Biochemical Pharmacology, Vol. 46, No. 11, pp. 2007–2020, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
Pergamon Press Ltd

ALTERED GENE EXPRESSION IN HUMAN LEUKEMIA K562 CELLS SELECTED FOR RESISTANCE TO ETOPOSIDE

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(*Received* 21 *May* 1993; *accepted* 10 *August* 1993)

Abstract—Sublines of K562 human leukemia cells were selected for resistance (30- to 80-fold) to etoposide by continuous exposure to 0.5 μM VP-16. Two etoposide-resistant cell lines, K/VP.5 and K/VP.5-1, showed a 5-fold reduction in levels of topoisomerase IIα protein compared with K562 cells. Northern analysis indicated a 2.5-fold reduction in topoisomerase IIα mRNA in etoposide-resistant cell lines, due in part to a 1.7-fold decrease in topoisomerase II mRNA stability with no change in transcription rate. Immunoblotting assays of electrophoresed cell lysates from VP-16-treated cells revealed less drug-induced covalent topoisomerase II/DNA adducts in resistant than in sensitive cells, suggesting a functional alteration in resistant cell topoisomerase II. Recent reports of specific topoisomerase II DNA binding sites near the promoter sites of growth response genes and alterations of gene expression in cells treated with topoisomerase II inhibitory drugs led to experiments to determine if the apparent functional alterations of topoisomerase II were accompanied by changes in the regulation of these genes. Therefore, the expression of several growth response genes was compared by northern analysis in parental K562 and both VP-16-resistant cell lines. Basal levels of c-*myc* were comparable for all three cell lines, but levels of c-*jun* and c-*fos* were elevated 2- to 4-fold in VP-16-resistant cell lines. Increased levels of c-*fos* and c-*jun* were not a result of altered rates of transcription, as determined by nuclear run-off assays. Exposure of both sensitive and resistant cells to 200 μM VP-16 for 5 hr resulted in no further changes in topoisomerase II mRNA levels but caused an additional 2- to 3-fold elevation in the level of c-*jun* mRNA, indicating that altered basal levels of this gene were not due to deregulation of this gene. Acquired VP-16 resistance in K/VP.5 and K/VP.5-1 cells was accompanied by reduced levels and altered activities of DNA topoisomerase II as well as changes affecting the expression of genes important for growth and differentiation.

Key words: topoisomerase II, etoposide, oncogenes, drug resistance

The nuclear protein DNA topoisomerase II (topoisomerase II)† occurs as a homodimer and accomplishes the topological alteration of DNA [1–3]. Two isoforms of topoisomerase II have been identified [4]; the α-isoform (170 kDa) is more abundant than the β-isoform (180 kDa) in proliferating hematopoetic cells [5, 6]. Topoisomerase II binds to double-stranded DNA forming a reversible, covalent protein–DNA complex that introduces breaks on both DNA strands [1]. Next, in an ATP-stimulated reaction, an intact double strand of DNA passes through the transient break, and the cleaved strand is resealed by ligation at each active site [7]. Turnover of topoisomerase II is accomplished by the hydrolysis of ATP and subsequent dissociation of topoisomerase II from the DNA [8]. The conformational changes that result from strand passage are presumably required to relieve torsional stress incurred during DNA replication [9, 10] and RNA transcription [11–13], and allow for the segregation of chromosomes during mitosis [14].

Topoisomerase II levels are usually found to be higher in proliferating than in quiescent cells [15–18]. Differentiation-inducing drugs have been shown to reduce topoisomerase II activity [19–22], and topoisomerase II inhibitory drugs have been demonstrated to induce differentiation in murine [21, 23] and human [22, 24] leukemia cell lines. Also, differences in topoisomerase II expression between normal and transformed cells have been reported [6, 25]. Collectively, these observations suggested a role for topoisomerase II in regulating cell growth and differentiation. Recent studies of the progression of cells between conditions characteristic of proliferation and those of differentiation have focused on the importance of transcriptional regulation, particularly accompanying changes in expression of the transcription regulatory genes, c-*myc*, c-*jun* and c-*fos* [reviewed in Ref. 26]. Transcriptional activation of c-*jun* and c-*fos* expression in response to DNA-damaging agents has also been reported, and a

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† Abbreviations: topoisomerase II, DNA topoisomerase II; m-AMSA, 4′-(9-acridinylamino)methane-sulfon-*m*-anisidide; β_2m, beta-2-microglobulin; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; HEPES, *N*-2-hydroxymethylpiperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propane-sulfonic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; and VP-16, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside).

role for these genes in a cell signal pathway associated with apoptotic cell death has been proposed [27–29]. The involvement of topoisomerase II in the transcriptional regulation of c-*fos*, c-*myc*, and other genes has been suggested by several studies reporting: (1) the location of specific topoisomerase II binding sites in sequences that control transcription of these genes [30–32]; (2) the activation of oncogene expression in response to topoisomerase II inhibitory drugs such as etoposide [29]; and (3) the interaction of topoisomerase II proteins with transcription regulatory proteins [33].

Acquired resistance to clinically useful DNA topoisomerase II inhibitors have included reports of decreased topoisomerase II expression [34–39] or alteration in the activity of the enzyme [40–43]. In several resistant leukemia cell lines, point mutations have been identified in the gene coding for topoisomerase IIα [44–50]. We recently characterized acquired resistance to the topoisomerase II inhibitory drug VP-16 in a human leukemia K562 cell line.* Compared with K562 cells, the VP-16-resistant cell line K/VP.5 exhibits more rapid reversal of VP-16-induced DNA damage, decreased stability of drug-induced topoisomerase II–DNA covalent complexes, and reduced *in vitro* topoisomerase II catalytic activity, suggesting a functional alteration of topoisomerase II in these cells. K/VP.5 cells also have reduced levels of both the α and the β isoforms of topoisomerase II protein and mRNA. Recent sequencing data suggest that topoisomerase II may be transcriptionally regulated [51]. Thus, it would be reasonable to expect that a reduction in the rate of transcription may represent a mechanism for acquiring resistance to a topoisomerase II inhibitory drug.

In this study, we quantitated the expression of several genes in K562 and K/VP.5 cells to address the following questions. Is resistance to VP-16 correlated with the level of expression of topoisomerase II? Is altered transcription a mechanism for reducing levels of topoisomerase II in VP-16-resistant cells? Do reduced levels or altered DNA-binding activity of resistant cell topoisomerase II affect the transcription of genes hypothesized to be regulated by this enzyme? Our results indicate that topoisomerase II levels do not predict the magnitude of resistance to VP-16 in resistant K562 cells. We also present evidence that the rates of topoisomerase II mRNA synthesis are identical for K562 and VP-16-resistant K/VP.5 cells but that post-transcriptional degradation of topoisomerase II mRNA is accelerated in K/VP.5 cells. In addition, we found increased constitutive expression of the growth and differentiation sensitive genes, c-*jun* and c-*fos*, but not c-*myc* for VP-16-resistant compared with -sensitive K562 cells. Finally, we demonstrated that short-term VP-16 incubation increases the expression of c-*jun* in both VP-16-sensitive and -resistant cells, suggesting that exposure to VP-16 alters the transcriptional regulatory mechanism(s). The results of this study suggest that other targets in addition to topoisomerase II are affected by exposure to VP-16. Results are discussed in relation to understanding mechanisms of resistance to topoisomerase II inhibitory drugs and the resulting alterations of gene expression.

* Ritke MK, Roberts D, Allan WP, Raymond J, Bergoltz VV and Yalowich JC, Altered stability of etoposide-induced topoisomerase II/DNA complexes in resistant human leukemia K562 cells. Manuscript submitted for publication.

MATERIALS AND METHODS

Chemicals and reagents. Etoposide (VP-16) was provided by the Bristol-Myers Squibb Co. (Wallingford, CT). 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (m-AMSA) was obtained from the Drug Investigation Branch of the National Cancer Institute, and vinblastine, actinomycin D, and cycloheximide were obtained from the Sigma Chemical Co. (St. Louis, MO). These drugs were dissolved in 100% dimethyl sulfoxide (DMSO) as concentrated stocks (made fresh weekly) and added to cells so that final DMSO concentrations did not exceed 0.2%. Rabbit antiserum (IID3) to the carboxyl-terminus of recombinant human DNA topoisomerase II was a gift from Dr. L. F. Liu (Johns Hopkins University, Baltimore, MD). Alkaline phosphatase conjugated goat anti-rabbit IgG antibody and alkaline phosphatase detection reagents were obtained from Promega Biotech (Madison, WI). Restriction enzymes, Klenow fragment of *Escherichia coli* DNA polymerase I, and random hexanucleotides were obtained from Boehringer Mannheim (Indianapolis, IN) or Bethesda Research Laboratories (BRL, Gaithersburg, MD). Radiolabeled dCTP, GTP, and leucine were obtained from New England Nuclear (DuPont, Wilmington, DE) and ribonucleotides and deoxyribonucleotides were obtained from the Sigma Chemical Co.

Plasmids. Plasmids used in this study included pSP65 (Promega, Madison, WI), pA1 (containing 2 kb chicken actin; obtained from Dr. R. Ragout, University of Tennessee, Memphis, TN), pβ_2m (containing 0.5 kb of the human β_2 microglobulin gene; obtained from Dr. K. B. Tan, SmithKline Beecham, King of Prussia, PB), pHSR-1 (containing the full-length human c-*myc* gene; ATTC, Rockville, MD), pHJ (containing 2 kb of human c-*jun*; obtained from Dr. D. Bohmann, European Molecular Biology Laboratory, Heidelberg, FRG), pT7fos (containing 1.3 kb 3′ c-*fos*; obtained from Dr. I. M. Verma, Salk Institute, San Diego, CA), and pC15 (containing 1.8 kb of the 3′ end of human topoisomerase II cDNA; obtained from Dr. L. F. Liu, Johns Hopkins University).

Cell characteristics. The VP-16-resistant K/VP.5 cell line was derived by first periodic and then continuous exposure of K562 cells to 0.5 μM VP-16 for 1 year and was cloned by limiting dilution. K/VP.5-1 cells were selected from K/VP.5 cells after an additional 1 year of continuous exposure to 0.5 μM VP-16 and have remained stably resistant to VP-16 in the absence of drug for 1 year. All cell lines were maintained as suspension cultures in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and 1 mM glutamine in an atmosphere of 5% CO_2. For experimental purposes,

cells were maintained in the absence of VP-16 for a minimum of 3 days. To confirm that cells were free of Mycoplasma contamination, culture medium from mid-log cells was assayed using a Gen-Probe Mycoplasma rRNA hybridization kit (Fisher Scientific, Pittsburgh, PA).

Topoisomerase II levels: western analysis of whole cell lysates. Two to five million cells at mid-log phase (5×10^5 cells/mL) were pelleted, washed twice with phosphate-buffered saline (PBS), and resuspended in 50 μL PBS. Cells were lysed with the addition of 50 μL of 2× sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample loading buffer (20% glycerol, 2% SDS, 0.1 M Tris, pH 6.8, 0.5 M β-mercaptoethanol, 0.2 mg/mL bromophenol blue). Lysates were boiled for 5 min and the DNA was sheared by using a sonicator (Tekmar, Woodstock, CT) for 3 sec at 40 W. Protein concentrations were determined in triplicate using Bio-Rad Protein Assay Dye (Bio-Rad, Richmond, CA) and bovine serum albumin (BSA) standards. Proteins (2–10 μg per lane) were electrophoresed through SDS–6% polyacrylamide gels and transferred to nitrocellulose [52] using a Transphor Electrophoresis Unit (Hoefer, San Francisco, CA). Topoisomerase II was labeled by sequential incubation with topoisomerase II specific rabbit polyclonal antibodies and goat anti-rabbit IgG conjugated to alkaline phosphatase. The antibody-labeled topoisomerase II was visualized by the addition of 5-bromo-4-chloro-3-indolyl *p*-toluidine and nitroblue tetrazolium chloride as recommended by the supplier (Promega Biotech, Madison, WI). For quantitation, blots were scanned using an LKB laser densitomer and the GSXL gel scan program (Pharmacia, Piscataway, NJ). Only bands in the molecular weight range of 170–180 kDa were scanned since the presence of lower molecular weight bands was variable and of unknown identity, due to the polyclonal nature of the antisera used to detect topoisomerase II. Various quantities of protein from K562 cells were electrophoresed and transferred for each blot being quantitated. The topoisomerase II signals (area under scanned peak) obtained with various amounts of K562 protein served as a standard curve from which signals for other lanes of interest were quantitated. For comparisons of topoisomerase II levels between cell lines, results were expressed as relative levels compared with K562 cells.

RNA purification and northern analysis. RNAs were extracted from 0.5 to 1.0×10^8 cells [53], and poly(A) RNA was selected by chromatography through columns of oligo-dT-cellulose (Type II, Collaborative Research, Inc., Bedford, MA) in 1.0-mL micropipette tips using buffer conditions suggested by the supplier. Total RNA (10–20 μg) or 5–8 μg poly(A) RNA was resuspended in 50% formamide/2 M formaldehyde/40 mM 3(*N*-morpholino)propane-sulfonic acid (MOPS), pH 7.0, containing 1 μg/mL ethidium bromide (to allow visual inspection of the integrity of the RNA before northern transfer). RNA was denatured by heating to 65° for 5 min, then electrophoresed through 1% agarose gels containing 0.6% formaldehyde and 1× MOPS-acetate (pH 7.0) electrophoresis buffer, immobilized to Nytran nylon membranes (Schleicher & Schuell, Keene, NJ) by capillary transfer, and baked for 2 hr at 80°.

Radioactive labeling of probes. Templates for *in vitro* synthesis of radiolabeled probes were gel purified from restriction enzyme digested plasmids. Radiolabeled probes were prepared by extension of random primers $p(dN_6)$ using the Klenow fragment of *E. coli* DNA polymerase I in the presence of [α-^{32}P]dCTP [54]. For labeling β_2m probes, the [α-^{32}P]dCTP was diluted 1:5 with unlabeled dCTP (3 μM). Unincorporated nucleotides were separated from labeled probe by chromatographic separation through 5-mL Sephadex G50 columns (Pharmacia) equilibrated with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Detection of specific mRNAs: hybridization of probes to northern blots. RNA blots were hybridized with ^{32}P-labeled DNA probes for 16–24 hr at 47° in hybridization buffer containing 50% formamide, 1 M NaCl, 50 mM Tris–HCl, pH 8.0, 1% SDS, and 1 μg/mL sonicated and denatured salmon sperm DNA. Hybridized blots were washed three times at room temperature in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) containing 1% SDS and three times at 65° in 0.1× SSC containing 1% SDS. Blots were autoradiographed using Kodak XAR-5 film and intensifying screens at −70°. Autoradiographic signals were quantitated by densitometric scanning and the results expressed as the ratio of the area of the peak of interest to the area of the β_2m peak. Only autoradiographic signals determined to give a linear response relative to RNA amounts loaded were used for this quantitation.

Nuclear run-off assay. Nuclei isolated from 1×10^8 mid-log cells [55] were gently resuspended in 0.5 mL nuclei wash buffer [10 mM Tris–HCl, pH 8.0, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT)], and pelleted for 5 min at 250 *g* in a Sigma-202MK microcentrifuge (Polymedco, Inc., Yorktown Heights, NY). Washed nuclei were resuspended in a storage buffer (50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 40% glycerol) and adjusted to a final concentration of $1–2 \times 10^7$ nuclei/0.1 mL. For *in vitro* transcriptions, nuclear suspensions (125 μL) were added to 50 μL of 4× transcription buffer (50 mM Tris–HCl, pH 7.5, 7 μM $MgCl_2$, 0.4 M potassium glutamate, 1 μM $MnCl_2$, 1 mM DTT, 2.8 mM ATP, 1.4 mM each UTP and CTP, and 25 μL [α-^{32}P]GTP (250 μCi; 810 Ci/mmol). The use of radiolabeled GTP, instead of the more conventional UTP, allowed for fresh supplies of this extremely labile nucleotide, circumventing one potential limitation to maximum elongation and labeling of nascent RNAs. After a 5-min incubation on ice, the nuclei mixture was incubated for 30 min at 30°. Reactions were terminated by the addition of 3 mL of 4 M guanidinium isothiocyanate and RNA was extracted (as described above). After ethanol precipitation, RNA was resuspended in 0.2 mL TES (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1% SDS), heated for 5 min at 65° and chromatographed through a 5-mL Sephadex G50 column equilibrated with TES. The leading peak fraction(s) was collected and quantitated, and equivalent counts (usually 10^7 cpm/10^7 nuclei) were hybridized to 5 μg dot blots of plasmids containing the genes of interest (target

saturation for any of the probes used was > 0.5 μg). Hybridization was carried out in a buffer containing 5 × SSPE (0.73 M NaCl, 0.05 M $NaH_2PO_4 \cdot H_2O$, 5 mM EDTA, pH 7.5), 1% SDS, 50 μg/mL sonicated and denatured salmon sperm DNA, 25% formamide at 50° for 70–80 hr, followed by three washes each in 1× SSC/1% SDS and 0.1× SSC/1% SDS at 65°. Signals were obtained by autoradiography for 3–5 days at −70° using an intensifying screen.

DNA dot blots. Pst 1 linearized plasmid DNA (30 μg) was denatured for 5 min in 0.4 M NaOH, and applied directly (6 μg/dot) to a Nytran membrane (prewashed in 6 × SSC) using a dot blot manifold (Bio-Rad Laboratories). The membrane was then immersed in neutralization buffer (0.5 M Tris, pH 7.5, 0.5 M NaCl) for 2 min, and baked for 2 hr at 80°. The resulting immobilized plasmid DNA was used to detect specific RNAs labeled in nuclear run-off assays (described above).

Measurements of protein synthesis: [^{14}C]leucine incorporation. K562 and K/VP.5 cells were pelleted, washed once with cold saline, and resuspended in an isotonic buffer (110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 25 mM HEPES, 10 mM glucose, pH 7.4) at 37° at a concentration of 0.4 to 1 × 10^6 cells/mL. To measure the rate of protein synthesis, [U-^{14}C]leucine (318.5 mCi/mmol) was added to a final specific activity of 0.5 μCi/mL. At various times, 0.2 mL of cells was removed in triplicate and quenched in 5 mL of cold saline. Pelleted cells were resuspended in 0.1 mL saline and precipitated by the addition of 5 mL of 10% trichloroacetic acid (TCA). After 15 min on ice, TCA-precipitable radioactivity was collected on Whatman GF/B glass fiber filters (Fisher Scientific) and quantitated by liquid scintillation counting. For measurements of protein synthesis after drug treatments, some cells were exposed to either cycloheximide (40 μM) or VP-16 (0–200 μM) for 0, 2.5 or 5.0 hr. Drug treatments were terminated by adding 0.25-mL aliquots of cell suspensions (2 × 10^6 cells/mL) to 10 mL of ice-cold saline. TCA-precipitable radioactivity was collected and counted as described above.

RESULTS

Cell characteristics. Continuous growth of human leukemia K562 cells in 0.5 μM VP-16 for 1 year resulted in the selection of the resistant subcloned cell line K/VP.5. Maintenance of K/VP.5 cells in 0.5 μM VP-16 for an additional year resulted in the selection of the cloned line K/VP.5-1. During the course of these studies, K/VP.5 and K/VP.5-1 cells were 28- and 78-fold resistant to the growth inhibitory effects of VP-16, respectively, compared with parental K562 cells (not shown). Growth rates (doubling times) were comparable for K562 (16 hr), K/VP.5-1 (18 hr) and K/VP.5 (22 hr). Flow cytometric analysis showed no difference in the cell cycle distribution between K562 and K/VP.5 cells (data not shown). Both VP-16-resistant cell lines showed comparable levels of resistance (10- to 20-fold) to m-AMSA, comparable sensitivity to a variety of microtubule inhibitors and antimetabolites, and no overexpression of the P150–180 kDa P-glycoprotein.* Although K/VP.5 cells accumulate VP-16 to the same intracellular concentration as parental K562 cells, the higher-fold resistant K/VP.5-1 cells exhibit a 2-fold reduction in steady-state VP-16 accumulation [56]. Therefore, selection of higher-fold resistant K/VP.5-1 cells from K/VP.5 cells results in an additional phenotypic expression of drug resistance, namely reduction in drug accumulation not associated with overexpression of the P-glycoprotein pump [57, 58]. Similar observations were previously reported for VP-16-resistant KB cells selected by stepwise exposure to drug [35]. No additional phenotypic changes have been observed for K/VP.5-1.

Topoisomerase II levels. Human tumor cells express both the α-isoform and the β-isoform of topoisomerase II; the α-isoform is usually predominant [6]. In this report only the 170 kDa topoisomerase II (α-isoform) was examined. Levels of 170 kDa topoisomerase II were measured using polyclonal antisera (IID3) specific for topoisomerase II and western blots of cell lysates from K562, K/VP.5 and K/VP.5-1 cells. The protein content of each cell line, determined from cell lysates was 62 ± 6 pg/cell (N = 13) for K562, 56 ± 5 pg/cell

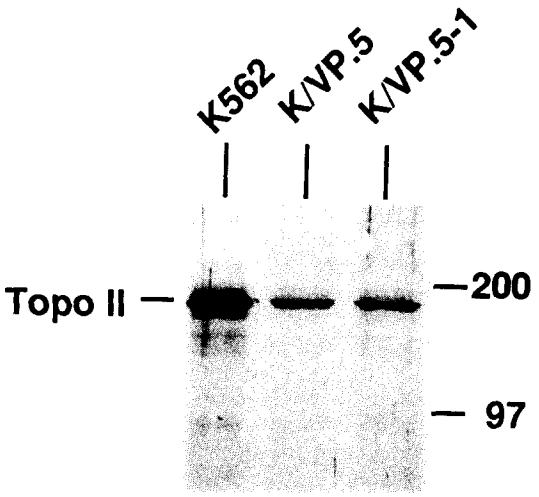

Fig. 1. Topoisomerase II (170 kDa) protein levels from VP-16-sensitive and -resistant cells. PBS-washed cells were lysed, electrophoresed through SDS–polyacrylamide gels, electroblotted to nitrocellulose, and labeled first with topoisomerase II specific polyclonal antisera and then with alkaline phosphatase-conjugated goat anti-rabbit antibody. Each lane contained 10 μg of protein from lysates of K562, K/VP.5, or K/VP.5-1 cells respectively, corresponding to the amount of protein from 1.5 × 10^5 cells. Numbers to the right of the figure indicate the positions of molecular size markers. Quantitations were performed, as indicated in Materials and Methods. Compared with the K562 cell topoisomerase II level (1.00), topoisomerase II levels in K/VP.5 and K/VP.5-1 cells were reduced to 0.19 ± 0.03 and 0.19 ± 0.02, respectively (mean ± SEM from 7 to 10 separate determinations; $P < 0.01$, Wilcoxon's signed ranks test).

* Meyers M and Yalowich JC, unpublished observations.

(N = 13) for K/VP.5, and 69 ± 13 pg/cell (N = 5) for K/VP.5-1 cells (P = 0.1 and 0.7, respectively; paired Student's *t*-test). Because the amount of protein from the VP-16-resistant cells was not significantly different from that of K562, 10 μg protein (equivalent to 1.5×10^5 cells) from these cells was used to quantitate topoisomerase II levels by western blotting (Fig. 1). Topoisomerase II signals were reduced in the resistant cells (last 2 lanes) compared with the parental K562 cells. Topoisomerase II levels in VP-16-resistant cells compared with the parental cell line were determined subsequent to electrophoresis of various amounts (2–10 μg or $0.3–1.5 \times 10^5$ cells) of total protein from K562 cell lysates through SDS–PAGE gels (not shown). Binding of topoisomerase II antisera was quantitated from all lanes by densitometric scanning. The signals for K562 topoisomerase II were used to construct a standard curve which was then utilized to determine the relative levels of topoisomerase II in 10 μg cell lysates of VP-16-resistant cells. The results of these quantitations showed that topoisomerase II levels were reduced 5-fold for both VP-16-resistant lines (Fig. 1 legend).

Topoisomerase II mRNA levels. Northern analysis was performed using RNA isolated from all three cell lines to determine if topoisomerase II levels in VP-16-resistant cells were altered at the level of transcription. Total RNA isolated from mid-log phase cells was electrophoresed through denaturing formaldehyde gels, blotted to nylon membranes, and hybridized to a mixture of ^{32}P-labeled cDNA probes specific for topoisomerase II and β_2m. A hybridized blot shown in Fig. 2 illustrates a mRNA of *ca.* 6 kb, corresponding to the size previously reported for human topoisomerase II mRNA [59] and suggesting that no substantial alterations (insertions, deletions or alternative processing) of the topoisomerase II gene occurred during selection for VP-16 resistance. Relative levels of topoisomerase II mRNA were obtained subsequent to densitometric scanning of autoradiographs, using the 1.0 kb β_2m mRNA as an internal control and revealed that topoisomerase II mRNA levels were reduced about 2.5-fold in both K/VP.5 and K/VP.5-1 as compared with K562 cells (Fig. 2 legend).

Rates of transcription. Nuclear run-off assays were performed to determine if the reduction of topoisomerase II mRNA was due to a reduced rate of initiation of transcription. The level of *in vitro* transcription of topoisomerase II was low compared with that of the other genes examined (Fig. 3), suggesting either a slower rate of transcription or initiation of transcription at very few topoisomerase II sites. In either case, visual inspection of three similar assays indicated little or no difference in the rate of initiation of topoisomerase II transcription in K562 or K/VP.5 cells. Therefore, the next experiments compared the stability of topoisomerase II mRNA from K562 and K/VP.5 cells exposed for various times (0.5–6 hr) to 20 μM actinomycin D, a concentration that inhibits uridine incorporation > 95%. During the course of actinomycin D treatment, >96% of both K562 and K/VP.5 cells retained the ability to exclude trypan blue, cellular DNA remained intact, and β_2m mRNA signals remained constant. Linear regression analyses of the first-order decay curves of topoisomerase II mRNA from K562 and K/VP.5 cells (Fig. 4) indicated that this mRNA was 1.8-fold less stable in K/VP.5 cells than in K562 cells. Averaging the results from 5 independent experiments the $T_{1/2}$ for topoisomerase II mRNA was 111 ± 19 and 63 ± 16 min in K562 and K/VP.5 cells, respectively (P < 0.001, paired Student's *t*-test). These results suggest a post-transcriptional alteration in the

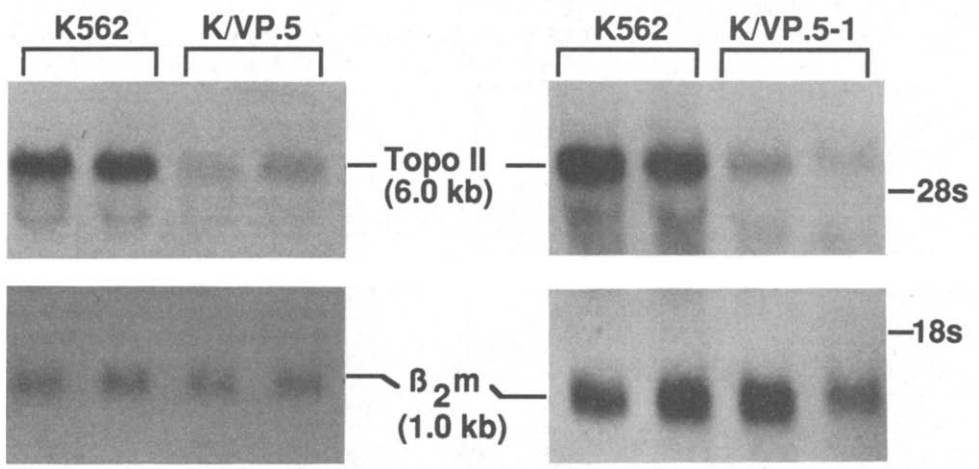

Fig. 2. Topoisomerase II mRNA levels in VP-16-sensitive (K562) and -resistant (K/VP.5, K/VP.5-1) cells. RNA (10 μg) was extracted from mid-log phase cells, electrophoresed through formaldehyde-containing agarose gels, blotted and hybridized to ^{32}P-labeled probes for topoisomerase II and β_2 microglobulin (β_2m), as detailed in Materials and Methods. Duplicate samples are shown for each cell line. Topoisomerase II mRNA levels in K/VP.5 and K/VP.5-1 cells were reduced significantly to 0.44 ± 0.12 and 0.42 ± 0.11 that of K562 cells, respectively (mean ± SEM from 3 to 5 separate determinations; P < 0.01, Wilcoxon's signed ranks test).

resistant cell topoisomerase II mRNA affecting its stability and/or the activation of internal RNase(s) in VP-16-resistant cells.

Altered accumulation of topoisomerase II–DNA covalent complexes. Exposure of cells to topoisomerase II inhibitory drugs causes stabilization and accumulation of topoisomerase II–DNA covalent complexes [2, 3]. Since topoisomerase II–DNA complexes cannot electrophorese through polyacrylamide gels, the accumulation of drug-induced topoisomerase II–DNA covalent complexes may be indirectly assessed by a "band depletion" assay measuring the decrease of electrophoretically mobile topoisomerase II [60]. This assay was used to compare topoisomerase II–DNA covalent complex accumulation in VP-16-treated sensitive and resistant cells. Lysate protein (10 μg) from cells treated for 5 hr with 200 μM VP-16 or 25 μM m-AMSA was electrophoresed followed by quantitation of topoisomerase II by western blotting. The formation of covalent topoisomerase II–DNA adducts after exposure of cells to VP-16 resulted in the "depletion" of topoisomerase II in all three cell lines (Fig. 5); however, the mean depletion of topoisomerase II was at least 2-fold less for both K/VP.5 and K/VP.5-1 cells compared with K562 cells (Table 1). These results suggest that not only is topoisomerase II reduced quantitatively in K/VP.5 and K/VP.5-1 cells (Fig. 1) but, in addition, enzyme that is produced exhibits an alteration affecting VP-16-induced topoisomerase II binding to DNA. m-AMSA was not as effective as VP-16 in depleting topoisomerase II and the extent of depletion was similar for K562 and K/VP.5. However, m-AMSA treatment of K/VP.5-1 resulted in less depletion of topoisomerase II signal (Table 1), which may indicate additional alterations affecting topoisomerase II in this more resistant line.

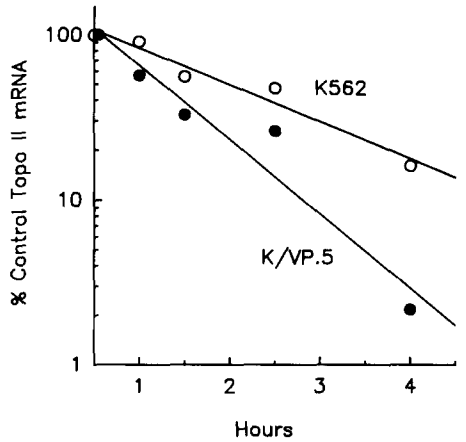

Fig. 4. Stability of topoisomerase II mRNA in K562 and K/VP.5 cells. RNA was extracted at various times after the addition of 20 μM actinomycin D to mid-log phase cultures of K562 and K/VP.5 cells. RNA was subjected to northern analysis as in Fig. 2, and signals were scanned and quantitated, as described in Materials and Methods. A representative first-order decay curve is presented in which the $T_{1/2}$ of topoisomerase II mRNA was determined to be 81 min for K562 cells (○) and 45 min for K/VP.5 (●).

Effect of VP-16-induced resistance on gene expression. Recent evidence suggested potential roles for DNA topoisomerase II in regulation of transcription [11–13, 30–33] and in altered gene expression in cells treated with DNA-damaging agents [27–29, 61]. These studies prompted us to determine whether quantitative and/or functional alterations of topoisomerase II in K/VP.5 and

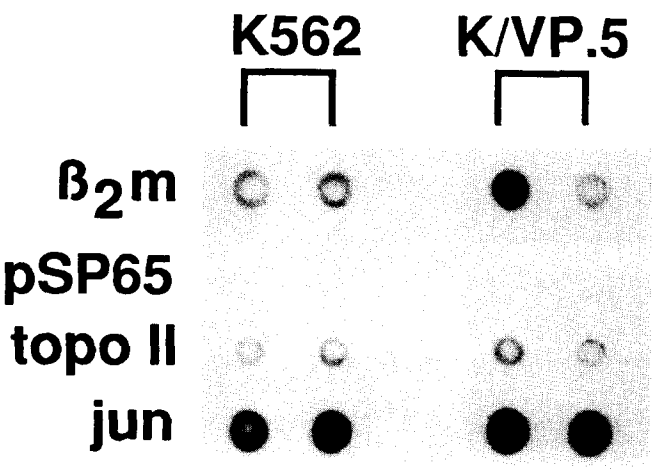

Fig. 3. Transcription rates in nuclei of K562 and K/VP.5 cells (nuclear run-off assays). Nuclei from 2×10^7 cells were allowed to transcribe *in vitro* in the presence of [α-^{32}P]GTP. In the experiment illustrated here, 6×10^7 cpm (K562) and 8×10^7 cpm (K/VP.5) representing 36–48 pmol of GTP were incorporated. For quantitation of specific initiated RNAs, 5×10^7 cpm were hybridized for 70 hr to dot blots of plasmids containing the cloned inserts to each of the genes indicated in the figure and were processed, as described in Materials and Methods.

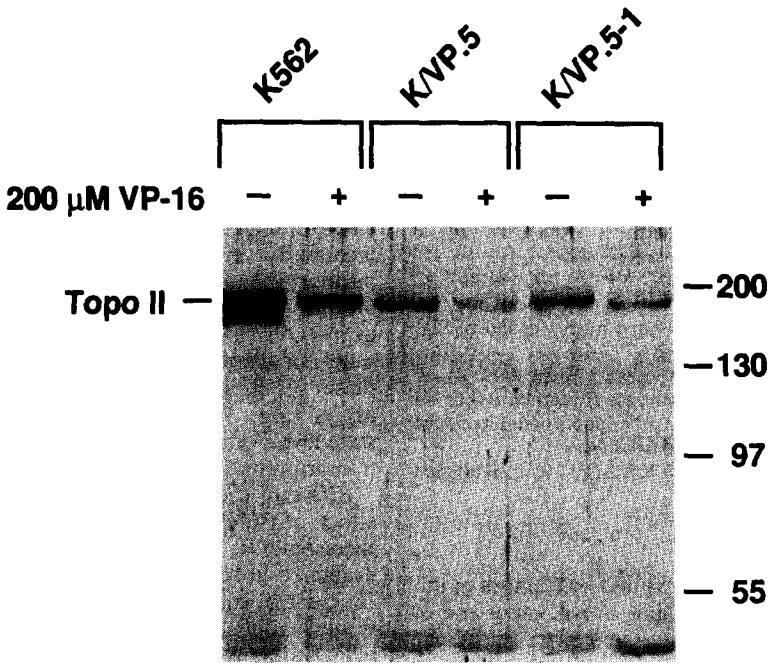

Fig. 5. Topoisomerase II depletion in cells treated for 5 hr in the presence of 0.1% DMSO (−) or 200 μM VP-16 (+). After drug treatment, PBS-washed cells were lysed and 10 μg protein/lane was electrophoresed through 6% SDS–polyacrylamide gels, blotted to nitrocellulose, and labeled with topoisomerase II specific, polyclonal antibody as for Fig. 1. Molecular size markers are indicated on the right of the figure.

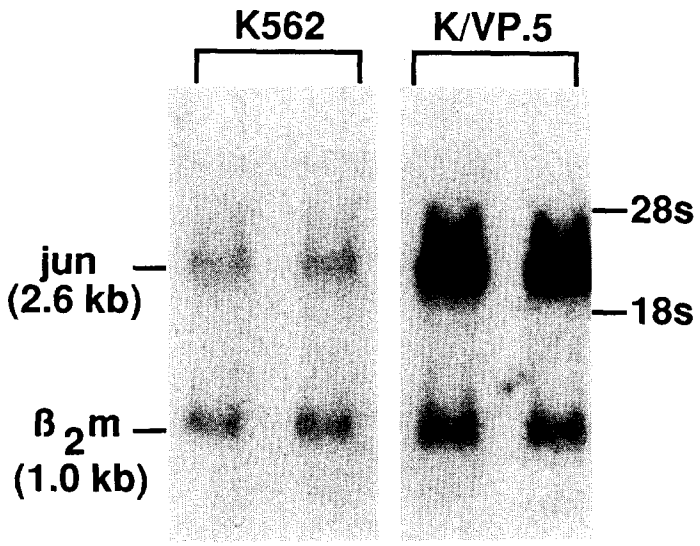

Fig. 6. c-*jun* mRNA levels in VP-16-sensitive (K562) and -resistant (K/VP.5) cells. Total cellular RNA (20 μg) was extracted, electrophoresed, and subjected to northern analysis as in Fig. 2, using ^{32}P-labeled probes for c-*jun* and β_2m, as detailed in Materials and Methods. Basal levels of c-*jun* were elevated 3.82 ± 0.66-fold and 2.72 ± 0.37-fold in K/VP.5 and K/VP.5-1 cells, respectively, compared with K562 cells (mean ± SEM of 5–7 separate determinations; $P < 0.01$, Wilcoxon's signed ranks test).

Table 1. Topoisomerase II depletion in VP-16- or m-AMSA-treated cells*

Cell line	Percent topoisomerase II depletion†	
	200 μM VP-16	25 μM m-AMSA
K562	56.2 ± 4.6 (5)	34.0 ± 12.3 (4)
K/VP.5	25.0 ± 14.1 (2)	28.1 ± 15.0 (2)
K/VP.5-1	16.3 ± 11.5‡ (5)	16.3 ± 3.4‡ (4)

* Cells were treated with VP-16 or m-AMSA. Topoisomerase II from cell lysates was quantitated from western blots prepared as described in the legend of Fig. 1.

† Results are expressed as mean ± SEM ($N \geq 4$) or mean ± range ($N = 2$) topoisomerase II reduction relative to cells treated with 0.1% DMSO. Numbers of independent experiments (N) are given in parentheses.

‡ Significantly different from K562 ($P < 0.05$, Student's paired *t*-test).

K/VP.5-1 cells were accompanied by changes in the expression of genes (including c-*myc*, c-*jun* and c-*fos*) demonstrated to be regulators of transcription, sensitive to perturbations of cell growth and differentiation, or responsive to DNA damage. The basal levels of c-*jun* were elevated 2- to 4-fold in VP-16-resistant cells compared with parental K562 cells ($P < 0.01$; Wilcoxon's signed ranks test; see also Fig. 6). Because c-*fos*, another tightly regulated early response gene and c-*jun* have been demonstrated to act cooperatively in the regulation of transcription of some genes [62], we also examined the levels of expression of c-*fos* in VP-16-sensitive and -resistant cells. Results from two independent poly(A) RNA preparations revealed that, similar to c-*jun*, c-*fos* was elevated approximately 2.5-fold in VP-16-resistant cells (Fig. 7). The c-*myc* levels in K/VP.5 cells were identical to those of K562 cells (0.99 ± 0.15, mean ± SEM from 5 determinations; $P = 0.86$, Wilcoxon's signed ranks test). In contrast, c-*myc* mRNA levels were slightly (but significantly) increased in K/VP.5-1 cells (1.44 ± 0.17, mean ± SEM from 5 determinations; $P = 0.006$, Wilcoxon's signed ranks test). The increased expressions of c-*jun* and c-*fos* in VP-16-resistant cells were not due to an altered rate of transcription of these genes, as determined from nuclear run-off assays (Fig. 3), suggesting that increased steady-state levels of c-*jun* and c-*fos* are a result of a post-transcriptional mechanism.*

Effects of VP-16 treatment on gene expression. The effect of VP-16 treatment on the expression of topoisomerase II, c-*myc*, and c-*jun* mRNAs was examined in cells treated for 5 hr with 200 μM VP-16. Control and drug-treated cells remained > 96% viable, as determined by the ability to exclude trypan blue, and cellular DNA remained intact (not shown). After a 5-hr exposure to VP-16, expression of topoisomerase II mRNA was not changed significantly (Table 2); however c-*jun* expression was elevated not only in K562 cells but also in the VP-16-resistant cell lines (Table 2, Fig. 8). The 2-fold VP-16-induced increase of c-*jun* mRNA in K/VP.5 and K/VP.5-1 cells indicates that c-*jun* mRNA regulation remained responsive to external stimuli in these cells even though basal levels of expression in these lines were increased compared with those of the parental K562 line. Drug-induced reduction of c-*myc* mRNA levels was consistently observed only in K562 cells (Table 2). The effect of VP-16

* Ritke MK, Bergoltz VV, Allan WP and Yalowich JC, Increased c-*jun* (AP-1) expression in VP-16 resistant human leukemia K562 cells. Manuscript submitted for publication.

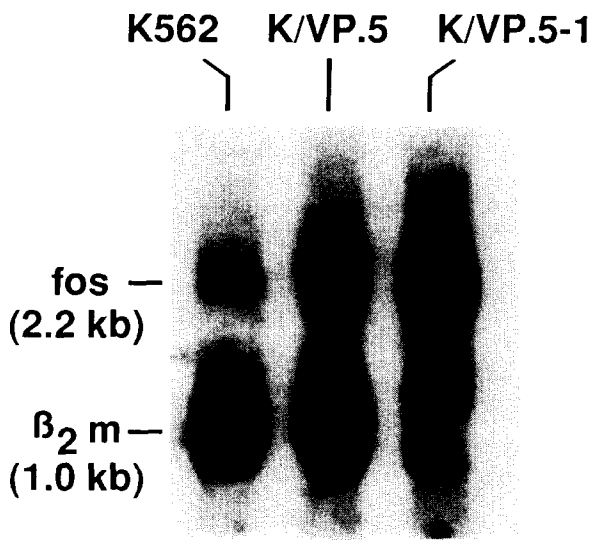

Fig. 7. c-*fos* mRNA levels in VP-16-sensitive (K562) and -resistant (K/VP.5 and K/VP.5-1) cells. Poly (A) selected RNA (8 μg) was electrophoresed and subjected to northern analysis as in Figs. 2 and 6, using ^{32}P-labeled probes for c-*fos* and β_2m, as detailed in Materials and Methods. The autoradiograph pictured here was intentionally overexposed to more clearly show the c-*fos* signal in K562 cells.

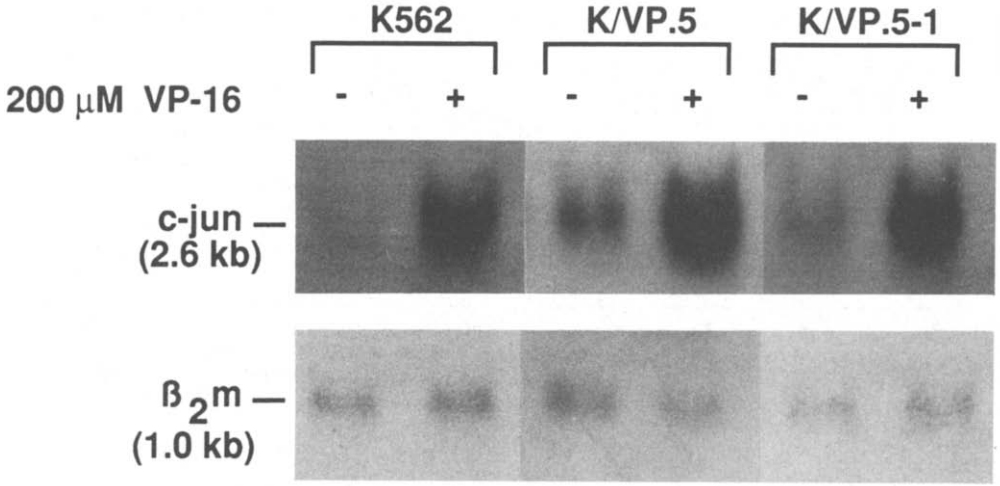

Fig. 8. Effect of VP-16 treatment on c-*jun* mRNA levels in VP-16-sensitive (K562) and -resistant (K/VP.5 and K/VP.5-1) cells. Prior to isolating RNA, mid-log phase cultures of cells were incubated in the presence (+) or absence (−) of 200 μM VP-16 for 5 hr. RNA (10 μg) was electrophoresed, blotted and hybridized to c-*jun* and β_2m probes, as described in Materials and Methods.

treatment on c-*myc* mRNA levels in K/VP.5 or K/VP.5-1 cells was quite variable; thus, no significant change in expression of this gene was observed.

Rates of protein synthesis. Since the expression of some genes is known to be affected, both positively and negatively, by agents that inhibit translation [63, 64], it is possible that the observed alterations in gene expression were due to VP-16-mediated inhibition of protein synthesis [65]. Therefore, we compared the rates of protein synthesis (measured by incorporation of [U-^{14}C]leucine) in VP-16- and cycloheximide-treated cells. No significant inhibition of protein synthesis was observed in cells treated for 2.5 or 5.0 hr with 25 μM VP-16 (Table 3), conditions that also induce c-*jun* expression (not shown). At 200 μM VP-16, protein synthesis inhibition in K562 cells approached that observed in the presence of 40 μM cycloheximide at both 2.5 and 5.0 hr. Inhibition of protein synthesis was less in K/VP.5 compared with K562 cells, yet there was comparable stimulation of c-*jun* expression under these conditions (Table 2). Protein synthesis rates were similar in K562 and K/VP.5 cells (mean ratio of rates = 1.08 ± 0.08 (SEM), P = 0.8 for 5 separate experiments). Together these data indicate that c-*jun* stimulation in response to VP-16 treatment was not the result of protein synthesis inhibition.

DISCUSSION

Acquired resistance to VP-16 was examined in K/VP.5 and K/VP.5-1 cells, two VP-16-resistant sublines of human leukemia K562 cells, focusing on quantitative and qualitative changes in topoisomerase II and effects on the expression of the tightly regulated genes, c-*myc*, c-*fos* and c-*jun*. These observations indicate that acquired resistance to VP-16 is accompanied by a number of phenotypic changes in addition to quantitative reduction of topoisomerase II expression.

In the presence of 200 μM VP-16, covalent binding of topoisomerase II to DNA was decreased in VP-16-resistant compared with -sensitive cells, as measured by depletion of the electrophoretically mobile 170 kDa protein. Under these conditions, 56% of K562 topoisomerase II, but only 16–25% of

Table 2. Effect of VP-16 on gene expression in sensitive and resistant cells*

	Ratio of mRNA levels†		
mRNA	K562	K/VP.5	K/VP.5-1
Topoisomerase II	0.96 ± 0.04 (5)	0.79 ± 0.17 (3)	1.09 ± 0.13 (5)
c-*jun*	3.01 ± 0.65‡ (4)	2.33 ± 0.41‡ (4)	2.03 ± 0.51‡ (7)
c-*myc*	0.64 ± 0.18‡ (5)	1.11 ± 0.40 (2)	0.99 ± 0.59 (4)

* RNA was purified from cells treated with 200 μM VP-16 for 5 hr and from cells treated with DMSO alone. Northern analysis was performed and quantitated, as detailed in Materials and Methods.

† Results are expressed as the ratio of gene specific mRNA in drug-treated cells to that of DMSO-treated cells. Values are means ± SEM (N ≥ 3) or range (N = 2); numbers of independent experiments (N) are given in parentheses.

‡ Significantly different from untreated cells (P < 0.04, Wilcoxon's signed ranks test).

Table 3. Protein synthesis ([U-^{14}C]leucine incorporation) in cycloheximide- and VP-16-treated K562 and K/VP.5 cells*

	Percent incorporation†			
	2.5 hr		5.0 hr	
Drug treatment	K562	K/VP.5	K562	K/VP.5
VP-16 (25 μM)	96 ± 11 (3)	101 ± 9 (3)	94 ± 10 (3)	97 ± 17 (3)
VP-16 (200 μM)	60 ± 8 (5)	86 ± 17 (5)	37 ± 10 (5)	84 ± 12‡ (5)
Cycloheximide (40 μM)	47 ± 12 (5)	43 ± 7 (5)	29 ± 5 (5)	23 ± 5 (5)

* Cells were exposed to the indicated concentrations of cycloheximide or VP-16 for 2.5 or 5.0 hr, washed free of drug, and pulse labeled for 15 min with L-[U-^{14}C]leucine.

† TCA-precipitable radioactivity was measured and expressed as percent incorporation in drug-treated cells compared with untreated control cells. Values are means ± SEM; numbers of independent experiments are given in parentheses.

‡ Significantly different from K562 ($P = 0.05$; paired Student's *t*-test).

either K/VP.5 or K/VP.5-1 topoisomerase II, was depleted from the total pool of this enzyme (Fig. 5, Table 1). These observations suggest that a qualitative change(s) in topoisomerase II in terms of its interaction with VP-16 or its binding to DNA has occurred in the resistant cells. This interpretation is consistent with our observations of altered nucleotide stimulation of VP-16-induced DNA–protein cross-linking, DNA single-strand breaks, and decreased stability of VP-16-induced topoisomerase II–DNA covalent complexes in resistant K/VP.5 cells.*

Reductions of topoisomerase II protein (Fig. 1) paralleled reductions of the topoisomerase II mRNA (Fig. 2, Table 1) in VP-16-resistant cells. However, the levels of topoisomerase II mRNA and protein in the 78-fold resistant K/VP.5-1 cells were identical to those of the 28-fold resistant K/VP.5 cells. Thus, the extent of reduction of topoisomerase II was not a predictor of the magnitude of resistance to VP-16. The cause of the decreased stability of topoisomerase II mRNA in VP-16-resistant cells is not clear. Studies focusing on determinants of mRNA stability may provide mechanisms to explain a less stable mRNA (reviewed in Ref. 66). First, a structural alteration in the topoisomerase II gene itself may exist. Two structural motifs have been demonstrated to affect mRNA stability of other genes. An $A(U)_3A$ motif in the 3′ non-coding region of mRNAs has been demonstrated to contribute to the relative instability of c-*myc* and c-*fos* transcripts [67–70]. Specific destabilizing sequences have also been identified within the coding region of these genes [63, 69]. When cloned within normally stable genes these sequences destabilize the resulting chimeric mRNA, presumably by providing a suitable target for RNases. None of these previously defined instability determinants have been found in human topoisomerase II cDNA sequences. A more precise description of the stability determinant(s) in the coding regions of c-*fos*, c-*myc*, or other genes may reveal additional targets for selective degradation of mRNAs that may be applicable to topoisomerase II. A second possible mechanism for decreased topoisomerase II mRNA stability is suggested by studies of mRNA levels for β-globin [71] and tubulin [72], in which it was determined that the rate of translation of these genes determines the stability of their mRNAs. Thus, a high rate of translation of a specific mRNA may enhance [71] or decrease [72] the stability of its mRNA. If this mechanism were applicable to topoisomerase II, one would expect the rate of topoisomerase II translation in K/VP.5 and K/VP.5-1 cells to be less than in K562 cells.

Recent *in vitro* studies reported the presence of specific topoisomerase II binding sites near the promoter regions of several early response genes that regulate the long-term responses (proliferation or differentiation) of cells to external stimuli [30–32]. These studies prompted us to examine whether altered levels and/or a functionally altered topoisomerase II have an effect on cellular transcription of some of these genes. The elevated expressions of c-*jun* and c-*fos* (Figs. 6 and 7) in VP-16-resistant cells were suggestive of a change in post-transcriptional regulation in these genes, similar to results reported in a previous study in which cells induced to differentiate exhibited an elevated level of c-*fos* expression [73]. The modest elevation of c-*myc* levels in K/VP.5-1 may be related to additional changes occurring subsequent to the continuous exposure to VP-16 used to select this subline [56], but these levels were not comparable with those of other studies in which c-*myc* elevation due to deregulation of the gene was reported elevated several-fold [74].

The overexpression of c-*jun* and c-*fos* in VP-16-resistant cells was particularly interesting but was not accomplished by complete deregulation of these genes since treatment with 25 μM VP-16 (not shown) or 200 μM VP-16 (Table 2, Fig. 8) induced an additional 2- to 3-fold increase in c-*jun* in these cell lines. The increased basal levels of c-*jun* in K/VP.5 or K/VP.5-1 cells were not accomplished by a change

* Ritke MK, Roberts D, Allan WP, Raymond J, Bergoltz VV and Yalowich JC, Altered stability of etoposide-induced topoisomerase II/DNA complexes in resistant human leukemia K562 cells. Manuscript submitted for publication.

in transcription rates as determined by nuclear run-off assays (Fig. 3); thus, it seemed most likely that mRNA stability was increased. Consistent with this idea, recent results (not shown) indicate that c-*jun* mRNA from K/VP.5 cells is 2-fold more stable than that from K562 cells. Previous reports of specific topoisomerase II binding sites near the regulatory regions of c-*myc* [31] and c-*fos* [30] suggested a role for topoisomerase II in the expression of these genes; however, our nuclear run-off assays do not support this role in intact cells, at least in the context of initiation of transcription. Because c-*fos* and c-*jun* were elevated similarly in both resistant cell lines, but the levels of c-*myc* were somewhat higher in K/VP.5-1 than in K/VP.5, the regulation of c-*myc* and c-*jun* or c-*fos* appeared to be dissociated, consistent with previous reports [75].

The relationship between decreased topoisomerase II expression, altered VP-16-induced topoisomerase II/DNA binding, and increased basal levels of c-*jun* and c-*fos* in our VP-16 selected K562 resistant cell lines is currently unclear. Topoisomerase II expression is reduced in differentiating cells [5, 19, 20] and increased expression of c-*fos* and c-*jun* has been identified as an early event in the differentiation of cells treated with retinoic acid [76] and phorbol ester [77]. In addition, overexpression of c-*fos* and c-*jun* leads to differentiation of some cell lines [78, 79]. Thus, the increased levels of c-*jun* and c-*fos* in K/VP.5 and K/VP.5-1 cells may reflect an initial step toward a differentiated phenotype, characterized by reduced levels of topoisomerase II. Recently, VP-16-induced c-*jun* and c-*fos* overexpression was found to be associated with early events of apoptotic cell death [27, 29]. Also, c-*jun* and c-*fos* were selectively activated secondary to DNA damage caused by ultraviolet radiation and hydrogen peroxide [80]. These results suggest that the elevated expression of c-*jun* and c-*fos* may be related to a cell signalling pathway associated with DNA damage inflicted, in this case by VP-16, via topoisomerase II.

C-*jun* and c-*fos* comprise a transcription regulatory complex, AP-1 [81, 82]. Both Jun and Fos proteins have been shown to be autoregulative [83, 84]. A common feature of AP-1 proteins and several other DNA binding proteins including topoisomerase II is a conserved protein dimerization motif containing a leucine zipper [82, 85, 86]. Kroll *et al.* [33] recently demonstrated that topoisomerase II interacts with c-*jun*, c-*fos*, and other transcription regulatory proteins, although this interaction was not dependent on the leucine zipper itself. Destabilization of topoisomerase II interactions with these transcriptional proteins through a topoisomerase II dimerization site would be expected to alter protein function. This putative dimerization motif may represent a common target for both the VP-16-induced changes in regulation of c-*jun* and c-*fos* transcription and the altered DNA binding characteristics of topoisomerase II.

Based on the presented studies of VP-16-resistant K/VP.5 and K/VP.5-1 cells, we conclude that acquired resistance to VP-16 in K562 cells results in alterations not limited to changes in topoisomerase II. The investigation of drug effects at other loci has been encouraged [35, 87] and may be of import in elucidating the cytotoxic mechanisms of drugs such as VP-16. Our observation of increased expression of the transcriptional regulatory genes c-*fos* and c-*jun* in response to VP-16 treatment may contribute to a better understanding of additional resistance mechanisms as well as expanding knowledge of the biological activities of topoisomerase II.

Acknowledgements—The authors thank Dr. Stephen Phillips, Department of Molecular Genetics and Biochemistry, University of Pittsburgh, for productive technical suggestions for nuclear run-off assays, Mr. William P. Allan for expert technical assistance and Ms. Linda Levine, Ms. Bonnie Schiavone and Ms. Carolyn Smith for help in preparation of this manuscript. This work was supported by Grant DHP-49 from the American Cancer society and Grant CA-51657 from the National Cancer Institute, NIH, Bethesda, MD. M.K.R. is the recipient of a Pittsburgh Cancer Institute Postdoctoral Fellowship.

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