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INCREASED c-*jun*/AP-1 LEVELS IN ETOPOSIDE-RESISTANT HUMAN LEUKEMIA K562 CELLS

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Abstract—C-*jun* mRNA and AP-1 levels were examined in etoposide (VP-16)-sensitive (K562) and -resistant (K/VP.5) human leukemia cell lines. Previously, we reported that K/VP.5 cells have increased basal levels of mRNA for the protooncogene c-*jun* (Ritke MK and Yalowich JC, *Biochem Pharmacol* **46**: 2007–2020, 1993). In this study, we show that the 3-fold increase in c-*jun* transcripts in K/VP.5 cells was accompanied by a 2-fold increase in the stability of the mRNA for this gene and a nearly 2-fold increase in AP-1 DNA binding activity compared with parental K562 cells. Treatment of K562 and K/VP.5 cells with 50–200 μM VP-16 resulted in 3- to 10-fold stimulation of c-*jun* transcripts, which peaked 90–150 min after addition of drug and remained elevated up to 5 hr. In contrast, amsacrine stimulated the levels of c-*jun* mRNA only 3-fold in both cell lines, and its c-*jun* stimulatory effects were decreased at concentrations greater than 50 μM. VP-16 stimulation of c-*jun* mRNA levels resulted in a 2-fold increase in AP-1 binding activity in K562 but not in K/VP.5 cells. Taken together, these results suggest that posttranscriptional changes in c-*jun* mRNA regulation may be associated with acquired resistance to VP-16.

Key words: topoisomerase II; etoposide; c-*jun*; AP-1; K562 cells

The products of c-*jun* and c-*fos* (JUN and FOS) form a complex that activates [1] or represses [2] transcription by binding a regulatory promoter element, the AP-1† site [3]. C-*jun* mRNA levels are increased by extracellular stimulation from growth factors [4–6], differentiation-inducing drugs [7, 8], and cytotoxic agents [9–13]. The orderly regulation of AP-1 responsive genes is presumed to be a role for FOS–JUN dimers in the multiple changes of gene expression required for cells to progress through stages of proliferation and differentiation [14].

Increased constitutive levels of c-*jun* and c-*fos* mRNAs have been reported in MDR cells compared with drug-sensitive parental lines [15, 16]. In addition, treatment of cell cultures with the DNA topoisomerase II inhibitor etoposide (VP-16) and the DNA topoisomerase I inhibitor camptothecin has also been found to increase the rate of c-*jun* and c-*fos* transcription concomitant with the appearance of endonucleolytic DNA cleavage associated with programmed cell death [12, 17]. These studies suggest additional roles for the protein product of c-*jun* in the signal pathways(s) associated with the acquisition of multidrug resistance and DNA damage. Topoisomerase II, a target for VP-16 [18], is a DNA-binding protein that accomplishes conformational changes in DNA required for replication, transcription, and separation of chromatids during mitosis [19–27]. Topoisomerase II binds to DNA forming a transient double-strand break allowing, in an ATP-requiring step, subsequent passage of a second DNA duplex [21]. Like other topoisomerase II inhibitory drugs, VP-16 stabilizes the covalent cleavable complex between topoisomerase II and the 5′terminus of the cleaved DNA, inhibiting religation and enzyme turnover, and resulting in the accumulation of DNA strand breaks [28]. Mapping of topoisomerase II cleavage sites in the transcriptional regulatory sites of some genes [29, 30], and the activation of oncogene expression in cells exposed to DNA-damaging agents [9–12] suggest a role for topoisomerase II in the regulation of transcription.

We have reported previously increased basal levels of c-*fos* and c-*jun* mRNAs in a cloned subline of K562 cells (K/VP.5) selected for resistance to VP-16 [31]. The 30-fold-resistant K/VP.5 cells contain 5-fold less topoisomerase II protein and exhibit a 2.5-fold reduction of topoisomerase II mRNA levels due, in part, to a less stable topoisomerase II mRNA [31]. VP-16 (200 μM) was observed to increase the c-*jun* mRNA levels in both K562 and K/VP.5 cells, suggesting that c-*jun* mRNA levels are affected by extracellular signals in both cell lines [31].

In this study, we characterize in more detail the

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† Abbreviations: AP-1, activator protein 1; β_2m, beta-2-microglobulin; DTT, dithiothreitol; DMEM, Dulbecco's minimal essential medium; amsacrine, mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; MDR, multiple drug resistance; OCT-1, octamer-binding protein; TCA, trichloroacetic acid; topoisomerase II, DNA topoisomerase II (M_r 170,000 isoform); and VP-16, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylene-β-D-glucopyranoside).

levels of c-*jun* transcripts in K562 and K/VP.5 cells in the presence and absence of VP-16. We found that increased levels of c-*jun* in VP-16-resistant cells are due to posttranscriptional changes affecting the stability of c-*jun* mRNA and that the resulting increased levels of c-*jun* transcripts correlate with increased AP-1/DNA binding. In addition, AP-1 binding activity was found to be greater in VP-16 (100 μM) treated K562 cells but not in similarly treated K/VP.5 cells. Together with our previous observations of reduced levels of topoisomerase II mRNA in VP-16-resistant cells [31], the data presented in this study suggest that VP-16 differentially affects posttranscriptional regulation of c-*jun* and topoisomerase II mRNAs in K562 cells. Furthermore, VP-16-mediated changes in posttranscriptional regulation of these and perhaps other genes may play a role in the development of drug resistance.

MATERIALS AND METHODS

Chemicals and reagents. Etoposide (VP-16) was provided by the Bristol–Myers Squibb Co. (Wallingford, CT). Amsacrine (mAMSA) was obtained from the Drug Investigation Branch of the National Cancer Institute, and actinomycin D was obtained from the Sigma Chemical Co. (St. Louis, MO). Enzymes were obtained from either Boehringer Mannheim (Indianapolis, IN) or Bethesda Research Laboratories (BRL, Bethesda, MD). Cloned cDNAs for probes were provided by Dr. D. Bohmann (European Molecular Biology Laboratory, Heidelberg, Germany; c-*jun*), Dr. K. B. Tan (SmithKline Beecham Pharmaceuticals, King of Prussia, PA; β_2m), Dr. I. Verma (The Salk Institute, San Diego, CA; c-*fos*), Dr. L. F. Liu (UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ; topoisomerase II), and the American Type Culture Collection (Rockville, MD; c-*myc*). ^{32}P-labeled probes were synthesized as previously described [32].

Cells. The VP-16-resistant K/VP.5 cell line was derived by periodic and then continuous exposure of K562 cells to 0.5 μM VP-16 for 1 year, followed by cloning. K/VP.5 cells are 30-fold resistant to VP-16, 13-fold resistant to mAMSA, and have remained stably resistant for 2 years off drug [33]. K/VP.5 cells were cultured in the absence of drug for at least 3 days prior to experiments. Experiments were performed using mid-log phase cultured cells (4–8 $\times$ 10^5/mL) that were centrifuged and resuspended to a final concentration of 0.8 to 1.0 $\times$ 10^6/mL in DMEM containing the appropriate drug or 0.1% DMSO. Viability of drug-treated and control cells was assessed by staining cells with 0.2% Trypan Blue. Under the conditions used in the experiments reported here, greater than 95% of drug-treated cells excluded Trypan Blue.

Northern analysis. Total cellular RNA was purified from cells [34], and electrophoresed through formaldehyde-containing 1% agarose gels [31]. RNAs were blotted to Nylon membranes by capillary transfer and probed with radiolabeled cDNA; mRNAs for c-*jun* and β_2m were quantitated by densitometric scanning of autoradiographs. To correct for variable RNA content between gel lanes, autoradiographic signals for c-*jun* were normalized to those for β_2m.

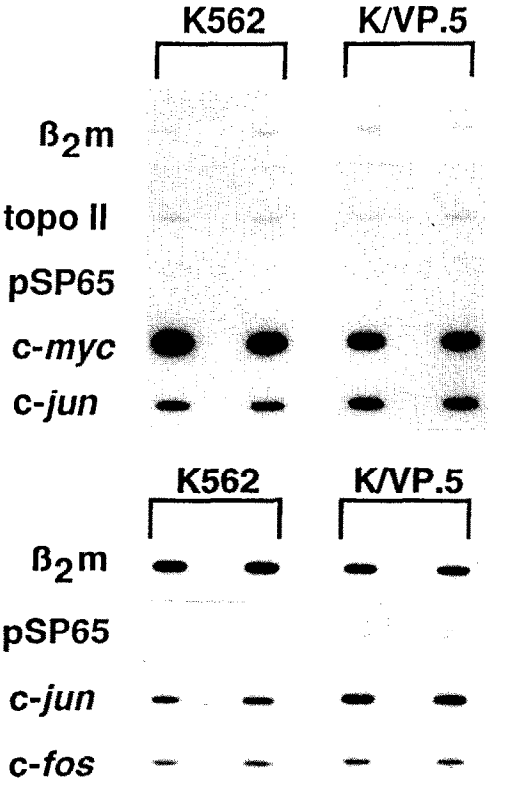

Fig. 1. Nuclear runoff assays in K562 and K/VP.5 cells. RNA was isolated from 5 $\times$ 10^7 nuclei incubated for 30 min in the presence of 250 μCi[α-^{32}P]GTP and hybridized for 3 days to duplicate slot blots of pSP65 plasmid DNA (background control) and cDNA for β_2m, c-*myc*, topoisomerase II, and c-*jun* and c-*fos*. Shown are two independent nuclear runoff assays.

Uridine incorporation. Rates of total RNA synthesis were determined by quantitating uridine incorporation into TCA-precipitable RNA. Mid-log phase cells (1 $\times$ 10^7) were incubated 0–15 min in DMEM containing 0.5 μCi/mL [5, 6-^{3}H]uridine (4.2 Ci/mmol). At various times, uridine incorporation was halted by the addition of 10 vol. of ice-cold saline after which cells were washed three times, resuspended in 10% TCA, incubated on ice for 10 min, collected on Whatman GF/B filters (Fisher Scientific, Pittsburgh, PA), and quantitated by liquid scintillation counting.

Transcription rates. Rates of c-*jun*, topoisomerase II, c-*fos*, c-*myc*, and β_2m mRNA synthesis were measured by nuclear runoff assays. Isolated nuclei from 1 $\times$ 10^8 mid-log cells were resuspended in 0.5 mL nuclei wash buffer (10 mM Tris–HCl, pH 8.0, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT), and pelleted for 5 min at 250 *g* in a Sigma-202MK microcentrifuge. Washed nuclei were resuspended in 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM

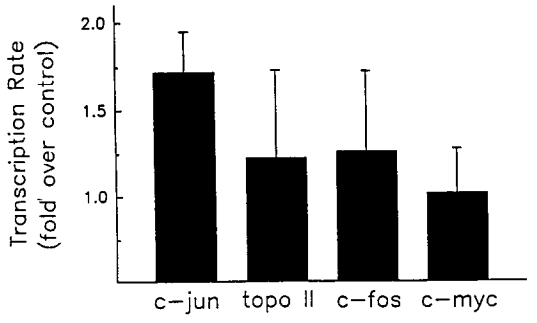

Fig. 2. Transcription rates in K562 and K/VP.5 cells. Relative transcription rates were quantitated from nuclear runoff assays (such as illustrated in Fig. 1) by densitometric scanning. Autoradiographic signals for c-*jun*, topoisomerase II, c-*fos*, and c-*myc* were normalized to that of β_2m. Results are presented as fold increase or decrease in transcription rates in K/VP.5 compared with K562 nuclei and represent the means ± SEM from three independent experiments.

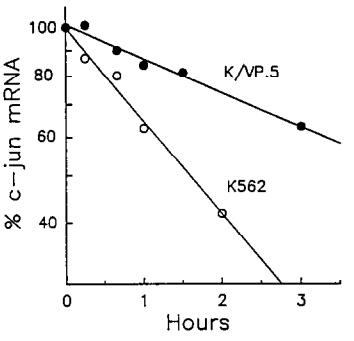

Fig. 3. Stability of c-*jun* mRNA in K562 and K/VP.5 cells. RNA was extracted at various times after the addition of 20 μM actinomycin D to mid-log phase cultures of K562 and K/VP.5 cells. RNA was electrophoresed and subjected to northern analysis; autoradiographic signals were scanned and quantitated as described in Materials and Methods. A representative first-order decay curve is presented in which the $T_{1/2}$ of c-*jun* mRNA was determined to be 91 min for K562 cells and 271 min for K/VP.5.

EDTA, 0.5 mM DTT, 40% glycerol to a final concentration of 1–2 × 10^7 nuclei/0.1 mL. 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 nuclei mixture was incubated for 5 min on ice and then 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 at 65° in 0.2 mL TES (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1% SDS), and chromatographed through a 5-mL Sephadex G50 column equilibrated with TES. The leading peak fraction from each column was collected and quantitated, and equivalent counts (usually 10^7 cpm/10^7 nuclei) were hybridized to 2 μg slot blots of plasmids containing the genes of interest. Hybridization was carried out at 50° for 70–80 hr in 5× SSPE (0.73 M NaCl, 0.05 M $NaH_2PO_4 \cdot H_2O$, 5 mM EDTA, pH 7.5), containing 1% SDS, 50 μg/mL sonicated and denatured salmon sperm DNA, 25% formamide, followed by three washes each in 1× SSC (0.15 M sodium chloride + 0.015 M sodium citrate)/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.

AP-1 assays. Nuclear extracts containing AP-1 were prepared from 5–10 × 10^7 cell nuclei with a high salt buffer (0.42 M NaCl), as described by Dignam *et al.* [35]. Ten nanograms of a 21 base pair oligonucleotide containing one copy of an AP-1 binding site (5′-$(N)_6$TGACTCA$(N)_8$-3′) was end-labeled using [α-^{32}P]dCTP (2000 Ci/mmol) and Klenow fragment and purified from unincorporated nucleotides by passage through Sephadex G-50 (Pharmacia, Inc., Piscataway, NJ) spin columns [36]. For binding assays, 10–20 μg of nuclear protein was incubated at room temperature for 30 min with 10,000 cpm of ^{32}P-labeled AP-1 oligonucleotide [1]. AP-1 bound oligonucleotides were resolved from unbound oligonucleotides by separation on 6% polyacrylamide/1× Tris–borate–EDTA (TBE) gels. Gels were dried and autoradiographed overnight, and "shifted" bands were excised and counted. As an internal control for nuclear protein content, OCT-1 binding was similarly assayed. The plasmid pUC-OCT-1 (obtained from Dr. Paul Robbins, University of Pittsburgh, Pittsburgh, PA) was digested with *Hin*dIII and *Eco*RI and 20 ng was end-labeled as described for AP-1 (above). For binding assays, 1–2 μg nuclear protein was incubated with 20,000 cpm of ^{32}P-labeled OCT-1 cDNA.

RESULTS

C-jun mRNA levels in K562 and K/VP.5 cells. Previously, we reported that c-*jun* and c-*fos* mRNA levels are constitutively elevated *ca.* 3-fold in VP-16-resistant K/VP.5 cells, which exhibit reduced levels of topoisomerase II mRNA compared with parental K562 cells [31]. For this study, we chose to focus our analysis on c-*jun* transcripts since the protein product of c-*fos* forms a biologically active AP-1 complex as a heterodimer with other AP-1 proteins such as the c-*jun* product [1, 37, 38]. In addition, c-*fos* mRNA is induced by a wide variety of stimuli but does not always result in subsequent formation of a biologically active AP-1 complex [14]. During the course of the experiments reported in this study, basal c-*jun* mRNA levels in K/VP.5 were elevated 3.42 ± 0.63-fold (mean ± SEM from 7 determinations; P = 0.008, Student's paired *t*-test), compared with K562 cells.

RNA synthesis rates. The relative rate of total RNA synthesis in K562 and K/VP.5 cells was determined by measuring [5,6-^{3}H]uridine incorporation into TCA-precipitable RNA after a 15-min incubation with cells at 37°. The ratio of RNA synthesis rates for K/VP.5 compared with K562 cells

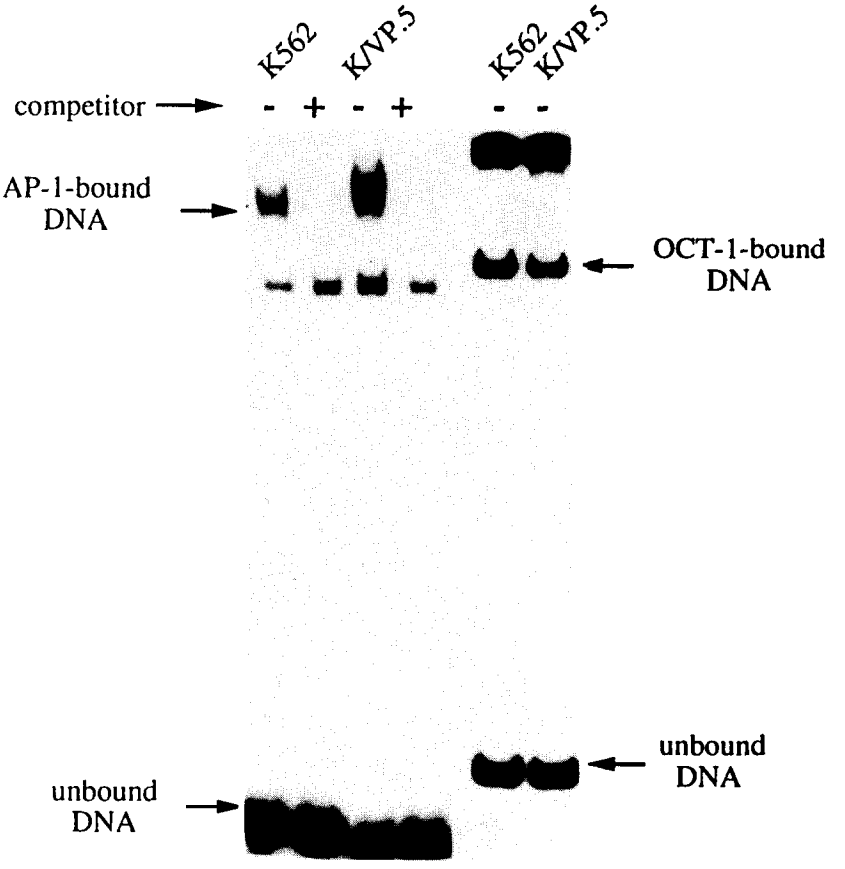

Fig. 4. AP-1 binding activity in K562 and K/VP.5 cells. Nuclear extracts were analysed for AP-1 binding activity in a gel shift assay using ^{32}P-labeled oligonucleotides containing an AP-1 binding site or cDNA containing the OCT-1 binding site (described in Materials and Methods). As a competitor, 1 ng unlabeled oligonucleotide was used (lanes labeled+).

was 0.98 ± 0.29 (mean ± SEM from five independent experiments). Therefore, the altered levels of expression of some mRNAs were not attributable to changes in bulk RNA synthesis.

We next isolated nuclei from K562 and K/VP.5 cells allowing nascent RNAs to elongate in the presence of [α-^{32}P]GTP (nuclear runoff). Hybridization of ^{32}P-labeled nuclear RNA to slot blots of cDNAs of β_2m, c-*fos*, c-*jun*, c-*myc*, topoisomerase II, and pSP65 (a negative hybridization control) allowed for quantitation of autoradiographic signals by densitometric scanning. After normalizing the signals to those for β_2m, we found no significant differences in the rates of transcription for c-*fos*, c-*jun*, c-*myc*, or topoisomerase II between K562 and K/VP.5 cells (Figs. 1 and 2). The 1.7-fold greater rate of c-*jun* transcription in K/VP.5 compared with K562 cells approached significance ($P = 0.089$, paired Student's *t*-test; Fig. 2). However, additional experiments indicated that the regulation of c-*jun* in these cells is more likely to be posttranscriptional (see below).

C-jun *mRNA stability*. RNA was isolated at various times after cells were exposed to 20 μM actinomycin D, and c-*jun* mRNA was quantitated by northern blot analysis. Over 95% of actinomycin D-treated cells retained the ability to exclude Trypan Blue up to 24 hr after drug addition; β_2m mRNA levels remained constant, but uridine incorporation was inhibited greater than 80% after 1 hr and greater than 95% after 2.5 hr (data not shown). First-order decay curves indicated that the stability of c-*jun* mRNA from K/VP.5 cells was 2-fold greater compared with K562 cells (Fig. 3). Averaging the results from 5 separate experiments, the $T_{1/2}$ of c-*jun* mRNA in actinomycin D-treated cells was 80 ± 9 min for K562 and 160 ± 41 min for K/VP.5 cells (mean ± SEM, $P = 0.034$, paired Student's *t*-test). In contrast to the results obtained for c-*jun*, K/VP.5 topoisomerase II mRNA is nearly 2-fold *less* stable than K562 topoisomerase II mRNA [31]. Together, these results suggest that phenotypic changes have occurred in K/VP.5 cells that differentially affect the stability of some mRNAs.

AP-1 activity. We next quantitated the change of a functional c-*jun* product (JUN), which is a component of the AP-1 complex [37–40]. Levels of AP-1 were assessed by mobility shift assays using a ^{32}P-labeled synthetic oligonucleotide containing an internal AP-1 binding site. To control for total

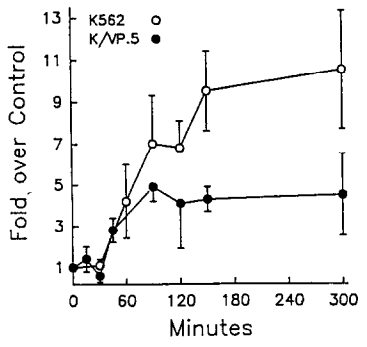

Fig. 5. VP-16-induced c-*jun* expression in K562 and K/VP.5 cells. RNA was isolated from cells incubated for various times with 200 μM VP-16; c-*jun* mRNA levels were quantitated from northern blots as described in Materials and Methods. Results are presented as fold increase in c-*jun* levels compared with 0.1% DMSO-treated controls; each data point represents the mean ± SEM from at least three separate experiments.

extractable nuclear proteins, similar assays were performed using a ^{32}P-labeled cDNA containing the consensus site for the constitutive transcription factor, OCT-1 [41] (Fig. 4). As shown in Fig. 4, incubation of radiolabeled oligonucleotide with nuclear extracts resulted in three bands subsequent to electrophoresis. Free (unbound) DNA electrophoresed most rapidly. The mobility of protein bound DNA was shifted to the top of the gel. To identify the AP-1 bound DNA, 50-fold excess unlabeled oligonucleotide was added to some assays (+competitor). The identity of the band below AP-1 is unknown, but is commonly observed with mobility shift assays using nuclear extract from human cells [42]. Because unlabeled oligonucleotide did not reduce the intensity of the lower band, the proteins in this unknown complex did not compete with AP-1 for binding to the oligonucleotide. The results shown in Fig. 4 indicated that extractable AP-1 binding activity in K/VP.5 cells was increased 1.9 ± 0.1-fold (mean ± SEM from five nuclear extract preparations, P = 0.0003, paired Student's *t*-test) compared with K562 cells. Because JUN is a major component of the AP-1 complex [38, 39], these results suggest that AP-1 binding is elevated in K/VP.5 cells as a consequence of increased c-*jun* mRNA levels.

C-jun mRNA levels in VP-16-treated cells. Increased levels of c-*jun* mRNA in K562 and K/VP.5 cells treated with 200 μM VP-16 began 30–60 min after addition of drug, reached a maximum at 90 min (K/VP.5) or 150 min (K562), and remained elevated for at least 5 hr (Fig. 5). In K562 cells, c-*jun* mRNA returned to baseline levels 12 hr after addition of VP-16 (not shown). Measurements of total RNA synthesis in both cell lines showed that uridine incorporation was inhibited by VP-16 in a concentration-dependent manner and to the same extent (up to 80%) in both K562 and K/VP.5 cells (Fig. 6A). This inhibition was reversible upon removal of drug from cells (Fig. 6B), in agreement with previous studies demonstrating that VP-16 interferes with uridine transport [43]. Thus, VP-16 induced an elevation of c-*jun* mRNA levels under conditions where total RNA synthesis was inhibited.

Both K562 and K/VP.5 cells exhibited a concentration-dependent increase in c-*jun* transcripts with increasing concentrations of VP-16 (Fig. 7A). At all VP-16 concentrations examined, c-*jun* mRNA levels were increased to a greater extent in K562 than in K/VP.5 cells. In contrast, the topoisomerase II inhibitor amsacrine (mAMSA) [44] stimulated c-*jun* levels similarly in both cell lines, was less effective than VP-16 in increasing c-*jun* levels, and was self-limiting at concentrations greater than 50 μM (Fig. 7B). No effect on c-*jun* transcripts was observed with 30 μM cisplatin or 10–20 μM actinomycin D (not shown).

*Transcription rate of c-*jun *in VP-16-treated cells.* Using nuclear runoff assays (Fig. 8), the relative rates of c-*jun*, topoisomerase II, c-*fos*, and c-*myc* transcription were determined in cells treated with or without 100 μM VP-16 for 2.5 hr (conditions under which maximal c-*jun* levels were observed; see Figs. 5 and 7A). The greatest change in the c-*jun* transcription rate occurred in VP-16-treated K562 cells (2.4-fold increase); however, this change was not statistically significant (P = 0.08, paired Student's *t*-test). Furthermore, rates of c-*jun* transcription in VP-16-treated K562 or K/VP.5 cells, as measured by nuclear runoff assays, were not comparable to the 3- to 10-fold increase in c-*jun* mRNA found in these cells when treated with VP-16 (Fig. 7).

AP-1 binding activity in VP-16-treated cells. AP-1 binding activity in cells treated for 3 hr with 100 μM VP-16 or 0.1% DMSO was measured using proteins extracted from nuclei exactly as for Fig. 4. AP-1 binding activity was increased by VP-16 treatment of K562 but not K/VP.5 cells (not shown). Results of four independent experiments indicated that AP-1 activity was increased significantly 2.3 ± 0.4-fold in VP-16-treated K562 cells compared with DMSO-treated controls (mean ± SEM; P = 0.018, paired Student's *t*-test), but was comparable in VP-16 treated K/VP.5 cells (0.9 ± 0.1, mean ± SEM).

DISCUSSION

The VP-16 resistant K562 cell line (K/VP.5), which shows reduced levels and activity of DNA topoisomerase II [33], also exhibits increased basal levels of the proto-oncogene mRNAs for c-*jun* and c-*fos* [31]. In addition, levels of c-*jun* transcripts were increased further after a 5-hr exposure of both K562 and K/VP.5 cells to VP-16 [31]. In this study we have characterized in more detail the resistance-associated and VP-16-induced levels of c-*jun* mRNA and AP-1 in VP-16-sensitive and -resistant K562 cells.

Increased levels of c-*jun* mRNA in K/VP.5 cells were due to posttranscriptional changes affecting mRNA stability (Figs. 1–3) and resulted in 2-fold increased AP-1 binding activity compared with K562 cells (Fig. 4). Our findings suggest that *increased* c-*jun* mRNA levels and *decreased* topoisomerase II mRNA levels [31] in K/VP.5 cells result from changes

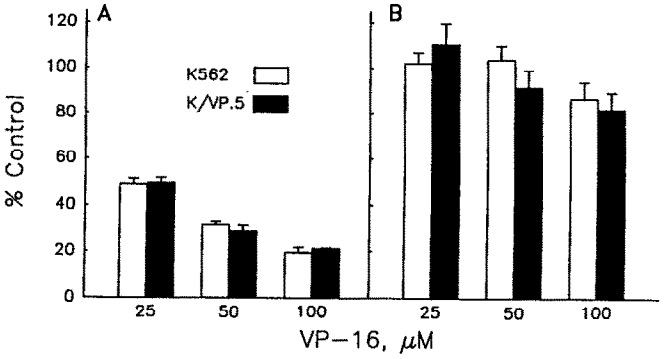

Fig. 6. Uridine incorporation in K562 and K/VP.5 cells incubated with VP-16. (A) K562 and K/VP.5 cells were incubated for 2.5 hr in the presence of 0–100 μM VP-16 and then were pulse-labeled for 10 min with 1 μCi/mL [5,6-^{3}H]uridine. (B) Cells were washed free of VP-16 prior to pulse labeling. TCA-precipitable counts were obtained as described in Materials and Methods. Results are shown as percent incorporation in VP-16-treated compared with 0.1% DMSO-treated control cells and represent the means ± SEM from 3–5 separate experiments. The control rate of uridine incorporation in K562 and K/VP.5 cells was 1080 ± 60 and 1170 ± 190 cpm/min/10^6 cells, respectively (mean ± SEM from four separate experiments).

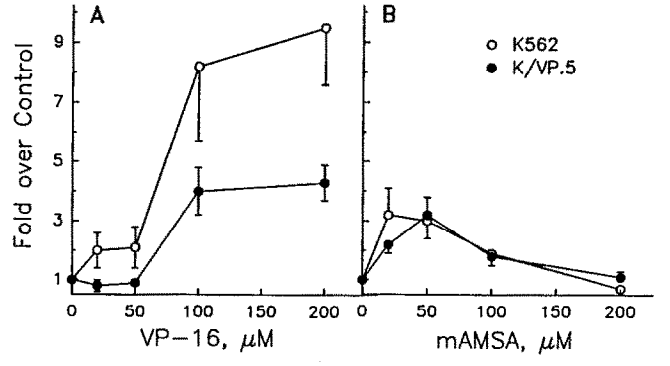

Fig. 7. Concentration-dependent induction of expression of c-*jun* by VP-16 (A) and mAMSA (B) in K562 and K/VP.5 cells. RNA was isolated from cells incubated for 2.5 hr in the presence 0–200 μM VP-16 or mAMSA, and c-*jun* mRNA was quantitated exactly as for Fig. 5. Results represent the means ± SEM from at least three separate experiments.

in posttranscriptional regulation that differentially affect mRNA stability. Based on information available regarding the determinants of mRNA stability, continuous exposure to VP-16 during the selection of K/VP.5 cells may have resulted in changes in the levels or activities of proteins that control mRNA processing, transport from nucleus to cytoplasm, or degradation [45–47].

Although reports of transient elevations of c-*fos* and c-*jun* mRNA levels in response to extracellular stimuli are common [1, 4–13], *constitutive* overexpression of c-*fos* and c-*jun* is an unusual observation [15, 16, 48]. Elevated basal levels of c-*jun* and c-*fos* (and ultimately AP-1) in K/VP.5 and other drug-resistant lines may play an essential role in regulating the expression of a gene or multiple genes needed for the establishment of anticancer drug resistance. For example, increased constitutive levels of c-*fos* mRNA have been observed in cisplatin-resistant tumor cells, which suggests a role for this gene in DNA repair [48]. Also, basal levels of c-*jun* and c-*fos* were observed to be increased in several multidrug-resistant cell lines [15, 16]. Finally, the presence of an AP-1 binding site in the promoter region of the P-glycoprotein gene supports a role for AP-1 in MDR [49]. Since VP-16-resistant K/VP.5 cells contain reduced levels of topoisomerase II [31, 33], increased levels of AP-1 may be responsible for regulation of topoisomerase II expression in these cells. However, we found that the transcription rate of topoisomerase II in K/VP.5 cells is the same as for K562 cells (Fig. 1) [31]. Furthermore, sequencing of the promoter region of the human topoisomerase II gene revealed several transcription regulatory elements but no AP-1 binding sites [50]. Therefore, it is unlikely that increased levels of FOS and/or JUN play a role in regulating topoisomerase II expression in resistant K/VP.5 cells.

It is not clear, therefore, what role increased levels of AP-1 play in acquired resistance to VP-16. It is

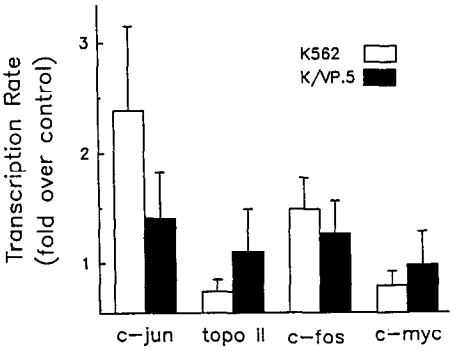

Fig. 8. Transcription rates in VP-16-treated K562 and K/VP.5 cells. Nuclear runoff assays were performed exactly as described for Fig. 1 after exposure of cells to 100 μM VP-16 for 2.5 hr. Relative transcription rates were quantitated as described for Fig. 2. Transcription rates for c-*jun*, topoisomerase II, c-*fos*, and c-*myc* in VP-16-treated cells are expressed as fold increase or decrease compared with 0.1% DMSO-treated controls and represent the means ± SEM from three separate experiments.

possible that increased levels of c-*jun*, c-*fos*, and AP-1 protein observed in K/VP.5 cells are the result of a stochastic event that occurred during the selective conditions used to derive and clone this cell line. To determine if increased c-*jun* expression plays a role in resistance to VP-16, we are currently introducing vectors that express c-*jun* into K562 cells and using c-*jun* antisense oligonucleotides to reduce c-*jun* levels in K/VP.5 cells.

VP-16 treatment increased c-*jun* mRNA levels to a greater extent (>2-fold) in K562 than in K/VP.5 cells (Figs. 5 and 7A). After accounting for the 3.4-fold higher constitutive level of c-*jun* mRNA in K/VP.5 compared with K562 cells, these results indicate that total c-*jun* levels in sensitive and resistant cells may be nearly the same after incubation with 100–200 μM VP-16. Also, VP-16 (100 μM) increases AP-1 binding (2.3-fold) in K562 but not in K/VP.5 cells. This normalizes for the 2-fold greater constitutive AP-1 binding activity in K/VP.5 cells (Fig. 4). Therefore, the results from acute treatments with high concentrations of VP-16 suggest that there may be a maximal stimulation of c-*jun* levels and AP-1 activity by VP-16, which may be independent of the action of this drug at the level of topoisomerase II and which may be independent of the resistant phenotype.

In contrast to VP-16, mAMSA was less effective in stimulating c-*jun* levels (Fig. 7), while cisplatin and actinomycin D failed to induce c-*jun* expression (not shown). Thus, the c-*jun* stimulatory effect of VP-16 in K562 cells is not a general response to drug-induced DNA damage and may depend on intracellular drug targets that ultimately regulate c-*jun* levels. Though both mAMSA and VP-16 are topoisomerase II inhibitors, only mAMSA is a DNA intercalator. Amsacrine has also been demonstrated to bind to extranuclear sites [51]. Therefore, the lack of similar responses of K562 and K/VP.5 cells to VP-16 and mAMSA, in terms of stimulation of c-*jun* levels, may relate to different intracellular binding characteristics and/or mechanism(s) of resistance. For example, we have demonstrated that VP-16-induced topoisomerase II/DNA binding is less stable in K/VP.5 than in K562 cells [33]. In contrast, mAMSA-induced topoisomerase II/DNA complexes dissociated at similar rates in both K562 and K/VP.5 cells.* Thus, 30-fold VP-16 resistance in K/VP.5 cells is likely due to both quantitative and qualitative changes in topoisomerase II [31], while the lower 13-fold cross-resistance to mAMSA may be due only to the quantitative decrease in topoisomerase II levels in K/VP.5 compared with K562 cells. The different mechanism(s) of resistance to VP-16 and cross-resistance to mAMSA in K/VP.5 cells may result in differential c-*jun* stimulation in response to these agents.

The concentrations of VP-16 required for appreciable stimulation of c-*jun* (greater than 50 μM) were at least 100-fold greater than those required for stimulation of c-*jun* in HL-60 cells in a previous study [12]. In addition, oligonucleosomal DNA cleavage, shown in HL-60 cells to be associated with drug-induced apoptosis [52] and to be temporally related to induction of c-*jun* expression [12, 17], was absent in K562 and K/VP.5 cells treated with up to 200 μM VP-16.† These observations indicate that the gene regulatory and apoptotic effects of cytotoxic drugs such as VP-16 are cell line dependent.

A role for topoisomerase II in the regulation of transcription of some genes has been proposed [26, 29, 30]; however, results from the nuclear runoff experiments presented here suggest that exposure of K562 or K/VP.5 cells to VP-16 (which induces topoisomerase II/DNA binding) did not affect the initiation of transcription of c-*jun* or the highly regulated genes c-*myc* and c-*fos* (Fig. 8). The lack of stimulation of c-*jun* transcription rates in VP-16-treated K562 cells contrasts with previous reports demonstrating increased rates of transcription of c-*jun* in HL-60 and/or U937 cells treated with several DNA-damaging agents [9, 10–12, 17]. For example, Rubin *et al.* [12] demonstrated increased rates of c-*jun* transcription in the nuclei of VP-16-treated HL-60 cells, with no detectable c-*jun* transcription observed in nuclei from untreated HL-60 cells. Yet, we could readily detect initiation of this transcript in nuclei from K562 and K/VP.5 cells in the absence of VP-16 (Fig. 1). Thus, the lack of further stimulation of the c-*jun* transcription rate in K562 or K/VP.5 compared with HL-60 cells [12] after VP-16 treatment may be attributable to different signal transduction pathways in these cell lines.

A myriad of events has been associated with the induction of c-*jun* and c-*fos* by growth stimulatory and inhibitory agents [14, 38]. In most cases, the c-*jun* stimulatory effects of a cytotoxic drug observed during a brief incubation period are short-lived. The early induction of c-*jun* following incubation with toxic concentrations of drugs such as VP-16 would be expected to involve a signaling pathway much different from that associated with establishment of drug resistance. Therefore, assigning a more precise

* Yalowich JC and Allan WP, unpublished observation.

† Ritke MK, Rusnak J, Allan WP, Dive C, Lazo JS and Yalowich JC, manuscript submitted for publication.

role for c-*jun* in the development and/or maintenance of resistance to topoisomerase II inhibitory drugs, such as VP-16, will require monitoring c-*jun* and AP-1 expression during the establishment of acquired drug resistance rather than under conditions where short exposure times and high drug concentrations are utilized.

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