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Expression of c-*fos* in Human and Murine Multidrug-Resistant Cells

ALOK BHUSHAN, ROBIN ABRAMSON, JEN-FU CHIU, and THOMAS R. TRITTON

Departments of Pharmacology (A.B., R.A., T.R.T.), Biochemistry (J.-F.C.), and Cell and Molecular Biology (R.A., J.-F.C., T.R.T.), The University of Vermont College of Medicine, Burlington, Vermont 05405

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SUMMARY

In both mouse sarcoma 180 and human KB cells selected for the multiple drug resistance (MDR) phenotype, there is an elevation in the steady state mRNA level of c-*fos*. There is no detectable gene amplification for c-*fos*, nor is there any significant change in the rate of mRNA transcription or degradation, suggesting that other factors are responsible for the increased expression level in resistance. Cells selected for resistance to methotrexate, a drug not in the MDR group, do not have an increase in c-*fos* mRNA expression. When drug-sensitive cells are exposed for 30 min to an ED_{50} concentration of vinblastine, Adriamycin, colchicine, or VP-16, but not to methotrexate or cisplatin, there is a 3–6-fold induction in the level of c-*fos* message. Because the former drugs are members of the MDR class and the latter are not, the results are consistent with the hypothesis that induction of c-*fos* by low levels of cytotoxic drugs may be an early event in the acquisition of the MDR phenotype. If this were the case, then c-*fos* would be expected to act in concert with c-*jun* to control transcription by binding to a specific DNA regulatory site. Consistent with this explanation is the existence of an AP-1 sequence in the promotor region for the P-glycoprotein gene (*mdr1*), as well as the fact that c-*jun* is also overexpressed in MDR cells.

Cells in culture that are exposed to a variety of antiproliferative drugs become resistant to the selecting agent and, simultaneously, to many other toxic compounds (1, 2). Elevated expression of a particular plasma membrane protein, termed P-glycoprotein, is necessary and sufficient to induce this MDR phenotype. Drug-sensitive cells that have never been exposed to selecting agents but that express P-glycoprotein, as a result of either transfection or cell fusion (3, 4), also acquire the set of characteristics that operationally define MDR, i.e., cross-resistance to an assortment of structurally unrelated drugs, reduced intracellular accumulation of the drugs, and the ability to be modulated to higher drug sensitivity by agents like verapamil and trifluoperazine (5, 6). Thus, expression of this single protein appears to be the dominant characteristic that governs whether a cell will express the primary MDR attributes.

MDR cells may also express a wide variety of additional phenotypic properties that are seemingly unrelated to P-glycoprotein. These include the increase in c-*fos* expression reported here, altered levels of cellular proteins like cytochrome P-450 (7), topoisomerase II (8), sorcin (9), and glutathione-*S*-transferase (10), and cell surface changes in lipid composition (11, 12), membrane fluidity (13), and display of receptors for epidermal growth factor (14). The functional relationship between any of these changes in cells and the expression of the MDR phenotype is not clear, but it is evident that, when cells are selected for MDR, they are simultaneously selected for a variety of additional characteristics that are not explainable solely by reference to P-glycoprotein. The function of the P-glycoprotein, purportedly as an efflux pump, also does not universally explain the intracellular concentrations of cytotoxic agents in a way that closely links uptake or efflux with drug action. For example, our laboratory has shown, in a series of S180 cells of progressively increasing P-glycoprotein content and decreasing Adriamycin sensitivity, that the amount of intracellular drug is unrelated to the degree of drug cytotoxicity (15). Similarly, Sonka *et al.* (16) showed by flow cytometry that extrusion of Adriamycin was not sufficient to ensure drug resistance, and Lothstein and Horwitz (17) found that cells that were allowed to revert to normal drug sensitivity had lowered P-glycoprotein but retained the ability to maintain lowered intracellular levels. Thus, the relationship between intracellular drug levels and the relative sensitivity or resistance of a cell is not always straightforward.

Our laboratory is interested in the possibility that signal transduction pathways may act to link the expression of P-glycoprotein with other biochemical responses seen in MDR cells. Many of the proteins involved in cellular growth control and signaling systems are coded for by proto-oncogenes, so we have examined the expression of selected proto-oncogenes in MDR cells. In this paper, we report a consistent association of

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ABBREVIATIONS: MDR, multidrug-resistant; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

elevated c-*fos* expression with the MDR phenotype. Because the presumed function of c-*fos* is to act as a regulator of gene transcription, the results suggest a model whereby this proto-oncogene may control the expression of the various factors that constitute the MDR phenotype.

Materials and Methods

Cell lines. S180A1, S180A3, and S180A10 cells were selected by exposing S180 mouse sarcoma cells to increasing concentrations of Adriamycin and are 6-, 11-, and 125-fold resistant, respectively. The cells were grown in modified McCoy's medium (GIBCO) with 10% heat-inactivated horse serum (GIBCO), at 37°, in an atmosphere of 95% air/5% CO_2. Cells were grown in plastic tissue culture dishes obtained from Corning Glass Works (Corning, NY). S180A1, S180A3, and S180A10 were continuously maintained in 0.05, 0.2, and 2.0 μM Adriamycin, respectively. Medium was changed every 3–4 days.

Human KB cells were grown as monolayers in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% fetal calf serum, 1% L-glutamine, and 100 U/ml penicillin/streptomycin, at 37°, in 5% CO_2. KB-A1 cells and KB-A10 cells (obtained from Dr. M. Gottesman, National Cancer Institute) were maintained in 1 μg/ml Adriamycin and 10 μg/ml Adriamycin, respectively, and are 100- and 1000-fold resistant. GRC1 cells (KB cells transfected with a single copy of the *mdr1* gene) were obtained from Dr. Igor Roninson (University of Illinois); these cells were maintained as described above, except that 8 ng/ml colchicine replaced Adriamycin in the medium.

Highly methotrexate-resistant KB cells (>1000-fold resistant) were obtained from Dr. Bruce Dolnick (Roswell Park Memorial Institute). These cells were grown as monolayers in RPMI 1640 with 5% fetal calf serum and were maintained in 50 μM methotrexate and 30 μM thymidine.

RNA preparation. Cell lines were maintained in drug-free medium for at least 1 week before RNA was isolated. Total cellular RNA was isolated by the method of Glisin *et al.* (18). Briefly, cells were washed two times with phosphate-buffered saline and suspended in 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M mercaptoethanol, pH 7.0. The RNA was then purified by one of two methods in different experiments. In method 1, used for S180 cells, the lysate was passed five times through a 27-gauge needle to shear DNA, layered on a 5.7 M CsCl cushion, and centrifuged at 120,000 × *g* for 14 hr at 20°, in a Beckman ultracentrifuge (model L8-M). The pellet was solubilized in TE buffer, extracted once with phenol and once with chloroform/isoamyl alcohol (24:1), and precipitated with ethanol. In method 2, used for KB cells, the lysate was extracted with phenol and chloroform/isoamyl alcohol, and the resulting aqueous layer was precipitated with isopropanol. The pellet was resuspended in guanidinium solution and reprecipitated with isopropanol. After suspension of the pellet in RNase-free water, the RNA was treated with DNase and proteinase K and was extracted with phenol and chloroform/isoamyl alcohol. The RNA was then precipitated with ethanol. After centrifugation, RNA was resuspended in TE buffer and quantified spectrophotometrically.

Northern blot analysis. Denatured total RNA was electrophoretically fractionated on a 1% formaldehyde agarose gel, transferred to nitrocellulose in 20× SSC overnight, and baked at 80° for 2 hr. The blots were prehybridized in 50% formamide, 5× SSC, 0.25 mg/ml denatured salmon sperm DNA, 5× Denhardt's reagent (1 mg/ml each of Ficoll, bovine serum albumin, and polyvinylpyrrolidone), 50 mM phosphate buffer, pH 6.5, 0.1% SDS, 2 mM EDTA, overnight at 42°, and were hybridized (19) in 50% formamide, 5× SSC, 1× Denhardt's reagent, 0.1 mg/ml salmon sperm DNA, 10 mM phosphate buffer, pH 6.5, 0.1% SDS, 2 mM EDTA, 10% dextran sulfate. The blots were washed twice with 2× SSC, 0.1% SDS, for 5 min at room temperature, and twice with 0.2× SSC, 0.1% SDS, for 15 min at 55°, followed by autoradiography. The films were scanned using a Shimadzu dual-wavelength scanner (model CS-930). We have found that the β-actin probe that is commonly used to control for equal nucleic acid loading is not suitable for this system, because its quantity varies with the degree of drug resistance and the proliferation status of the cells. Consequently, all experiments shown used both A_{260} values and ethidium staining as an approximate test for equal gel loading. We have also used the 28 S rRNA probe pI-19 as a test of gel loading (see Fig. 1). In addition, we note that, whereas in the experiments reported here c-*fos* and c-*jun* are increased, other proto-oncogenes are decreased (c-*myc*) or unchanged (c-*src*) in MDR cells[1]; this argues against any systematic error in gel loading or an unusual mRNA instability, compared with the control rRNA.

Run-on transcription assay. Nuclei were prepared as described by Greenberg and Ziff (20), except that the final nuclear pellet was resuspended in 200 μl of nuclei freezing buffer [50 mM Tris·HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 M EDTA]. The run-on assay was performed using modifications of previously described methods (21). Nuclei from 1×10^7 cells were thawed at room temperature and incubated in 300 μl of reaction buffer (10 mM Tris·HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 1 mM levels each of CTP, ATP, and GTP, 0.5 mM dithiothreitol, 1 mM aurin-tricarboxylic acid), with 100 μCi of [α-^{32}P]UTP (3000 Ci/mmol; ICN) for 30 min at 30°. Reactions were stopped by addition of RNase-free DNase I to a final concentration of 12 μg/ml, in 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris·HCl, pH 7.4, and incubation at 30° for 5 min. Three hundred microliters μl of 1× SET (0.01 M Tris·HCl, pH 7.4, 5 mM EDTA, 1% SDS, 1 mM aurin-tricarboxylic acid) were then added, along with 15 μl of 25 μg/ml RNase-free proteinase K, and the reaction was incubated at 37° for 15 min. The reaction was extracted with equal volumes of phenol and chloroform/isoamyl alcohol (24:1). The organic phase was back-extracted with an equal volume of 1× SET, and the two supernatants were pooled and precipitated with 2 volumes of 95% ethanol and 0.1 volumes of 3 M ammonium acetate. Unincorporated nucleotides were removed on a Sephadex G-50 column. Labeled RNA was heated at 60° for 15 min, and equal counts for each cell line were hybridized to linearized plasmids immobilized on nitrocellulose, in the presence of 50% formamide, for 48 hr. The filters were washed twice for 5 min each in 2× SSC, 0.1% SDS, at room temperature and twice in 0.1× SSC, 0.1% SDS, at 60° and were exposed for autoradiography.

mRNA half-life. Cells were treated with actinomycin D (Sigma), at a final concentration of 10 μg/ml, for specified times. This level of actinomycin D is at least 2 orders of magnitude higher than the LD_{50} for the resistant cell lines and inhibits overall transcription in both sensitive and drug-resistant cell lines. The total RNA was isolated and processed as described above, to determine the remaining fraction as a function of time after inhibition of RNA synthesis.

DNA isolation. DNA was isolated from cells by using an established procedure (22). After extraction, DNA was digested with *Eco*RI (BRL Laboratories) under conditions recommended by the manufacturer. Digested DNA was electrophoretically separated on 1% agarose and partially hydrolyzed by soaking of the gel in 0.25 M HCl; the DNA was denatured in 0.5 M NaOH, 1.5 M NaCl, at room temperature for 15 min. The gel was neutralized in 1 M Tris·HCl, pH 8.0, 1.5 M NaCl, and transferred to nitrocellulose in 10× SSC overnight. After transfer, the membrane was baked at 80° for 2 hr and prehybridized overnight at 68° in a solution containing 6× SSC, 5× Denhardt's reagent, 0.5% SDS, and 200 μg/ml denatured salmon sperm DNA. Hybridization was carried out in 6× SSC, 5× Denhardt's reagent, 0.5% SDS, 10 mM EDTA, 200 μg/ml denatured salmon sperm DNA, with nick-translated probe ($2–3 \times 10^6$ cpm/ml), for 24 hr at 68°. Blots were washed twice with 2× SSC, 0.5% SDS, at room temperature for 10 min and twice with 0.2× SSC, 0.5% SDS, at 55° for 30 min and were then autoradiographed.

Probes. The plasmids pc-fos(mouse)-1 and pc-fos(human)-1 were purchased from the American Type Culture Collection. The *Xho*I/*Nco*I fragment of the human pc-fos-1 plasmid was used. Human pEHJ-2 c-*jun* was a gift from Dr. Peter Vogt (University of Southern California);

[1] A. Bhushan and T. R. Tritton, unpublished observations.

the *PstI* fragment was used as a probe. The 28 SrRNA probe pI-19 was a gift from Dr. Muthu Periasamy (University of Vermont).

Results

Fig. 1 shows Northern blot analysis of total RNA extracted from S180 and KB cells and from resistant sublines. The cells were grown for a minimum of 1 week in the absence of drug before isolation of RNA, and each experiment was reproducible throughout numerous independent repetitions. With both the mouse and human MDR systems, there is a consistent increase in the expression of c-*fos* in MDR cells. The films were quantified by densitometry and gave the following typical relationship between the degree of Adriamycin resistance and the overexpression of c-*fos:* S180, 1:6:11:122/1:1.4:1.7:2.5; KB, 1:100:1000/1:3:3. Thus, although the expression of c-*fos* is not linearly proportional to the level of drug resistance, there is a systematic increase in c-*fos* with increasing MDR. GRC1 cells, which are MDR as a result of transfection of a single copy of the *mdr1* gene into KB-3-1 cells, express c-*fos* at levels similar to those in the parent cells (Fig. 1). These experiments have been repeated more than 12 independent times, with the increase in c-*fos* being seen in all cases. For S180 versus S180A10 cells the mean fold increase in c-*fos* is 2.3 ± 0.34, whereas for KB-3-1 versus KB-A1 cells the increase is 2.6 ± 0.74-fold.

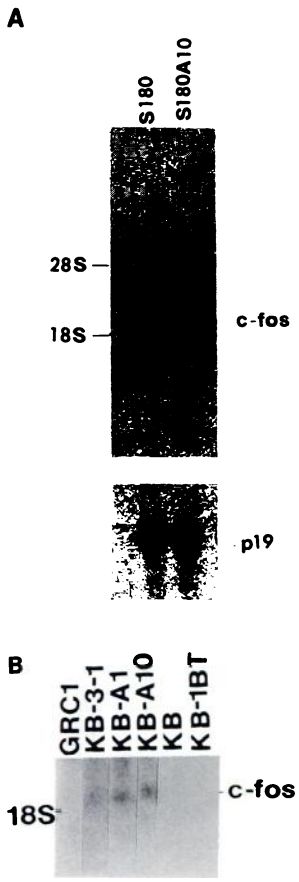

Fig. 1. Hybridization analysis of RNA from sensitive and resistant cells. A, Northern analysis (4 μg of total RNA) from S180 and S180A10 cells probed with the mouse pc-fos-1; the blots were stripped and rehybridized with the rRNA probe pI-19. The differences in c-*fos* expression were observed over a range of cell densities (300,000–900,000 cells/ml), suggesting that differences in proliferation status do not underlie the results. B, Northern analysis (20 μg of total cellular RNA) of KB-GRC1, KB-3-1, KB-A1, KB-A10, KB, and KB-1BT cells probed with the *XhoI*/*NcoI* fragment of pc-fos-1; the first four lanes are from a single experiment and the last two from a different one. KB cells are the KB-3-1 strain from which the KB-1BT cells were selected.

MDR cells often have amplified DNA sequences, most notably those coding for P-glycoprotein. To see whether amplified genomic DNA could account for the increased expression of c-*fos*, we isolated total DNA and compared the sensitive parent line with each resistant subline by Southern blotting, for both cell systems under study. The results (not shown) reveal that there is no apparent amplification of c-*fos* sequences in the S180- or KB-derived MDR cells.

In order to ascertain whether the higher levels of c-*fos* mRNA detected in MDR cells are due to an increased rate of transcription, we performed nuclear run-on assays, using a modification of previously described methods. In this experiment, one compares the rate of extension of previously initiated RNA transcripts, by hybridizing equal counts of the newly extended radiolabeled chains in each cell line to immobilized probes. The results shown in Fig. 2 for KB cells reveal that the c-*fos* transcriptional rate is similar in sensitive and resistant cells. Likewise, S180 MDR cells show no detectable differences in transcription rate from the sensitive parent line. In order to be sure that the overall rate of transcription is not measurably different between the sensitive and resistant cell lines, we measured total [^{32}P]UTP counts incorporated into the mRNA of each line and found that the general transcription rates were equal. This point was also verified by using histone H4 transcription rate as an internal control for each line (data not shown). Although the run-on assay is not sensitive enough to detect small changes in the transcription rate, we would be able to measure the 3-fold increase in transcript production if this mechanism accounted for the entire increase in steady state level. Thus, alterations in the rate of transcription are not likely to be the primary reason that the resistant cells show an increased amount of c-*fos* message.

A change in the degradation rate of c-*fos* message is another mechanism that could account for the increased expression of this gene in resistant cells. Because the basal steady state transcription level is relatively high in each cell line and because no change in the transcription rate is detected, we examined the message half-life of c-*fos* in each of the KB-derived cell lines (Fig. 3). KB-3-1, KB-A1, and KB-A10 cells have a $t_{1/2}$ of 25, 27, and 22 min, respectively. Although only a few data points were collected for each experiment, we performed four independent determinations of the half-life for each cell line, with an average standard deviation of 2.1 min. Therefore,

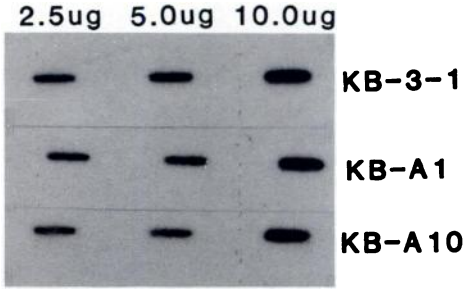

Fig. 2. Nuclear run-on assays of the transcription rate for c-*fos* in sensitive and resistant KB cells. No detectable levels of RNA polymerase II transcription were seen in the presence of 1 μg/ml α-amanitin, indicating that RNA synthesis catalyzed by other polymerases is not significant in this system. The pc-fos-1 probe did not hybridize to linearized pBR322 immobilized on the filter. Histone H-4 was used as an internal control, to ensure equivalent transcription rates between the cell lines.

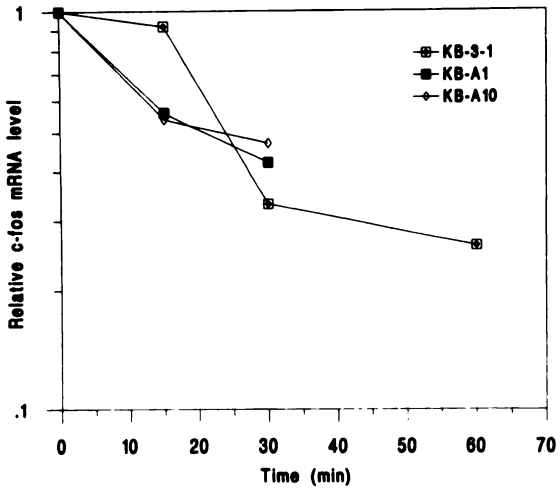

Fig. 3. c-*fos* message half-life measurements. The cells were incubated with 10 μg/ml actinomycin D for the indicated times. The blots from Northern analysis were scanned and plotted. Although it is not shown in this figure, we find that at short times (<15 min) the actinomycin treatment transiently increases c-*fos* expression before the decay process begins. Thus, the resistant cell time courses start at 15 min, so that true degradation rates can be determined.

Fig. 4. Northern blot analysis of 20 μg of total cellular RNA from KB-3-1, KB-A1, and KB-A10 cells. The blots were probed with the *Pst*I fragment of the human pEHJ-2 c-*jun* sequence.

changes in the $t_{1/2}$ of c-*fos* cannot, by itself, account for the increased expression level seen in MDR cells.

Because c-*fos* expression is increased in the human KB and mouse S180 MDR cells, we decided to analyze the expression of this gene in a cell line resistant to a drug not in the MDR group. KB-1BT cells were derived from KB-3-1 cells by selection in methotrexate and are >1000-fold resistant to this drug. These cells do not express detectable *mdr1* (data not shown). Furthermore, as seen in Fig. 1, KB-1BT cells do not have an increase in c-*fos* expression.

The cellular functioning of the c-*fos* protein is thought to be accomplished in combination with the protein encoded by another proto-oncogene, c-*jun*. We decided to determine whether c-*jun* expression was also modulated in the cell lines in which c-*fos* was elevated. The Northern blot shown in Fig. 4 indicates that c-*jun* is, indeed, elevated 2.7 ± 1.2-fold in the KB-A1 and 3.7 ± 1.6-fold in the KB-A10 cells. There is no increase in c-*jun* expression in the methotrexate-resistant KB-1BT cells (data not shown).

MDR sublines are generally selected by exposure of the

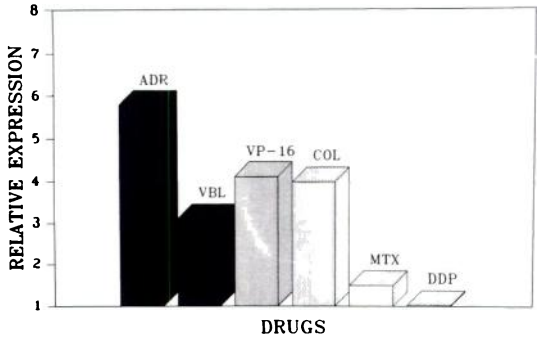

Fig. 5. Effect of cytotoxic drugs on the expression of c-*fos*. S180 cells were treated with 0.1 μM Adriamycin (*ADR*), 0.008 μM vinblastine (*VBL*), 0.5 μM VP-16 (*VP-16*), 0.25 μg/ml colchicine (*COL*), 0.04 μg/ml methotrexate (*MTX*), or 0.1 μg/ml *cis*-platinum (*DDP*), for 30 min. Total cellular RNA was isolated, analyzed by slot blot, and scanned. The values represent fold changes over untreated cells.

sensitive cells to subtoxic concentrations of cytotoxic agents. We wondered whether this level of exposure to drugs could, itself, induce the expression of c-*fos*, so we measured the level of the proto-oncogene message by slot blot hybridization, after exposure of sensitive parent cells to several cytotoxic agents. Fig. 5 shows that 30-min exposures to Adriamycin, vinblastine, VP-16, or colchicine, at the ID_{50} concentration, each induce higher levels of c-*fos*; these drugs are all members of the MDR group. In contrast, methotrexate and *cis*-platinum, drugs that are not in the MDR family, do not significantly increase the level of c-*fos* under the conditions used (low concentration for 30 min). Longer exposures, up to 24 hr, show qualitatively similar results, but the magnitude of the increase is smaller (~2-fold). These data do not prove that all MDR family drugs induce c-*fos* or that all non-MDR drugs do not (see Discussion), but the results are consistent with this classification scheme at the low concentrations used for selection of resistant cells. Verapamil, a Ca^{2+} channel blocker that can antagonize the MDR phenotype, also causes the level of c-*fos* to be elevated in exposed cells. Verapamil is also a cytotoxic agent, and recent results have shown that cells can be selected for resistance to it (24).[2]

Discussion

In MDR cells from two different species, we have shown that c-*fos* gene expression is increased, relative to that in drug-sensitive control cells. There is no increase in c-*fos* expression in a non-MDR, methotrexate-resistant cell line, nor is there an increase in KB cells that have acquired the MDR phenotype by transfection of the *mdr1* gene. The amount of increase is generally parallel to the degree of resistance and ranges up to severalfold for the most drug-resistant cells we have examined.

There are at least three commonly recognized mechanisms whereby the level of mRNA expression for a particular gene could be increased. One possibility is gene amplification, and this mechanism is known to occur in MDR cells in the gene coding for P-glycoprotein (25). In Southern blotting experiments using genomic DNA probed with a c-*fos* sequence, however, we detect no evidence of gene amplification, even in the most highly resistant cells. A second mode of increased gene expression involves escalation of the rate of transcription, either for overall RNA synthesis or for the particular gene in

[2] A. Bhushan, unpublished observations.

question (20). The nuclear run-on experiments show that neither the overall rate of RNA polymerase II-mediated transcription nor the rate of synthesis of the c-*fos* gene itself is detectably increased, even though the steady state levels of this message are higher in the MDR cells. The third potential mechanism that could account for the augmented level of c-*fos* message is increased stability of the particular mRNA, which has been observed for this message in other systems (26, 27). Using actinomycin D to inhibit RNA polymerase II, we find no change in the c-*fos* message half-life in any of the cells. It should be noted, however, that the methods used to determine the input and output for the mRNA pool are not highly accurate. Thus, if both transcription rate and degradation rate contribute approximately equally to the 3-fold increase in c-*fos* expression in KB MDR cells, their individual contributions would probably not be detectable by our methods, and this may be a plausible explanation for the results.

Other considerations that may affect the interpretation of the role of c-*fos* half-life in resistant cells are the cross-resistance of these cells to actinomycin D and our observation that, 15 min after actinomycin D treatment, c-*fos* expression actually increases transiently in KB-A1 and KB-A10 cells before it starts to decline. The standard concentration of actinomycin used is >41,000-fold higher than the LD_{10} for this drug in KB-3-1 cells, so this concentration should overcome the inherent cross-resistance. Also, because we do observe message degradation after actinomycin treatment, it is evident that this drug is effective at the concentration used. However, we cannot discount the possibility that the drug may be interacting differently in the MDR cells than in the parent line. We also cannot rule out the possibility that events like alternate splicing, nuclear transit time, or polymerase pausing could change the level of c-*fos* expression.

Although it is not proven that c-*fos* expression is functionally linked to the acquisition or expression of MDR, we have considered two ways to rationalize the overexpression of c-*fos* in MDR cells. Our original hypothesis in these experiments was that, although P-glycoprotein was necessary and sufficient to confer the MDR phenotype, other cellular changes might also be essential to explain the complex set of characteristics observed in cells selected for MDR. By this explanation, changes in the levels of c-*fos* (or, for that matter, any of the other changes reported by several laboratories) are associated with, but not the primary cause of, MDR. This does not necessarily imply that the cellular changes other than P-glycoprotein are not important in the pathways of resistance, particularly because MDR has been shown by numerous workers to be multifactorial (e.g., Refs. 28 and 29). In *mdr1* transfection experiments, there is considerable variation in the relative degree of drug resistance, as well as in the patterns of cross-resistance (3, 4), suggesting the involvement of additional cellular factors and/or post-translational modification of P-glycoprotein. It seems likely, then, that induction of P-glycoprotein is required to establish the setting for MDR but overexpression of the P-glycoprotein is recognized by the cell as an adaptive response to toxicity, which in turn sets in motion a series of additional changes that contribute to the overall spectrum of characteristics that typify MDR. The Fos protein could play a central role in this scenario, because this gene product activates the transcription of other genes. Thus, an elevation in c-*fos* (or other transcriptional factors) could be functionally linked to the elevation of additional gene products, exactly in the manner observed for classic MDR.

The second possible way in which c-*fos* could play a role in MDR is, if expression of this gene precedes P-glycoprotein induction, by controlling *mdr1* transcription. It is notable in this regard that activation of transcription of *mdr1* generally precedes gene amplification (25), suggesting a need for control at the transcriptional level. The function of c-*fos* is thought to be to regulate transcription by forming a complex with c-*jun* (30, 31) and binding to the specific regulatory sequence on DNA that has been identified as 5′ ... $TGA^G/_CTCA$ ... 3′. When we examined the published sequence (32) for the 5′ upstream region of the human *mdr1* gene, we identified this AP-1 binding site in the putative upstream promoter region, with the replacement of T for the central base. A perfect match to the consensus sequence has also recently been found in the mouse *mdr1* promoter (33). Proof for a functional role for this site, or even whether it can interact with the AP-1 complex, awaits experimental demonstration, but our results raise the possibility that the *fos/jun* transcriptional control element may participate in the regulation of P-glycoprotein expression. We cannot tell which protein is present in limiting amounts, but both c-*fos* and c-*jun* are elevated in MDR cells. Additionally, at least one of the other proteins commonly elevated in MDR (glutathione-*S*-transferase) has a consensus AP-1 binding site in the 5′ promoter region of its gene (34), so this too could be regulated by an elevation of c-*fos*. We also note that the AP-1 site activates genes regulated by the phorbol ester tetradecanoyl phorbol acetate (35), including the *fos* gene itself (36, 37). This framework of results predicts that tetradecanoyl phorbol acetate might facilitate the acquisition of MDR, and such an observation has, in fact, been reported by Fine *et al.* (38).

The additional finding that drugs like Adriamycin, vincristine, VP-16, and colchicine, which are used to select cells for MDR, also cause a rise in the level of c-*fos* message suggests a model wherein an early event in the acquisition of MDR is the boosting of the transcriptional machinery, which allows for increased expression of P-glycoprotein. In this model, it is not surprising that the KB-A10 cells do not have appreciably more c-*fos* message than do the less resistant KB-A1 cells, because the P-glycoprotein content is not higher,[3] and at increased levels of resistance other mechanisms may begin to play a role (39). Furthermore, the GRC1 cells would not require an elevation of c-*fos*, because the additional transfected *mdr1* gene is capable of supporting the production of more P-glycoprotein to confer the resistant phenotype.

Our current state of understanding does not allow us to discriminate between the possibility that elevated c-*fos* is a prerequisite to enhanced transcription of *mdr1* and the supposition that the c-*fos* increase is a later event that promotes the transcription of the many additional proteins that characterize the MDR phenotype. In fact, the two hypotheses are not mutually exclusive. It does seem clear, however, that there are a variety of mechanisms that may mediate the induction of c-*fos* in different circumstances. Binding of growth factors like platelet-derived growth factor or epidermal growth factor to their cell surface receptors induces the expression of c-*fos*, but this is a transient phenomenon, with the increase in transcription occurring within about 15 min of stimulation (20). Inhibitors of protein synthesis like cycloheximide cause a "superin-

[3] E. Dolci, unpublished observations.

duction" of c-*fos* (40), possibly by effecting the disappearance of a ribonuclease or transcription-controlling protein. Other drugs have also been shown to induce *fos* and/or *jun* proteins, including cytosine arabinoside (41), camptothecin (42), L-phenylalanine mustard (43), and etoposide (44). In a recent paper (45), Hollander and Fornace (45) showed that a wide variety of DNA-damaging agents with different mechanisms of action and resistance, as well as heat shock, increase c-*fos* mRNA in Chinese hamster ovary cells. The increase in this species reported here for MDR cells is different in two respects from most of these other system; 1) the elevated c-*fos* is not transient but a permanent characteristic of the resistant mutants, and 2) our conditions of minimal drug exposure were chosen to mimic the environmental milieu during selection of cells for drug resistance. Under more toxic conditions a wide variety of agents and treatments may induce c-*fos*, but under the more mild conditions used for genetic selection only drugs in the MDR class induce this gene. Taken together, these various observations suggest that the Fos protein may play multiple roles in determining both the growth status of cells and the mechanisms that cells use to become resistant to toxic agents that inhibit their growth.

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Send reprint requests to: Thomas R. Tritton, Department of Pharmacology, The University of Vermont College of Medicine, Burlington, VT 05405.