong and weak sites. A total of 31 strong sites and 25 weak sites were detected as indicated by solid and open triangles in both the gel pictures and Figures 14, 15, 16 and 17, in which sequences of exons 2, 3, 4 and 5 were shown. There are several areas in the gel (the

Figure 14. Map of teniposide-induced in vitro topoisomerase II cleavage sites and deletion/insertion mutations at exon 2 of the *aprt* gene. Open and solid triangles indicate weak and strong cleavage sites that were arbitrarily defined. Continuous and broken lines represent deletions and duplications, respectively. Since short direct repeats appear at nearly all of the deletion and duplication termini and one of the repeated sequences is retained, the endpoints are indeterminate within the repeats and both of them are shown with extended lines.

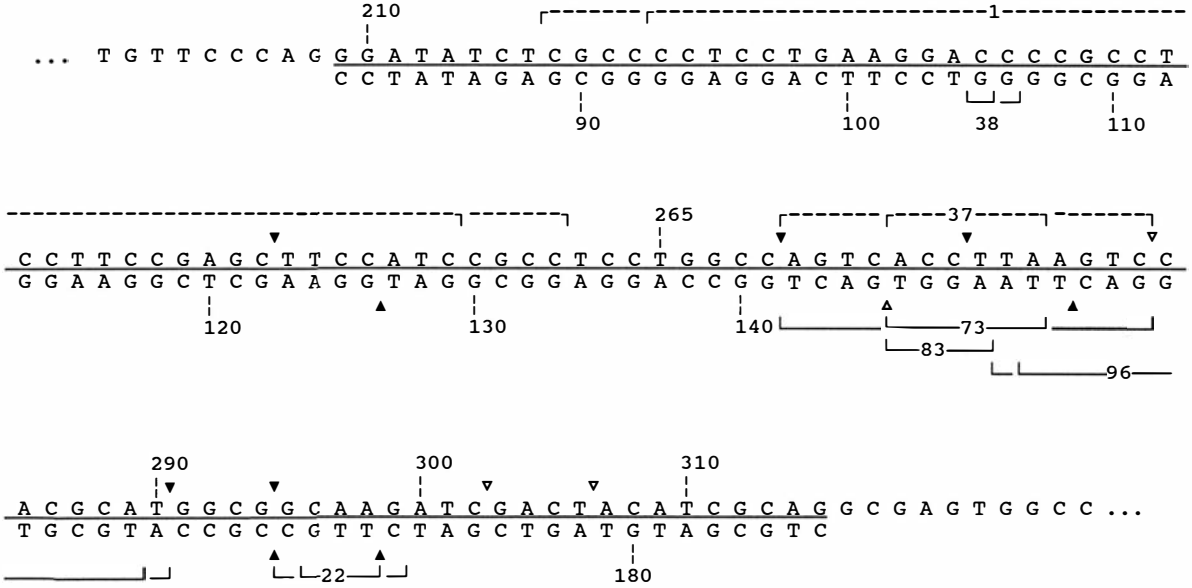

210
1
... T G T T C C C A G G G A T A T C T C G C C C C T C C T G A A G G A C C C C G C C T
C C T A T A G A G C G G G G A G G A C T T C C T G G G G C G G A
90
100
38
110
265
37
C C T T C C G A G C T T C C A T C C G C C T C C T G G C C A G T C A C C T T A A G T C C
G G A A G G C T C G A A G G T A G G C G G G A G G A C C G G T C A G T G G A A T T C A G G
120
130
140
73
83
96
290
300
310
A C G C A T G G C G G C A A G A T C G A C T A C A T C G C A G G C G A G T G G C C ...
T G C G T A C C G C C G T T C T A G C T G A T G T A G C G T C
22
180

Figure 15. Map of teniposide-induced *in vitro* topoisomerase II cleavage sites and deletion/insertion mutations at exon 3 of the *aprt* gene. Open and solid triangles indicate weak and strong cleavage sites that were arbitrarily defined. Continuous and broken lines represent deletions and duplications, respectively. Since short direct repeats appear at nearly all of the deletion and duplication termini and one of the repeated sequences is retained, the endpoints are indeterminate within the repeats and both of them are shown with extended lines.

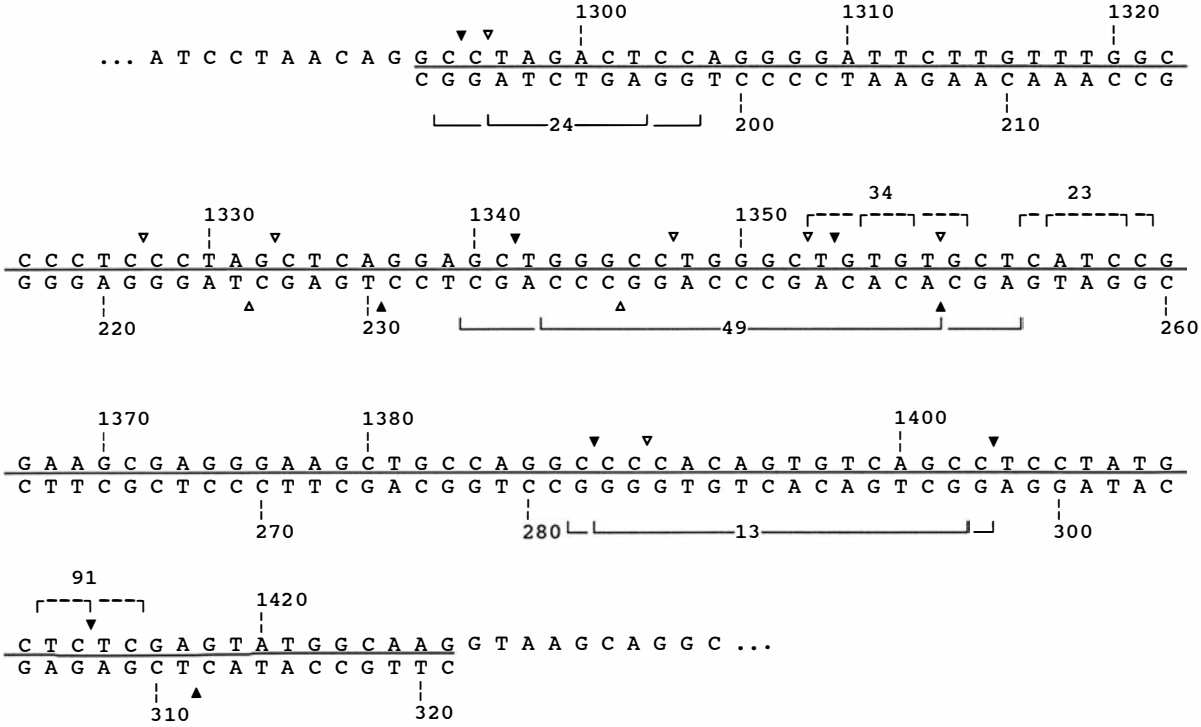

1300
1310
1320
... A T C C T A A C A G G C C T A G A C T C C A G G G G A T T C T T G T T T G G C
C G G A T C T G A G G T C C C C T A A G A A C A A A C C G
24
200
210
34
23
1330
1340
1350
C C C T C C C T A G C T C A G G A G C T G G G C C T G G G C T G T G T G C T C A T C C G
G G G A G G G A T C G A G T C C T C G A C C C G G A C C C G A C A C A C G A G T A G G C
220
230
49
260
1370
1380
1400
G A A G C G A G G G A A G C T G C C A G G C C C C A C A G T G T C A G C C T C C T A T G
C T T C G C T C C C T T C G A C G G T C C G G G G G T G T C A C A G T C G G A G G A T A C
270
280
13
300
91
1420
C T C T C G A G T A T G G C A A G G T A A G C A G G C ...
G A G A G C T C A T A C C G T T C
310
320

Figure 16. Map of teniposide-induced *in vitro* topoisomerase II cleavage sites and deletion/insertion mutations at exon 4 of the *aprt* gene. Open and solid triangles indicate weak and strong cleavage sites that were arbitrarily defined. Continuous and broken lines represent deletions and duplications, respectively. Since short direct repeats appear at nearly all of the deletion and duplication termini, and one of the repeated sequences is retained, the endpoints are indeterminate within the repeats and both of them are shown with extended lines.

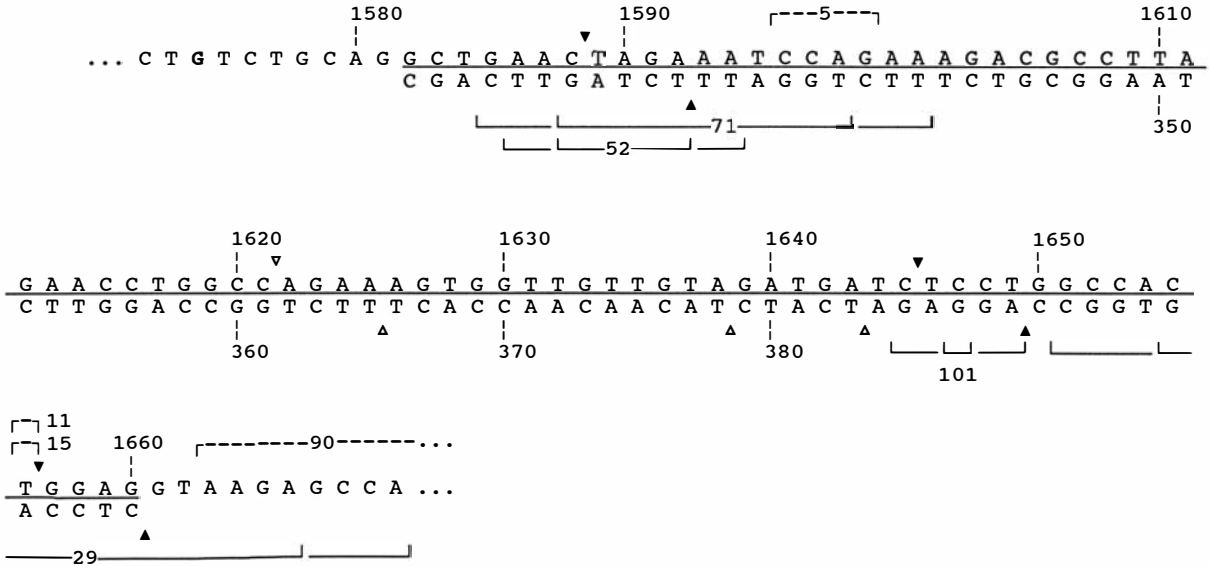
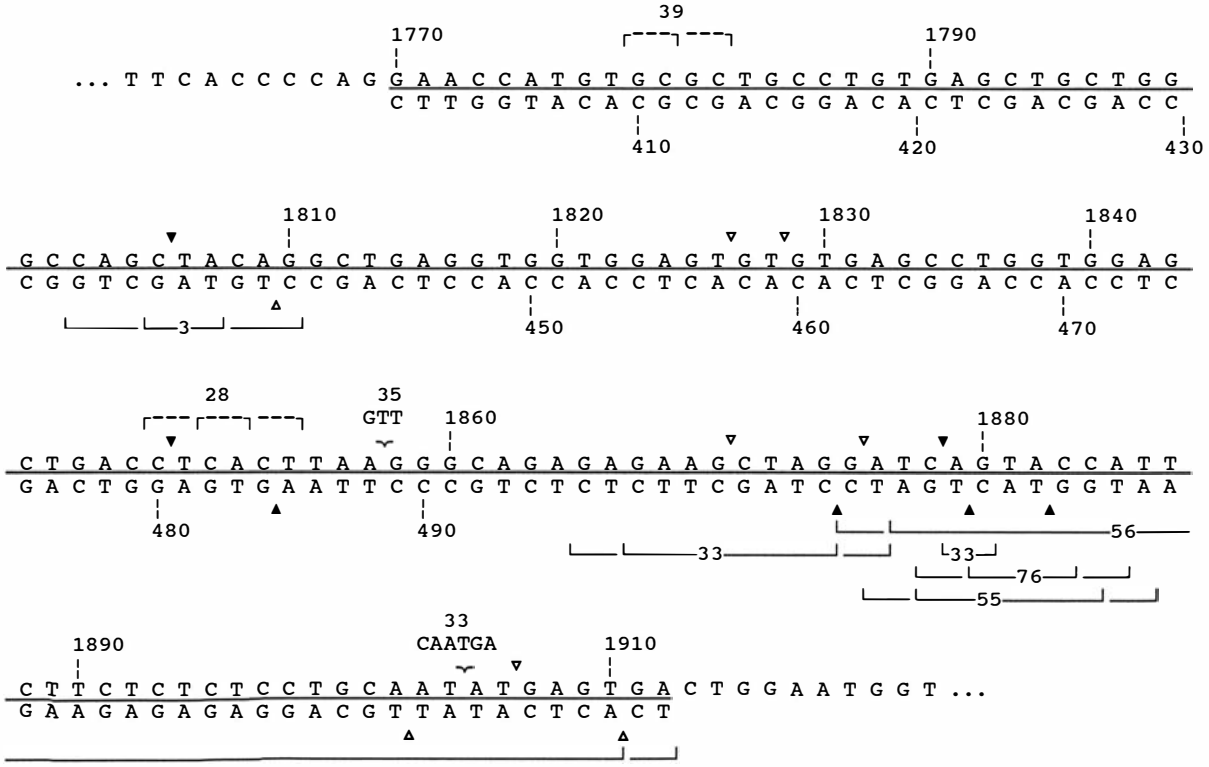

1580
1590
5
1610
... C T G T C T G C A G G C T G A A C T A G A A A T C C A G A A A G A C G C C T T A
C G A C T T G A T C T T T A G G T C T T T C T G C G G A A T
71
52
350
1620
1630
1640
1650
G A A C C T G G C C A G A A A G T G G T T G T T G T A G A T G A T C T C C T G G C C A C
C T T G G A C C G G T C T T T C A C C A A C A A C A T C T A C T A G A G G A C C G G T G
360
370
380
101
11
15
1660
90
T G G A G G T A A G A G C C A ...
A C C T C
29

Figure 17. Map of teniposide-induced *in vitro* topoisomerase II cleavage sites and deletion/insertion mutations at exon 5 of the *aprt* gene. Open and solid triangles indicate weak and strong cleavage sites that were arbitrarily defined. Continuous and broken lines represent deletions and duplications, respectively. Since short direct repeats appear at nearly all of the deletion and duplication termini, and one of the repeated sequences is retained, the endpoints are indeterminate within the repeats and both of them are shown with extended lines.


39
1770
1790
... T T C A C C C C A G G A A C C A T G T G C G C T G C C T G T G A G C T G C T G G
C T T G G T A C A C G C G A C G G A C A C T C G A C G A C C
410
420
430
1810
1820
1830
1840
G C C A G C T A C A G G C T G A G G T G G T G G A G T G T G T G A G C C T G G T G G A G
C G G T C G A T G T C C G A C T C C A C C A C C T C A C A C A C T C G G A C C A C C T C
3
450
460
470
28
35
GTT
1860
1880
C T G A C C T C A C T T A A G G G C A G A G A G A A G C T A G G A T C A G T A C C A T T
G A C T G G A G T G A A T T C C C G T C T C T C T T C G A T C C T A G T C A T G G T A A
480
490
56
33
33
76
55
33
CAATGA
1890
1910
C T T C T C T C T C C T G C A A T A T G A G T G A C T G G A A T G G T ...
G A A G A G A G A G G A C G T T A T A C T C A C T

first 20 nucleotides at the lower strand in exon 2, the first 14 nucleotides at the lower strand in exon 3 and the last 14 nucleotides at the upper strand in exon 5) where the background is too high to show DNA cleavage sites. As a result, mutations within these areas (mutants 1t, 24t, 56t) can not be analyzed with certainty.

In order to determine whether sequence co-specificity between *in vitro* cleavage sites and putative initial DNA lesions is present in this study, teniposide-induced deletions and duplications were also illustrated in Figures 14, 15, 16 and 17 by continuous and broken lines, respectively. Since many of the deletions and duplications have a short direct repeats at the ends, it was impossible to decide at which base pair a deletion or duplication began. So, extended lines were drawn to include both of the repeated sequences.

Some of the mutations were clearly associated with *in vitro* cleavage sites. However, the significance of such a relationship was not exclusive and needed to be distinguished from possible random coincidence. Two different analyses were done to fulfill this task. The basic assumptions behind these analyses were that teniposide mediated DNA strand breaks were the initial lesions and that the *in vitro* cleavage sites, to some extent, represent the DNA breakage points in the cells.

The first approach emphasized on the relative association of the teniposide-induced mutations within the strong and weak sites. As was described earlier, the *in vitro* cleavage sites were arbitrarily classified into 31 strong sites and 25 weak

sites. The number of strong or weak sites within and at the end(s) of each mutation sequences (including both repeated sequences) were summed and divided by the total number of base pairs of all the mutations (including both repeated sequences). The random background was determined by dividing the total numbers of strong or weak sites by the total number of base pairs in exons 2, 3, 4 and 5. The non-random background was determined by dividing the number of the strong or weak sites by the total number of base pairs in the mutation-free sequences.

Table 11 shows that the association between strong sites and the mutant sequences is more than 2 times greater than the random background and 17 times greater than the non-random background. On the other hand, the association between the weak sites and the mutant sequences is not significantly different from either of the backgrounds.

The second analysis is to compare the mutant association with the strong cleavage sites between teniposide-induced and spontaneous mutations. As is shown in Table 12, 82 % of the teniposide-induced mutants, while only 22 % of the spontaneous mutants that fell into exons 2, 3, 4 and 5 contain strong cleavage sites. Five of the 9 spontaneous deletion/duplication mutations are clustered in a region of exon 5 (Figure 18) where no double cleavage sites are observed.

Table 11. Association Between in vitro Strong and Weak Cleavage Sites and the Deletion/Duplication Sequences*

| | Strong Site | Weak Site |
|---|---|---|
| Mutant Sequence: | 0.160 | 0.072 |
| Total Coding Sequence: | 0.067 | 0.054 |
| Non-mutant Sequence: | 0.009 | 0.052 |

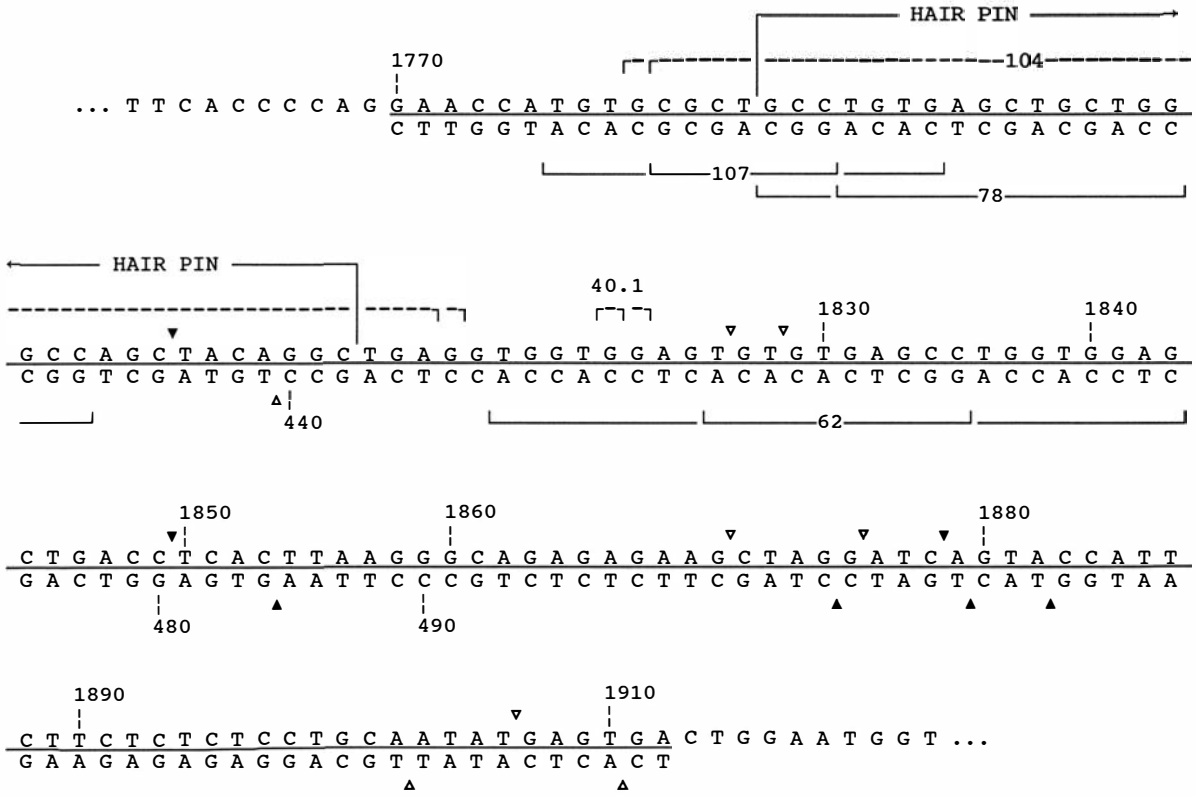
* The *in vitro* cleavage sites were arbitrarily classified into 31 strong sites and 25 weak sites. The association between strong or weak cleavage sites and the mutant sequences were calculated by dividing the total number of strong or weak sites within and/or at the end(s) of the deleted and duplicated sequences (49 strong sites and 22 weak sites) by the total number of base pairs in these sequences including repeats at the termini (306 base pairs). The random background was calculated by dividing the total number of strong or weak sites (31 strong sites and 25 weak sites) by the total number of base pairs in exons 2, 3, 4 and 5 (463 base pairs). The non-random background was calculated by dividing the total number of strong or weak sites within the non-mutant sequences (2 strong sites and 11 weak sites) by the total number of base pairs in these sequences (212 base pairs).

Table 12. Comparison of Association with Strong in vitro Cleavage Sites Between Teniposide-Induced and Spontaneous Mutant Sequences*

| | Teniposide-Induced | Spontaneous |
|---|---|---|
| Total mutations: | 28 | 9 |
| Mutations associated with cleavage site: | 23 | 2 |
| | 82 % | 22 % |

* Twenty-eight teniposide-induced and 9 Spontaneous deletion and duplication mutations were recovered in exons 2, 3, 4 and 5. Twenty-three teniposide-induced, but only 2 spontaneous deletions/duplications are associated with strong *in vitro* cleavage site(s).

Figure 18. A cluster of spontaneous deletions and duplications at exon 5 of the *aprt* gene. Continuous and broken lines represent deletions and duplications, respectively. Open and solid triangles indicate topoisomerase II mediated weak and strong cleavage sites that were arbitrarily defined. All of the deletions and duplications are framed by a short direct repeats and one of the repeated sequences is retained. Since the endpoints are indeterminate within the repeats, both of them are shown with extended lines. Region of a putative hair pin structure is also shown on the map.


HAIR PIN
1770
104
... T T C A C C C C A G G A A C C A T G T G C G C T G C C T G T G A G C T G C T G G
C T T G G T A C A C G C G A C G G A C A C T C G A C G A C C
107
78
HAIR PIN
40.1
1830
1840
G C C A G C T A C A G G C T G A G G T G G T G G A G T G T G T G A G C C T G G T G G A G
C G G T C G A T G T C C G A C T C C A C C A C C T C A C A C A C T C G G A C C A C C T C
440
62
1850
1860
1880
C T G A C C T C A C T T A A G G G C A G A G A G A A G C T A G G A T C A G T A C C A T T
G A C T G G A G T G A A T T C C C G T C T C T C T T C G A T C C T A G T C A T G G T A A
480
490
1890
1910
C T T C T C T C T C C T G C A A T A T G A G T G A C T G G A A T G G T ...
G A A G A G A G A G G A C G T T A T A C T C A C T

# DISCUSSION

## Mutability of the *aprt* locus by teniposide

Teniposide mutagenesis has been studied at the *tk* locus in mouse lymphoma cells (DeMarini et al., 1987), the *hgprt* locus in CHO cells (Singh and Gupta, 1983) and the *aprt* locus in CHO-D422 cells. The mutagenic responses at these loci to teniposide are different by more than two orders of magnitude. The maximum mutation frequency at the *aprt* locus is about 10 times lower than that at the *hgprt* locus and about 300 times lower than that at the *tk* locus. Different mutagenic responses at different genetic loci to strong clastogens has been noticed previously (DeMarini et al., 1987). It had also been observed that in the mouse lymphoma cells, the majority of the $tk^-$ colonies formed following treatment of a strong clastogen such as m-AMSA were small-size colonies (Moor et al., 1985). The frequency of the large $tk^-$ colonies was comparable to the mutation frequency at the *hgprt* locus (Pommier et al., 1985). It was proposed that large colony *tk* mutants represent single gene mutations which is probably also the type of mutation recovered at the *hgprt* locus following treatment of a strong clastogen. Small colony *tk* mutants, on the other hand, appear

to result from chromosomal events in which some of the surrounding genes that are essential for cell growth might have been affected (Hozier et al., 1985). The heterozygous nature of the *tk* locus enabled the recovery of small colony mutants since loss of the essential genes due to the large scale alterations could be partially compensated by functional alleles on the other copy of chromosome 11 (which is the chromosome bearing the *tk* gene) resulting in viable but slowly growing cells. However, for the hemizygous *hgprt* locus, loss of adjacent essential genes will lead to cell death. This hypothesis was strongly supported by the observation that in a mouse lymphoma cell line in which one of the two chromosome 11 homologues was lost, m-AMSA mutation frequency was reduced to the level that was observed at the *hgprt* locus (Evans and Mencl, 1986).

The same rationale appears to dictate the mutability of the *aprt* gene in CHO-D422 cells as well. Based on Southern blot analysis, this cell line carries a deletion of one copy of the *aprt* gene, rendering it hemizygous at this locus (Nalbantoglu, et al., 1983). This is presumably the main reason that would explain the low teniposide mutation frequency at the *aprt* locus. Such results also supported the idea that teniposide is a strong clastogen.

The *hgprt* gene is 44 kb which is about 20-fold larger than the *aprt* gene. This is probably one of the factors, if not the only factor, which caused the 10-fold difference in teniposide-induced mutation frequencies between these loci.

Due to the low level of *aprt* mutation frequency, the recovery of *aprt*$^-$ mutants tends to fluctuate more between replicate culture samples. Cultures with no mutant recovery are not uncommonly seen, which resulted in larger standard errors in mutagenesis data. On the other hand, cultures with very high mutant recovery (for example, mutation frequencies around 50 x $10^{-6}$) were occasionally observed, and such data were excluded from the data pool since these mutants were likely to be the progeny of preexisting *aprt*$^-$ cells which either were present in the initial inoculum or arose spontaneously shortly thereafter.

The most valuable merit of this system is that the hemizygosity and small size of the locus enabled us to sequence the entire coding region of the gene, so that the mutations could be revealed at the sequence level. The disadvantage of using CHO/*aprt* system to study teniposide mutagenesis is that the majority of the mutants recovered at this locus may not be the types of mutations induced by teniposide at other genetic loci, most of which are more similar to *hgprt* mutants in size. Only 10 % of the mutations recovered in this study were large scale alterations. However, selective recovery of certain types of mutations is almost inevitable in any mutagenesis system. This study opened another window for investigation of teniposide mutagenesis.

## Characteristics of Teniposide-Induced Mutations at the *aprt* Locus

Compared to the spontaneous mutation spectrum, teniposide induced more small deletions (29.4 % vs. 21.4 %), insertions (23.5 % vs. 7.1 %) and fewer base substitutions (36.7 % vs. 64.3 %). Moreover, 10 % of the teniposide-induced mutations appear to be large deletions and rearrangements while no such mutants were formed spontaneously.

Small deletions induced by teniposide are similar to spontaneous deletions in terms of size and the presence of short direct repeats at the mutation termini. A significant increase in the number of insertions was observed in teniposide-induced mutations. Eleven of the 16 insertions are duplications and eight of them are 4 base pairs or less. Five medium to large-sized insertions (41-330 bp) were found and two of them are duplications. Short direct repeats are also involved in most of the insertions.

Increased frequency of small deletions and insertions in the presence of teniposide raised the possibility that topoisomerase II may play a role in the formation of spontaneous deletions and insertions, since the clastogenic effect of teniposide is mediated by this enzyme. Furthermore, the involvement of short direct repeats in both spontaneous and teniposide-induced small deletions and insertions tempted the thoughts that these repeated sequences might serve as some sort of recognition signal for topoisomerase II binding to DNA. The majority of the spontaneous deletions and insertions that fell into exons 2, 3, 4 and 5 do not associate with strong *in vitro* cleavage sites (Figure 18), which is in sharp

contrast to the drug-induced small deletions and insertions. However, this result still does not address the question of whether the increased frequencies of small deletion and insertions in the presence of teniposide is due to the enhancement of a preexisting mechanism for the formation of spontaneous deletions and insertions, since the drug-stimulated cleavage sites may not be the same as sites of topoisomerase-mediated cleavage in the untreated cells.

Despite the unfavorable selection against large deletions and rearrangements in CHO/*aprt* system, several mutants of this type were recovered. Since no such mutants were found in the spontaneous group, it is almost certain that this type of mutation represents solely teniposide-induced DNA alterations. It would be more valuable if these mutants were analyzed at the sequence level, since they probably reflect more closely the type of DNA damage and repair events which occur in cells following teniposide exposure.

A predominance of G·C → A·T transitions in spontaneous base substitutions was observed previously (Phear et al., 1989; de Jong et al., 1988) and is also true in this study. But this type of base substitution is much less prevalent among teniposide-induced substitutions. Instead, transversions occurred more frequently than transitions. This may be due to the observed shift of frequency from G·C → A·T to G·C → C·G and G·C → T·A in the presence of teniposide. The prevalence of G·C → A·T transitions in spontaneous base substitution was suggested to reflect cytosine deamination (de Jong et al.,

1988). Enhancement of other types of base changes may be responsible for the shift of the base substitution spectrum.

The distribution of teniposide-induced base substitutions is not noticeably different from that of spontaneous base substitutions. In fact, several of the drug-induced substitutions fell at the same base pairs that were found in the spontaneous group. Teniposide-induced base substitutions did not appear to occur preferentially at sites of drug-stimulated cleavage, and the mechanism of their formation is unknown. One possibility is drug-induced interference with repair of endogenous DNA base lesions. Most of the drug-induced base substitutions targeted amino acids that are also targeted by spontaneous base substitutions. However, several amino acids alterations that have never been reported before were found in both the spontaneous and the teniposide-induced group.

## Correspondence Between *in vitro* Cleavage Sites and the Teniposide-Induced Mutant Sequences

By means of the two analyses shown in Tables 11 and 12, the relationship between teniposide-induced mutations and the *in vitro* cleavage sites was expressed in a quantitative way and a significant correspondence was indicated. The association between strong cleavage sites and the mutant sequences is more than 2 times greater than the random background and 17 times greater than the non-random background

(Table 11). It suggests that the strong sites are more likely to be the initial sites of DNA damage, whereas the weak sites may only play a minor role, if any, in the formation of the mutations. Twenty-three of the 28 teniposide-induced deletion/duplication mutations were found to associate with strong *in vitro* cleavage site(s) (Table 12) and most of these sites are 4 base staggered double strand cleavage sites.

These data imply that the *in vitro* cleavage sites may, to some extent, represent the sites of initial DNA lesions occurring in the cells following teniposide exposure, and a significant portion of teniposide-induced small deletions and duplications may have resulted from these DNA double strand breaks that were mediated by topoisomerase II. A previous study which was done by Ripley et al (1988) is clearly in favor of this presumption. In their work, frameshift hotspot sites for acridine-induced mutations in bacteriophage T4 were found to correspond to two very strong *in vitro* topoisomerase II cleavage sites. This observation not only implicated the acridine-induced, topoisomerase II mediated DNA cleavages as being responsible for the acridine-induced frameshift mutations, but also demonstrated that the topoisomerase II mediated *in vivo* cleavages were able to be reproduced in a cell free system with the same sequence specificity.

However, recent observations have indicated that the pattern of topoisomerase II mediated DNA cleavage in naked DNA is substantially different from that shown to occur in the *drosophila* KC cells (Udvardy and Schedl, 1991). Specifically,

cleavage sites within nucleosome core particles are suppressed while those in the internucleosome linker or in other nucleosome free areas are enhanced. As a result, the observed relationship between teniposide-induced mutations and *in vitro* cleavage sites may only partially reflect what had happened in the cells.

## Models for Possible Cellular Mutational Events

The topoisomerase II mediated DNA double strand break has been characterized as two single strand cleavages on opposite strands, separated by four base pairs, resulting in a 5'-overhang (Muller et al., 1988). In this study, we observed 31 strong *in vitro* cleavage sites in exons 2, 3, 4 and 5, among which 22 sites are positioned in such a way that double strand cleavage can occur. All of the 11 putative double cleavage sites are associated with at least one teniposide-induced deletion/duplication mutation. Two of these sites are each associated with four deletions and duplications. This unusual observation strongly suggests that topoisomerase II mediated DNA strand breaks, especially the double strand breaks, were the initial lesions which resulted in teniposide-induced mutations. This presumption enables us to try to address the question as how these mutations might be produced, which could lead to a better understanding of the mechanisms of DNA double strand break repair.

The formation of the mutations presumably occurred during

certain genetic activities such as replication, transcription and chromosomal segregation when topoisomerase II is required. The mutations are probably either the outcomes of incorrect DNA strand break repair or replication errors due to the interference with normal DNA synthesis by the drug-stabilized DNA-topoisomerase II complexes. By examining the positions of the double cleavage sites in the mutant sequences, no consistent pattern was found which could explain the formation of all the mutations. However, several models are presented below, each of which describes possible events occurring in the cells following teniposide exposure which could have resulted in observed mutations.

(1) Ripley's Model:

Ripley et al (1988) proposed that a topoisomerase II mediated DNA strand break would expose a free 3' hydroxyl end which could be acted on by two different kind of enzymes: a 3'-exonuclease could trim off a few nucleotides and covalent rejoining of the ends could form a deletion; DNA polymerase could fill in the single stranded 5' overhang and subsequent rejoining of the ends could form a duplication. Five mutants in this study (mutants 34t, 91t, 28t, 22t, 83t) could be explained by this model (Figure 19).

(2) Nonhomologous Recombination Model:

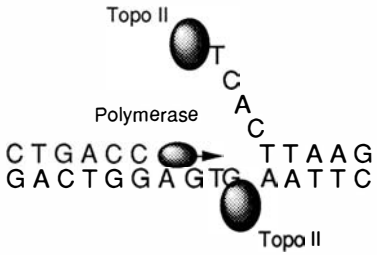
Nonhomologous recombination has been described by many investigators as an commonly seen mechanism of double strand

Figure 19. Ripley's Model. 1. *In vitro* teniposide-stimulated, topoisomerase II-mediated double cleavage site. 2. A strand displacement reaction is catalyzed by DNA polymerase. 3. A duplication is formed by rejoining of the ends.

# Ripley's Model

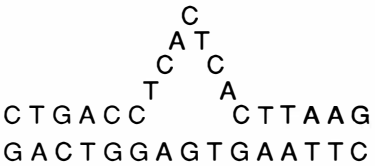
1.


2.





3.



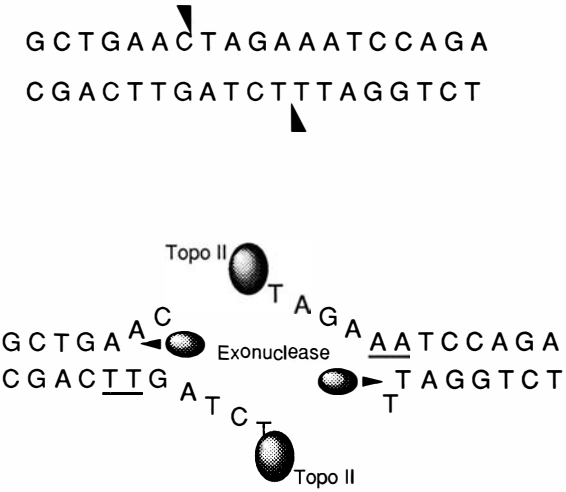

Mutant 28 t

break repair in mammalian cells. When linear DNA molecules with different ends were introduced into monkey cells, the ends were joined with limited sequence modifications despite the fact that little or no homology was present (Roth and Wilson, 1986). Figure 20 illustrates that one or both ends of a double strand break could be processed by exonucleases until some sequence homology is exposed. The cohesive ends are then rejoined and the gaps are filled. The observed short direct repeats which appear at nearly all of the deletion termini may be the outcome of this sequence homology searching process. But whether or not this short sequence homology is required for this type of double strand break repair or if there is some other mechanism involved is not clear at this point. Twelve mutants (73t, 49t, 71t, 52t, 101t, 29t, 56t, 76t, 55t, 96t, 3t and 22t) could be explained by this model. Among them, mutant 22t could also be explained by Ripley's model. Mutant 24t is located in the region where no lower strand cleavage sites could be recognized. The fact that one strong cleavage site is present at the upper strand makes it possible that mutant 24t could also be explained by this model. The most attractive feature of the nonhomologous recombination model is that it can account for both the predominance of strong cleavage sites within the deleted sequences, and the lack of any consistent positioning of these cleavage sites with respect to the deletion termini.

(3) Replication Blockage and Slippage Model:

Figure 20. Nonhomologous Recombination Model. 1. *In vitro* double cleavage sites. 2. Both 3' ends are acted on by exonucleases to expose short sequence homology (underlined sequence). 3. The ends are rejoined to form a deletion.

# Nonhomologous Recombination Model




Mutant 52 t

Four of the 11 duplications (1t, 37t, 23t, 39t) are not associated with *in vitro* cleavage sites in the way that the formation of these mutants could be explained by Ripley's model. These duplications are located at various distances from double cleavage sites. Short direct repeats are also present at the termini of these duplications and one of the repeated sequences is duplicated. These mutations could be DNA replication errors. Figure 21 illustrates that when the DNA replication complex is progressing along the leading strand and reaches the drug-stabilized topoisomerase II intermediate, the simple physical blockage may cause DNA polymerase to slip back. The short sequence of a direct repeat in the newly synthesized region could base pair with complimentary sequence at the opposite strand while the rest of it loops out. When the blockage is released, this part of the sequence is synthesized again and a duplication is formed.

(4) Subunit Exchange Model:

This model was first suggested by Ikeda (1986) for a unique kind of DNA gyrase mediated recombination. It was then generalized as illustrated in Figure 22: DNA topoisomerase II molecules bind to DNAs to form complexes, two such complexes associate with each other and lead to the exchange of DNA strands through the exchange of topoisomerase II subunits. But none of the mutants found in this study could be explained by this model.

Among all 17 deletions and 11 duplications examined, 8

Figure 21. Blockage and Slippage Model. 1. DNA replication Complex is progressing along the leading strand and reaches the drug stabilized topoisomerase II intermediate. 2. The physical blockage causes the replication complex to slip back and the newly synthesized sequence loops out. 3. A duplication is formed.

# Blockage and Slippage Model

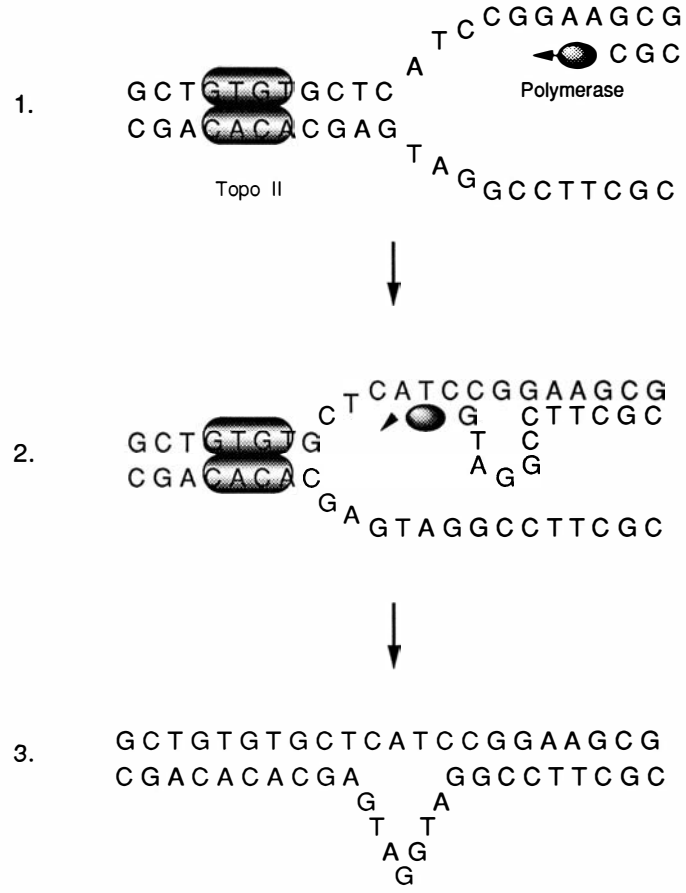
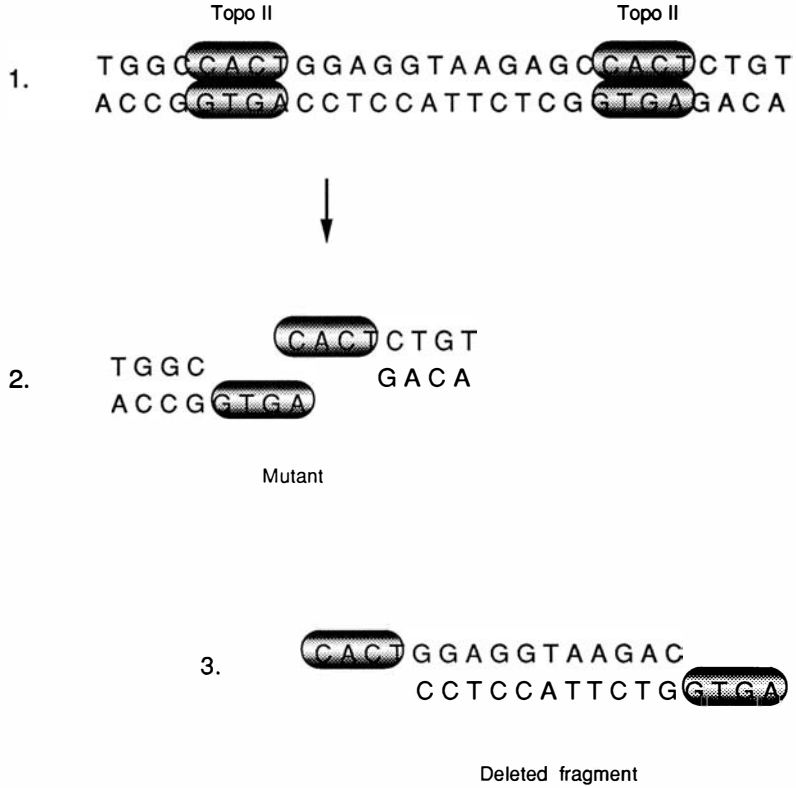


Figure 22. Subunit Exchange Model. 1. DNA-topoisomerase II complexes. 2. Two complexes associate with each other and lead to the exchange of DNA strands through the exchange of topoisomerase II subunits. 3. A deletion is formed.

# Subunit Exchange Model



mutants (38t, 5t, 11t, 15t, 13t, 33t, 90t) do not seem to fit any of the above models. At this point, there is no good explanation as how these mutations might be formed. Mutants 38t, 11t and 15t are single base pair deletion or duplications which may not be induced by teniposide, since these types of mutations could also be formed spontaneously. Mutant 33 has a complicated alteration in which two deletions and one insertion were found near the base of a putative stem loop structure at exon 5. The deletions cover several direct and inverted repeats which may play a role in the generation of the mutations. Mutant 90t carries multiple duplication/ insertion mutations (Figure 23) which start from the upstream splice junction and extend into intron 4. Such a complex form of mutation has never been reported before and may reflect a unique characteristic of the repair of topoisomerase II mediated double strand breaks.

This study provided little clue for the cause of DNA damages resulting in spontaneous deletions and duplications. Five of the spontaneous deletions and duplications are clustered around a putative hair pin structure in exon 5 (Figure 18). This is consistent with what was reported previously by other investigators that spontaneous deletions occur more frequently around hair pin structures (Phear et al., 1989). Although the cause of initial DNA damage might be distinct, the presence of short direct repeats in both spontaneous and teniposide-induced mutations suggests a similar mechanism of mutant formation.

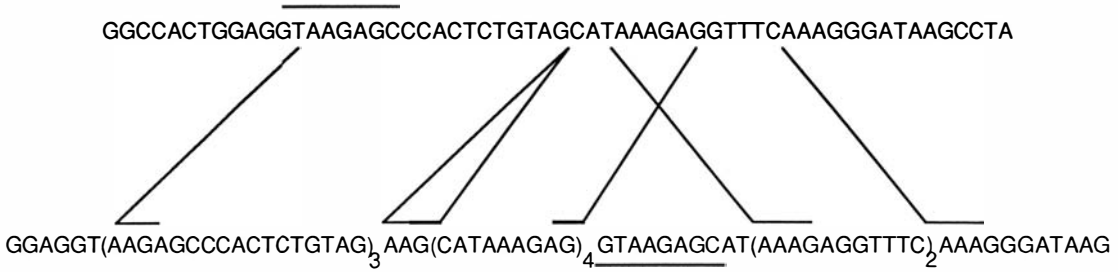



Figure 23. Sequence analysis of mutant 90t. The duplicated DNA segments are in parentheses. The short horizontal lines show the terminal repeats.

## Implications for Clinical Cancer Chemotherapy

Teniposide is currently used in the clinic as an effective cancer chemotherapeutic agent. However, a unique type of acute monocytic leukemia has been identified in recent years among long term cancer survivors who were treated with epipodophyllotoxins or certain other topoisomerase II inhibitors. This type of secondary leukemia are often found to have translocations involving chromosome 11q23. It is likely that certain specific genetic events were induced by the drug, which could trigger the process of transformation. However, most of the genetic events in secondary cancers are not known at the present time.

In this study, we analyzed the teniposide-induced mutations at the DNA sequence level in a mammalian mutagenesis model system. The observed evidence suggests that the majority of teniposide-induced mutations are probably resulted from cleavable complex, the DNA lesion which presumably cause cell death. Therefore no immediate hope of separating the two effects in one drug has emerged from this study.

However, the work does show that teniposide can induce mutations other than large deletions. As the genetic events in secondary cancers are discovered, such as which type of mutation is important in the development of secondary cancers, our results comprise a useful data base for comparison to determine whether teniposide was directly involved.

The exact relation between cleavable complex formation

and either cell death or mutagenesis is still not certain, nor is it known why some cleavable complexes produce actual chromosome breaks in the cell, or whether this breakage is important in cell death. Thus, it is possible that cleavable complexes with certain drugs may be less likely to produce frank DNA double strand breaks than those with other drugs. Whether this would increase or decrease mutagenesis relative to cell kill cannot be determined until mechanisms of both cell death and mutagenesis by these drugs are elucidated in more detail. The present study represents a small but essential step toward that goal.

## Future Studies

The present study has provided suggestive evidence for certain models to explain the mutagenic events occurring in the cells. But the evidence is still far from proving the validity of these models. Additional studies could be done in the future to try to address the remaining questions. (1) Since the intercalative topoisomerase II inhibitor m-AMSA has different and stronger sequence specificity, this drug could be used to induce *aprt*$^-$ mutants and stimulate the *in vitro* DNA cleavage to see if mutations also associate with strong sites. (2) Some of the models proposed above require DNA replication and some of them do not. It would be interesting to obtain mutants developed from stationary phase cells to see which types of mutants require DNA replication. For example, the

nonhomologous recombination model would not require replication. (3) The large deletions and insertions could be sequenced in the future to determine whether they are qualitatively similar to other mutants but simply involve larger segments of DNA. (4) To verify that if we are looking at double strand break repair events, a defined DNA substrate with a single topoisomerase II-linked break at a specific site could be made to determine how these breaks are processed in transfection assays or by nuclear extracts. This study could eventually lead to the elucidation of the details of double strand break repair mechanisms including isolation of enzymes involved.

# REFERENCES

## REFERENCES

Albain, K. S., Le Beau, M. M., Ullirsch, R., Schumacher, H. (1990) Implication of prior treatment with drug combinations including inhibitors of topoisomerase II in therapy-related monocytic leukemia with a 9;11 translocation. Genes, Chromosomes & Cancer 2:53-8

Allen, L. M. (1978a) Cellular pharmacokinetics of two epipodophyllotoxin glucopyranoside derivatives, VM-26 and VP-16: uptake in the presence and absence of human serum albumin. Proceedings of the American Association of Cancer Research 19:125

Allen, L. M. (1978b) The role of drug disposition kinetics on cellular transport of the anti-neoplastic agent VM-26. Drug Metabolism Reviews 8:119-35

Allen, L. M., Creaven, P. J. (1975) Comparison of the human pharmacokinetics of VM-26 and VP-16, two antineoplastic epipodophyllotoxin glucopyranoside derivatives. European Journal of Cancer 11:697-707

Allen, L. M., Shirley, M. (1977) Mechanism of cellular transport of two epipodophyllotoxin glucopyranoside derivatives, VM-26 and VP-16. Proceedings of the American Association of Cancer Research 18:22

Allen, L. M., Tejada, F., Okonmah, A., Nordqvist, S. (1982) Combination chemotherapy of the epipodophyllotoxin derivatives teniposide and etoposide. Cancer Chemotherapy and Pharmacology 7:151-6

Arnold, A. M. (1979) Podophyllotoxin derivative VP-16-213. Cancer Chemotherapy and Pharmacology 3:71-80

Ayres, D. C., Lim, C. R. (1982) Modification of the pendant ring of podophyllotoxin. Cancer Chemotherapy and Pharmacology 7:99-101

Bae, Y.-S., Kawasaki, I., Ikeda, H., Liu, L. F. (1988) Illegitimate recombination mediated by calf thymus DNA topoisomerase II in vitro. Proc Natl. Acad. Sci. USA 85:2076-80

Bailey-Wood, R., Dallimore, C. M., Littlewood, T. J., Bentley,

D. P. (1985) The effect of etoposide on human CFU-GM. British Journal of Cancer 52:613-7

Bertrand, R., Kohn, K. W., Pommier, Y. (1990) Calcium is required for the cytotoxicity of topoisomerase II-mediated DNA breaks induced by etoposide (VP-16) in Chinese hamster DC3F cells. Proc. Am. Assoc. Cancer Res. 31:435

Bodley, A., Wu, H.-Y., Liu, L. F. (1987) Regulation of DNA topoisomerases during cellular differentiation. NCI Monogr. 4:31-35

Bradley, W. E. C., Letovanec, D. (1982) High-frequency nonrandom mutational event at the adenine phosphoribosyltransferase (*aprt*) locus of Sib-selected CHO variants heterozygous for *aprt*. Somatic Cell Genetics 8:51-66

Breimer, L. H., Nalbantoglu, J., Meuth, M. (1986) Structure and sequence of Mutations induced by Ionizing Radiation at selectable losi in Chinese hamster ovary cells. J. Mol. Biol. 192:669-74

Budman, D. R., Shulman, P., Vinciguerra, L. et al. (1982) Anaphylactoid reaction to AZQ (letter). Cancer chemother. Pharmacol. 8:317

Capranico, G., Kohn, K. W., Pommier, Y. (1990) Local sequence requirements for DNA cleavage by mammalian topoisomerase II in the presence of doxorubicin. Nucleic Acid Research 18:6611-9

Clark, P. I., Slevin, M. L., (1987) The clinical pharmacology of etoposide and teniposide. Clinical Pharmacokinetics 12:223-52

Cornman, I., Cornman, M. E. (1951) The action of podophyllotoxin and its fractions on marine eggs. Annals of the New York Academy of Sciences 51:1443

Creaven, P. J., Allen, L. M. (1975) PTG, a new anti-neoplastic epipodophyllotoxin. Clinical Pharmacology and Therapeutics 18:227-33

D'arpa, P., Liu, L. F. (1989) Topoisomerase-targeting antitumor drugs. Biochimica et Biophysica Acta 989:163-77

DeBoer, J. G., Drobetsky, E. A., Grosovsky, A. J., Mazur, M., Glickman, B. W. (1989) The Chinese hamster *aprt* gene as a mutational target. Its sequence and an analysis of direct and inverted repeats. Mutation Research 226:239-44

DeJong, P. J., Grosovsky, A. J., Glickman, B. W. (1988) Spectrum of spontaneous mutation at the *APRT* locus of Chinese hamster ovary cells: an analysis at the DNA sequence level.

Proc. Natl. Acad. Sci. USA 85:3499-503

DeMarini, D. M., Brock, K. H., Doerr, C. L., Moore, M. M. (1987) Mutagenicity and clastogenicity of teniposide (VM-26) in L5178Y/TK+-3.7.2C mouse lymphoma cells. Mutat. Res. 187:141-49

DeVore, R., Whitlock, J., Hainsworth, J. D., Hohnson, D. H. (1989) Therapy-related acute nonlymphocytic leukemia with monocytic features and rearrangement of chromosome 11q. Ann. Intern. Med. 110:740-42

D'Incalci, M., Rossi, C., Urso, R., Zuchetti, M., Farina, P., et al. (1985) Pharmacokinetics of teniposide in patients with ovarian cancer. Cancer Treatment Reports 69:73-7

Drewinko, B., Barlogie, B. (1976) Survival and cycle-progression delay of human lymphoma cells in vitro exposed to VP-16-213. Cancer Treatment Reports 60:1295

Dye, D., Watkins, J. (1980) Suspected anaphylactic reaction to cremophor EL. Br. Med. J. 280:1353

Earnshaw, W. C., Halligan, B. D., Cooke, C. A., Heck, M. M. S., Liu, L. F. (1985) Topoisomerase II is a structural component of mitotic chromosome scaffolds. J. Cell. Biol. 100:1706-15

Evans, H. H., Mencl, J. (1986) Low mutagenicity of clastogens at the thymidine kinase locus in mouse L5178Y cells monosomic for chromosome 11. Environ. Mutagen. 8:27

Evans, W. E., Sinkule, J. A., Crom, W. R., Dow, L., Look, A. T. et al. (1982) Pharmacokinetics of teniposide (VM-26) and etoposide (VP-16-213) in children with cancer. Cancer Chemotherapy and Pharmacology 7:147-50

Fourth International Workshop on chromosome in leukemia, 1982 (1984) Secondary leukemias associated with neoplasia: Treated and untreated. Cancer Genet. Cytogenet. 11:319-21

Gellert, M. (1981) DNA topoisomerases. Annu. Rev. Biochem. 50:879-910

Gilette, J. R. (1973) Overview of drug protein binding. Annals of the New York Academy of Sciences 226:6-17

Gupta, R. S., Bromke, A., Bryant, D. W., Gupta, R., Singh, B., McCalla, D. R. (1987) Etoposide (VP16) and teniposide (VM-26): novel anticancer drugs, strong mutagenic in mammalian but not prokaryotic test systems. Mutagenesis 2:179-86

Gupta, R. S., Singh, B. (1982) Mutagenic responses of five

independent mutagenic loci in CHO cells to a variety of mutagens: development and characteristics of a mutagen screening system based on selection for multiple drug resistent markers. Mutat. Res. 94:449-66

Habibi, B., Lopez, M., Serdaru, M. et al. (1982) Immune hemolytic anemia and renal failure due to teniposide. N. Engl. J. Med. 306:1091-3

Hayes, F. A., Abromowitch, M., Green, A. A. (1985) Allergic reactions to teniposide in patients with neuroblastoma and lymphoid malignancies. Cancer Treatment Report 69:439-41

Holthuis, J. J. M. (1988) Etoposide and teniposide: Bioanalysis, metabolism and clinical pharmacokinetics Pharmaceutisch Weekblad Scientific Edition 10:101-5

Holthuis, J., de Vries, E., Postmus, P., Verleun, H., Sleijfer, D., et al. (1986) Pharmacokinetics of high dose teniposide (VM-26). Proceedings of the American Association of Cancer Research 27:177

Hozier, J., Sawyer, J., Clive, D., Moore, M. (1985) Chromosome 11 aberrations in small colony L5178Y/TK$^{+/-}$ mutants early in their clonal history. Mutation Res. 147:237-42

Hsiang, Y.-H., Wu, H.-Y., Liu, L. F. (1988) Proliferation-dependent regulation of DNA topoisomerase II in cultured human cells. Cancer Res. 48:3230-35

Huttel, M. S., Schou Olesen, A., Stoffersen, E. (1980) Complement mediated reactions to diazepam with cremophor as solvent. Br. J. Anaesth. 52:77-9

Issell, B. F., Crooke, S. T. (1979) Etoposide (VP-16-213). Cancer Treatment Reviews 6:107-24

Jardine, I., Strife, R. J., Koziowski, J. (1982) Synthesis, 470-MH$_2$ $^1$H NMR spectra, and activity of delactonised derivatives of anticancer drug etoposide. J. Medicinal Chemistry 25:1077-81

Jones, G. E., Sargent, P. A. (1974) Cell 2:43-54

Kalwinsky, D. K., Look, A. T., Ducore, J., et al. (1983) Effects of the epipodophyllotoxin VP-16-213 on cell cycle traverse, DNA synthesis and DNA strand size in cultures of human leukemic lymphoblasts. Cancer Res. 43:1592-7

Kantarjian, H. M., Keating, M. J. (1987) Therapy-related leukemia and myelodysplastic syndrome. Semin. Oncol. 14:435-42

Kaplan, I. W. (1942) Condylomata acuminata. New orleans Med. Surg. J. 94:388-90

Kelleher, J. K. (1977) Tubulin binding affinities of podophyllotoxin and colchicine analogues. Molecular Pharmacology 13:232-41

Keller-Juslen, C., Kuhn, M., Von Wartburg, A. (1971) Synthesis and antimycotic activity of glycosidic lignan derivatives related to podophyllotoxin. J. Med. Chem. 14:936-40

King, L. S., Sullivan, M. (1946) The similarity of the effect of podophyllin and colchicine and their use in the treatment of condylomata acuminata. Science 104:244-5

Krishan, A., Paika, K., Frei, E. III. (1975) cytoflurometric studies on the action of podophyllotoxin and epipodophyllotoxins (VM-26, VP-16-213) on the cell cycle traverse of human lymphoblasts. J. Cell Biol. 66:521-30

Kuhn, M., Keller-Juslen, C., Von Wartburg, A. (1969) Partialsynthese von 4'-demethylepipodophyllotoxin. Helv. Chim. Acta 52:944-7

Kuhn, M., Von Wartburg, A. (1969) Uber ein neues Glykosidierungsverfahren. Helv. Chim. Acta 52:948-55

Le Beau, M. M., Albain, K. S., Larson, R. A., Vardiman, J. W., Davis, E. M., Blough, R. R., Golomb, H. M., Rowley, J. D. (1986) Clinical and cytogenetic correlations in 63 patients with therapy-related myelodysplastic syndromes and acute nonlymphocytic leukemia: Further evidence for characteristic abnormalities of chromosomes no.5 and 7. J. Clin. Oncol. 4:325-45

Lerner, H. J. (1978) Acute myelogenous leukemia in patients receiving chlorambucil as long-term adjuvant chemotherapy for stage II breast cancer. Cancer Treat. Rep. 62:1135-8

Loike, J. D., Horwitz, S. B. (1976) Effects of VP-16-213 on the intracellular degradation of DNA in HeLa Cells. Biochemistry 15:5443-48

Loike, J. D., Horwitz, S. B., Grollman, A. P. (1974) Effects of VP-16-213 on HeLa cells. Pharmacologist 16:209

Long, B. H., Minocha, A. (1983) Inhibition of topoisomerase II by VP-16-213 (etoposide), VM-26 (teniposide), and structural congeners as an explanation for in vivo DNA breakage and cytotoxicity. Proc. Am. Assoc. Cancer Res. 24:321

Long, B. H., Musial, S. T., Brattain, M. G. (1984) Comparison of cytotoxicity and DNA breakage activity of congeners of

podophyllotoxin including VP16-213 and VM26: a quantitative structure-activity relationship. Biochemistry 23:1183-88

Long, B. H., Musial, S. T., Brattain, M. G. (1986) DNA breakage in human lung carcinoma cells and nuclei that are naturally sensitive to etoposide and teniposide. Cancer Research 46:3809-16

Long, B. H., Stringfellow, D. A. (1988) Inhibitors of topoisomerase II: Structure-activity relationships and mechanism of action of podophyllin congeners. Adv. Enzyme Regul. 27:223-56

Lowy, I., Pellicer, A., Jackson, J. F., Sim, G.-K., Silverstein, S., Axel, R. (1980) Isolation of transforming DNA: cloning the hamster aprt gene. Cell 22:817-23

Maraschin, J., Dutrillaux, B., Aurias, A. (1990) Chromosome aberrations induced by etoposide (VP-16) are not random. Int. J. Cancer 46:808-12

Moore, M. M., Clive, D., Howard, B. E., Batson, A. G., Turner, N. T., (1985) In situ analysis of trifluorothymidine-resistant ($TFT^r$) mutants of L5178Y/$^{+/-}$ mouse lymphoma cells. Mutation Res. 151:147-59

Moore, M. M., Doerr, C. L. (1990) Comparison of chromosome aberration frequency and small-colocy TK-deficient mutant frequency in L5178Y/$TK^{+/-}$-3.7.2C mouse lymphoma cells. Mutagenesis 5:609-14

Muggia, F. M., Selawry, O. S., Hansen, H. H. (1971) Clinical studies with a new podophyllotoxin derivative epipodophyllotoxin, 4'demethyl-9-(4,6,O-2-thenylidene-B-D-glucopyranoside) (NSC-122819). Cancer Chemother. Rep. 55:575-81

Muller, M. T., Spitzner, J. R., DiDonato, J. A., Mehta, V. B., Kiniko Tsutsuui, Ken Tsutsui (1988) Single-strand DNA cleavages by eukaryotic topoisomerase II. Biochemistry 27:8369-79

Nalbantoglu, J., Goncalves, O., Meuth, M. (1983) Structure of mutant alleles at the *aprt* locus of Chinese hamster ovary cells. J. Mol. Biol. 167:575-94

Nalbantoglu, J., Phear, G. A., Meuth, M. (1986a) Nucleotide sequence of the hamster adenine phosphoribosyl transferase (*aprt*) gene. Nucleic Acids Res. 14:1014

Nalbantoglu, J., Hartley, D., Phear, G., Tear, G., Meuth, M. (1986b) Spontaneous deletion formation at the *aprt* locus of

hamster cells: the presence of short sequence homologies and dyad symmetries at deletion termini. The EMBO Journal 5:1199-204

Nelson, E. M., Tewey, K. M., Liu, L. F. (1984) Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4'-(9-acridinylamino)-methanesulfon-*m*-anisidide. Proc. Natl. Acad. Sci. USA 81:1361-65

O'Dwyer, P. J., Alonso, M. T., Leyland-Jones, B., Marsoni, S. (1984) Teniposide: A review of 12 years of experience. Cancer Treatment Reports 68:1455-66

O'Dwyer, P. J., Leyland-Jones, B., Alonso, M. T., Marsoni, S., Wittes, R. E. (1985) Etoposide (VP-16-213): current status of an active anticancer drug. New England Journal of Medicine 312:692-700

Phear, G., Armstrong, W., Meuth, M. (1989) Molecular basis of spontaneous mutation at the *aprt* locus of hamster cells. J. Mol. Biol. (1989) 209:577-82

Pommier, Y., Zwelling, L. A., Kao-Shan, C.-S., Whang-Peng, J., Bradley, M. O. (1985) Correlations between intercalator-induced DNA strand breaks and sister chromatid exchanges, mutations, and cytotoxicity in Chinese hamster cells. Cancer Res., 45:3143-9

Pui, C. H., Behm, F. G., Raimondi, S. C., Dodge, M. S., George, S. L., Rivera, G. K., Mirro, J., Kalwinsky, D. K., Dahl, G. V., Murphy, S. B., Crist, W. M., Williams, D. L. (1989) Secondary acute myeloid leukemia in children treated for acute lymphoid leukemia. N. Engl. J. Med. 321:136-42

Rao, P. N. (1980) Mol. Cell Biol. 29:47-57

Radice, P. A., Bunn, Jr. P. A., Ihde, D. C. (1979) Therapeutic trials with VP-16-213 and VM-26: active agents in small cell lung cancer, non-Hodgkin's lymphomas and other malignancies. Cancer Treatment Reports 63:1231-9

Ratain, M. J., Kaminer, L. S., Bitran, J. D., Larson, R. A., Le Beau, M. M., Skosey, C., Purl, S., Hoffman, P. C., Wade, J., Vardiman, J. W., Daly, K., Rowley, J. D., Golomb, H. M. (1987) Acute nonlymphocytic leukemia following etoposide and cisplatin combination chemotherapy for advanced non-small-cell carcinoma of the lung. Blood 70:1412-7

Rivera, G., Avery, T., Pratt, C. (1975) 4'-demethyl-epipodophyllotoxin 9-(4,6-O-2-thenylidene-β-D-glucopyranoside) (NSC-122819; VM-26) and 4'-demethylepipodophyllotoxin 9-(4,6-O-ethylidene-β-D-glucopyranoside) (NSC-141540; VP-16-213) in

childhood cancer: preliminary observations. Cancer chemotherapy reports 59:743-9

Rivera, G., Dahl, G. V., Murphy, S. B., et al., (1982) VM-26 therapy in children with drug-refractory lymphocytic leukemia. Cancer Chemother. Pharmacol. 7:169-72

Ripley, L. S., Dubins, J. S., deboer, J. G., DeMarini, D. M., Bogerd, A. M., Kreuzer, K. N. (1988) Hotspot sites for acridine-induced frameshift mutations in bacteriophage T4 correspond to sites of action of the T4 type II topoisomerase. J. Mol. Biol. 200:665-80

Rodman, J., Abromowitch, M., Sinkule, J., Teresi, M., Rivera, G., et al. (1985) Interpatient pharmacokinetic variability of teniposide (VM-26) during prolonged continuous infusion in pediatric patients. Proceedings of the American Society of Clinical Oncology 4:49

Ross, W., Rowe, T., Glisson, B., Yalowich, J., Liu, L. F. (1984) Role of topoisomerase II in mediating epipodophyllotoxin-induced DNA cleavage. Cancer Res. 44:5857-60

Rossi, C., Zuchetti, M., Sessa, C., Urso, R., Mangioni, C., et al. (1984) Pharmacokinetic study of VM-26 given as a prolonged i.v. infusion to ovarian cancer patients. Cancer Chemotherapy and Pharmacology 13:211-4

Roth, D. B., Wilson, J. H. (1986) Nonhomologus recombination in mammalian cells: role for short sequence homologies in the joining reaction. Molecular and cellular Biology 6:4295-304

Rowe, T. C., Wang, J. C., Liu, L. F. (1986) In vitro localization of DNA topoisomerase II cleavage sites on Drosophila heat shock chromatin. Mol Cell Biol. 6:985-92

Rozencweig, M., von Hoff, D. D., Menney, J. E., Muggia, F. (1977) VM-26 and VP-16-213: a comparative analysis. Cancer 40:334-42

Singh, B., Gupta, R. S. (1983) Mutagenic responses of 13 anticancer drugs on mutation induction at multiple genetic loci and on sister chromatid exchanges in CHO cells. Cancer Res. 43:577-84

Sinkule, J. A. (1984) Etoposide: a semi-synthetic epipodophyllotoxin. Pharmacotherapy 4:61-73

Spitzner, J. R., Muller, M. T. (1988) Consensus sequence for cleavage by vertebrate DNA topoisomerase II. Nucleic Acid Research 16:5533-56

Stähelin, H. (1969) VM-26, a new podophyllotoxin glucoside derivative with anti-L-1210 activity. Proc. Am. Assoc. Cancer Res.10:86

Stähelin, H. (1970) 4'-Demethyl-epipodophyllotoxin thenylidene glucoside (VM-26), a podophyllum compound with a new mechanism of action. Eur. J. Cancer 6:303-11

Stähelin, H., Cerletti, A. (1964) Experimentelle ergebnisse mit den podophyllum-cytostatica SP-I und SP-G. Schweiz Med. Wochenschr. 94:1490-502

Stähelin, H., von Wartburg, A. (1991) The chemical and biological route from podophyllotoxin glucoside to etoposide: ninth Cain memorial award lecture. Cancer Res. 51:5-15

Stewart, D. J., Richard, M. T., Hugenholtz, H., Dennery, J., Nundy, D., et al. (1984) Penetration of teniposide (VM-26) into human intracerebral tumors. Journal of Neuro-Oncology 2:315-24

Sullivan, B. J., Wechsler, H. I. (1947) The cytological effects of podophyllin. Science 105:433

Sullivan, M. P., Van Eys, J., Herson, J., et al., (1979) Nonresponsiveness of brain tumors to VM-26 therapy in children. Cancer Treat. Rep. 63:155-6

Tewey, K. M., Chen, G. L., Nelson, E. M., Liu, L. F. (1984) Intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. J. Biol. Chem. 259:9182-7

Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D., Liu, L. F. (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226:466-8

Udvardy, A., Schedl, P. (1991) Chromatin structure, not DNA sequence specificity, is the primary determinant of topoisomerase II sites of action in vivo. Molecular and Cellular Biology 11:4973-84

Uemura, T., Ohkura, H., Adachi, Y. (1987) DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in S. pombe. Cell 50:917-25

Underberg, WJM. Kerkdijk, H. G., Holthuis, JJM., Beijnen, J. H. (1985) Analysis and degradation kinetics of etoposide (VP-16-213) in aqueous solution. (Abstract.) Pharmaceutisch Weekblad Scientific Edition 7:291

Vosberg, H. P. (1985) DNA topoisomerases: enzymes that control DNA conformation. Current topics in microbiology and

Immunology 114:19-88

Wang, J. C. (1985) DNA topoisomerases. Annu. Rev. Biochem. 54:665-97

Weiss, R. B. (1982) Hypersensitivity reactions to cancer chemotherapy. Semin. Oncol. 9:5-13

Yong, L., Rowe, T. C., Liu, L. F. (1985) Identification of DNA topoisomerase II as an intracellular target of antitumor epipodophyllotoxins in simian virus 40-infected monkey cells. Cancer Res. 45:5872-76

Yong, L., Wold, M. S., Li, J. J., Kelly, T. J., Liu, L. F. (1987) Roles of DNA topoisomerases in simian virus 40 DNA replication in vitro. Proc. Natl. Acad. Sci. USA 84:950-54

# Vita