

P-glycoprotein (*MDR1*) Expression in Leukemic Cells Is Regulated at Two Distinct Steps, mRNA Stabilization and Translational Initiation*

Received for publication, October 30, 2002, and in revised form, December 16, 2002
Published, JBC Papers in Press, January 13, 2003, DOI 10.1074/jbc.M211093200

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Multidrug resistance in acute myeloid leukemia is often conferred by overexpression of P-glycoprotein, encoded by the *MDR1* gene. We have characterized the key regulatory steps in the development of multidrug resistance in K562 myelogenous leukemic cells. Unexpectedly, up-regulation of *MDR1* levels was not due to transcriptional activation but was achieved at two distinct post-transcriptional steps, mRNA turnover and translational regulation. The short-lived (half-life 1 h) *MDR1* mRNA of naïve cells (not exposed to drugs) was stabilized (half-life greater than 10 h) following short-term drug exposure. However, this stabilized mRNA was not associated with translating polyribosomes and did not direct P-glycoprotein synthesis. Selection for drug resistance, by long-term exposure to drug, led to resistant lines in which the translational block was overcome such that the stabilized mRNA was translated and P-glycoprotein expressed. The absence of a correlation between steady-state *MDR1* mRNA and P-glycoprotein levels was not restricted to K562 cells but was found in other lymphoid cell lines. These findings have implications for the avoidance or reversal of multidrug resistance in the clinic.

MDR1 is the most common impediment to successful chemotherapy for a variety of cancers (1). The most frequent form of drug resistance in relapsed acute leukemia is overexpression of P-glycoprotein (2, 3). P-glycoprotein is a member of the ATP-binding cassette superfamily of active transporters and functions as an energy-dependent efflux pump that reduces the intracellular concentration of cytotoxic compounds and, hence, their toxicity. P-glycoprotein has a broad substrate specificity and can confer resistance to a wide range of different cytotoxic compounds (4).

Most pre-clinical and clinical efforts to overcome MDR aim to modulate P-glycoprotein activity. However, clinical trials of compounds that inhibit P-glycoprotein activity have had lim-

ited success and led to adverse pharmacokinetic side effects (1). It may, therefore, be more appropriate to target *MDR1* expression. Indeed, *MDR1* transcription has been targeted with Ecteinascidin 743 in pre-clinical studies (5) and more recently by modulation of the nuclear receptor SXR (6). Strategies involving antisense and transcriptional decoy (7) and the use of anti-*MDR1* mRNA hammerhead ribozymes have also been suggested (8).

Stresses such as short-term exposure to cytotoxic drugs results in the up-regulation of *MDR1* mRNA levels in many cell lines (9–13) and in human metastatic sarcomas *in vivo* (14). This is frequently due to transcriptional activation of the *MDR1* gene and has been reported in many cell lines after different physical and chemical stimulations and in cells selected for resistance to a variety of cytotoxic drugs (5, 9, 10, 15, 16). In cell lines selected for drug resistance, increased *MDR1* gene expression is also the result of amplification of the *MDR1* locus and the appearance of self-replicating episomes (4). Gene rearrangements that constitutively activate *MDR1* transcription have also been associated with refractory acute lymphocytic leukemia (17, 18).

Although regulation due to changes in the *MDR1* mRNA stability (19), P-glycoprotein turnover (20), or trafficking (21) have been suggested, transcriptional regulation is widely considered to be the key step accounting for the complex spatiotemporal pattern of expression *in vivo* (22, 23). It has also generally been assumed that up-regulation of *MDR1* mRNA leads to an increase in P-glycoprotein. For example, human renal carcinoma (16) and rat liver cells (24) up-regulate both *MDR1* mRNA and P-glycoprotein following different stresses and are consequently transiently resistant to vinblastine.

In this study, we show that in K562 leukemic cells the levels of *MDR1* mRNA increase in a dose- and time-dependent manner upon transient exposure to a variety of cytotoxic drugs. However, in contrast to the general prevailing models, we show that this is due to stabilization of mRNA and not because of transcriptional activation. Furthermore, the newly stabilized mRNA is not translated and so does not result in expression of P-glycoprotein and drug efflux. Only on subsequent long-term selection for drug resistance does the stabilized mRNA associate with polyribosomes, permitting translation of P-glycoprotein and drug efflux. The finding that drug resistance is a two-step post-transcriptional process, mediated by changes in both *MDR1* mRNA stability and translation, suggests new possibilities for treatment regimes to circumvent MDR in leukemia.

* This work was supported by the Medical Research Council UK and Cancer Research UK. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: MDR, multidrug resistance; RT, reverse transcription; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; C_T, threshold cycle; luc, luciferase; EBV, Epstein-Barr virus.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—K562, CCRF-CEM, and MANN cells were cultured in RPMI 1640 medium (Invitrogen) with 10% fetal calf serum and 2 mM L-glutamine; KB-V1 cells (25) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 20% fetal calf serum and 110 μ M vinblastine (Sigma). All drugs were obtained from Sigma. For transient drug treatments (inductions), exponentially growing cells were seeded at 1×10^6 cells/ml and incubated with drug for the indicated times. The drug concentrations used were determined previously to cause macroscopic changes in cell morphology, indicative of cytotoxic stress, such as swelling, and changes in shape and granularity, in greater than 50% of cells. Unless otherwise stated, drug inductions were for 3 days using 3.4 nM doxorubicin, 22 μ M vinblastine, 2.5 nM colchicine, 1.34 μ M colcemid, or 100 μ M cytarabine. Drug-resistant K562 sublines were obtained in a one-step selection by exposure to concentrations of the following cytotoxic drugs, which had been shown to result in 99.9% cell death after 14 days in culture in preliminary experiments: 40 pM colchicine, 30 pM doxorubicin, or 25 μ M 1- β -D-arabinofuranosylcytosine (cytarabine). Lines resistant to these concentrations of drug were designated KC40, KD30, and KA25 lines, respectively.

Determination of mRNA Expression by Semi-quantitative and Real-time RT-PCR—RNA was prepared from cells by RNeasy extraction (Biogenesys, Poole, United Kingdom), reverse-transcribed (Roche Molecular Biochemicals), and amplified by PCR. Semi-quantitative RT-PCR estimation of *MDR1* mRNA levels was performed as described (26). Real-time quantitative PCR (Taqman; PerkinElmer Life Sciences) used the following primers within the *MDR1* coding sequence: sense, 5'-TTGTTTCAGGTGGCTCTGGAT-3'; antisense, 5'-CTGTAGACAAACGATGAGCTATCACA-3'; and probe, 5'-AGGCCAGAAAGGTCGGACACCA-3'. Taqman Universal PCR Master Mix and control amplicons for *GAPDH* and 18 S ribosomal RNAs were used as recommended by the supplier (PerkinElmer Life Sciences). Results were collected and analyzed with an ABI Prism 7700 sequence detection system (PerkinElmer Life Sciences) as follows: the PCR cycle number that generated the first fluorescence signal above a threshold (threshold cycle, C_T ; 10 standard deviations above the mean fluorescence generated during the baseline cycles) was determined, and a comparative C_T method was then used to measure relative gene expression. The following formula was used to calculate the relative amount of the transcript in the sample and normalized to an endogenous reference (*GAPDH* or 18 S rRNA): $2^{-\Delta\Delta C_T}$, where ΔC_T is the difference in C_T between the gene of interest and *GAPDH* or 18 S rRNA, and $\Delta\Delta C_T$ for the sample = ΔC_T of the actual sample - ΔC_T of the lowest expressing sample.

Determination of RNA Half-lives—K562, KC40, and KD30 cells were incubated with 12.5 μ g/ml actinomycin D to inhibit transcription. This concentration of actinomycin D was determined empirically in a series of pilot experiments as that which inhibited greater than 95% of [3 H]uridine incorporation in both the naïve and drug-resistant K562 cell lines, and thus nascent transcription, within 1 h (27). Cells were harvested at different times after actinomycin D addition, and total RNA was isolated as above. Total RNA, or the poly(A) $^+$ fraction isolated by using an Oligotex kit (Qiagen), was used in real-time Taqman RT-PCR assays. In addition, Northern blots (28) of 10 μ g of total RNA from the above samples were hybridized with probes derived from gene-specific sequences from the *MDR1*, *GAPDH*, *Id2*, or *RAR- α* genes (29, 30). Hybridization signals were quantified with a PhosphorImager (Amersham Biosciences).

Genomic DNA and Southern Blotting—Genomic DNA was isolated from cells by proteinase K digestion and phenol/chloroform extraction, digested with *EcoRI*, Southern blotted, and hybridized to nick-translated radioactive probes by standard procedures (28). Probes were derived from the *MDR1* 5'-end region (975-bp *PstI* fragment comprising the transcription start point, 5'-untranslated region, and first intron) (31) and the β -globin locus (3.0-kb *EcoRI* fragment containing DNase I hypersensitive site V) (32). β -Globin to *MDR1* ratios were obtained by quantitation using a PhosphorImager.

Nuclear Run-on and Luciferase Transcription Assays—Nuclei were prepared from naïve, drug-induced, or drug-resistant K562 sublines as described (27). Nuclear run-on transcription and hybridization methods have been described elsewhere (33). For the *MDR1* promoter-luciferase transcriptional assays, K562, KC40, and KD30 cells were transiently transfected with p*MDR1*(-1202), carrying the luciferase reporter gene from pGL2B (Promega, Madison, WI) under the control of *MDR1* promoter (34), together with p*EF1acZ* carrying the bacterial β -galactosidase gene under the control of the *EF1 α* promoter. After 2 days, cells extracts were prepared, and luciferase activity was determined with a

luciferase assay system (Promega) and normalized against β -galactosidase expression (28). The AP-1-dependent luciferase reporter plasmid (p*API-luc*), containing four tandem copies of the TPA-responsive element consensus motif TGACTCA coupled to the -36 to +37 rat prolactin minimal promoter (35), was cotransfected with p*EF1acZ* into K562 and KD30 cells. When indicated, cells were stimulated with 20 ng/ml phorbol 12-myristate 13-acetate (Sigma) during the last 12 h, and luciferase and β -galactosidase expression were measured as above. Transfections were by electroporation using a Bio-Rad gene pulser (Bio-Rad) with 5×10^6 cells essentially as described (28).

Measurement of Cell Surface P-glycoprotein by Flow Cytometry—Analysis of surface P-glycoprotein expression was by flow cytometry using the fluorescently labeled monoclonal antibody UIC2 (Immunotech, Marseille, France), essentially as described (36), using a Becton Dickinson Flow Cytometer (BD Biosciences). Live cells were detected by exclusion of propidium iodide. Drug-induced cells were monitored 1, 2, 3, and 4 days after drug addition. Where indicated, single cells were sorted into 96-well plates after UIC2 staining at a density of one cell per well by a FACS Vantage (BD Biosciences) and clonally expanded.

Western Blot Analysis—Crude cell membrane fractions were prepared from 1×10^8 cells essentially as described (37), with minor modifications. Briefly, cells were lysed with a hypotonic buffer (50 mM mannitol, 50 mM Tris-HCl, pH 7.4, 2 mM EGTA) and centrifuged at a low speed ($500 \times g$) to pellet nuclei and associated membranes such as endoplasmic reticulum and Golgi apparatus (plasma membrane-depleted fraction). The supernatant from the low speed fractionation was further centrifuged at $100,000 \times g$ to obtain the plasma membrane-enriched fraction. Plasma membrane-enriched and -depleted fractions (200 μ g) were separated by SDS-PAGE, and proteins were transferred electrophoretically to Immobilon membranes (Millipore, Watford, United Kingdom). Filters were incubated overnight at 4 $^{\circ}$ C with 0.1 μ g/ml of the anti-P-glycoprotein monoclonal C219 (Cis-Bio International, Gif-Sur-Yvette, France) or 1:1000 anti-Na $^+$ /K $^+$ -ATPase α_2 subunit, clone IIDS, (Affinity Bioreagents Inc., Golden, CO) in 5 g/100 ml skimmed milk phosphate-buffered saline containing 0.1 g/100 ml Tween 20. Protein was visualized by enhanced chemiluminescence (Amersham Biosciences) after incubating with and horseradish peroxidase-conjugated goat and mouse IgG secondary antibody (1/1000) (Dako, Ely, United Kingdom).

De Novo Protein Synthesis—Incorporation of [35 S]Met in nascent proteins was determined by trichloroacetic acid precipitable counts, following standard procedures (38).

Sucrose Gradient Density Centrifugation and Detection of RNA across Polysome Profiles—Extracts of drug-induced K562, KC40, and MANN cells, sucrose gradient centrifugation, and RNA extraction followed standard procedures (39). Where indicated, buffers contained 20 mM EDTA. Isolated RNA was precipitated with 3 M LiCl and resuspended in 10 μ l of water. Detection of *MDR1* mRNA was by RT-PCR as described above.

RESULTS

***MDR1* mRNA Is Up-regulated in Drug-induced and Drug-resistant K562 Cells**—The myeloid leukemia-derived cell line K562 expresses very low levels of *MDR1* mRNA, barely detectable by standard RT-PCR assays (Fig. 1A), although readily determined by a more sensitive method like real-time RT-PCR using poly(A) $^+$ mRNA (data not shown). K562 cells responded to short-term exposure (drug induction) to several different cytotoxic drugs (doxorubicin, colchicine, colcemid, vinblastine, and cytarabine) by up-regulating *MDR1* mRNA levels (Fig. 1A). Real-time RT-PCR showed a 30- to 100-fold increase in *MDR1* mRNA levels in drug-induced cells compared with naïve (not exposed to drug) K562 cells (data not shown). This effect was due to the cytotoxic drug, because the increase in *MDR1* mRNA was both time- and dose-dependent (Fig. 1B).

To generate K562 sublines resistant to low levels of drugs, three cytotoxic drugs with different modes of action were used, colchicine (which binds tubulin and prevents mitosis), doxorubicin (a DNA intercalating agent), and cytarabine (a pyrimidine analogue). A one-step drug selection resulted in the generation of resistant pools of clones (see "Experimental Procedures"). These lines were called KC40, KD30, and KA25 (colchicine-, doxorubicin-, and cytarabine (araC)-resistant, respectively). *MDR1* mRNA was substantially up-regulated in

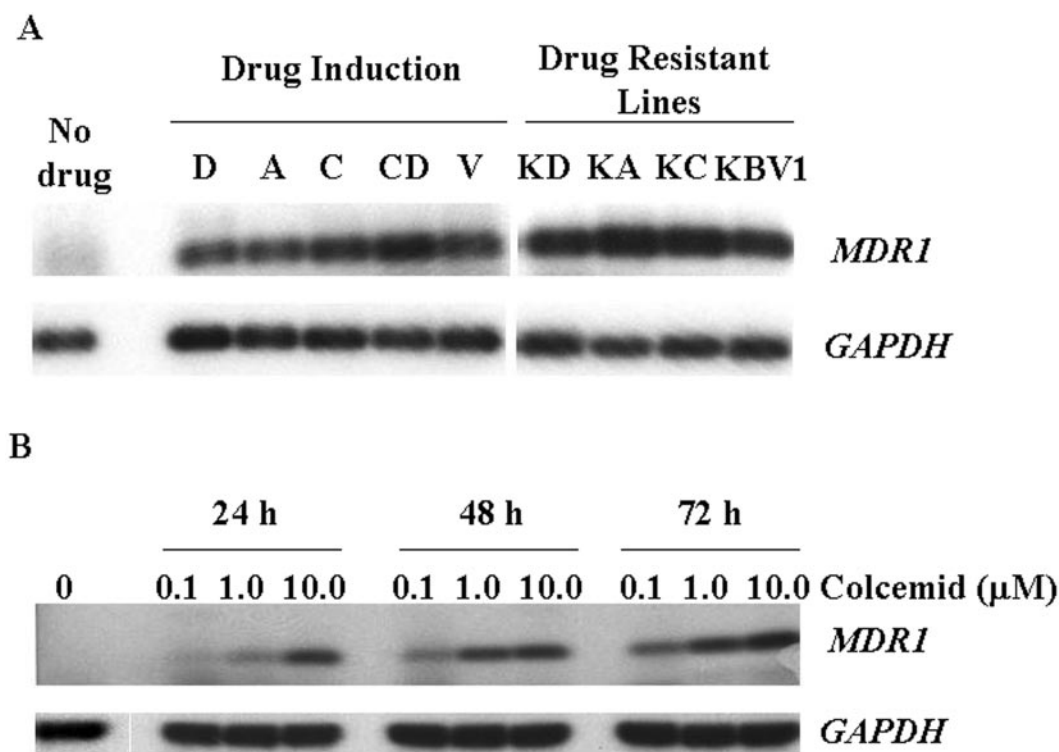


FIG. 1. *MDR1* mRNA is up-regulated in drug-induced and drug-resistant K562 cells. *MDR1* and *GAPDH* transcripts were amplified by RT-PCR and detected by Southern blotting and hybridization with specific internal oligonucleotide probes. *A*, left hand lane shows naïve K562 cells (not treated with drug), middle lanes show K562 cells transiently induced with doxorubicin (*D*), cytarabine (*A*), colchicine (*C*), colcemid (*CD*), or vinblastine (*V*) for 3 days; right hand lanes show K562 cells selected for resistance to doxorubicin (KD30, *KD*), cytarabine (KA25, *KA*), or colchicine (KC40, *KC*). *KBV1*, human KB epidermoid carcinoma line. *B*, dose-time response for induction of *MDR1* mRNA by colcemid using concentrations of 0.1, 1.0, and 10.0 μM.

each of these lines (Fig. 1A), shown by real-time RT-PCR to be between 2- and 5-fold greater than the levels found in the 3-day drug-induced cells (data not shown). Thus, both drug induction and selection for drug resistance result in substantial increases in steady-state *MDR1* mRNA levels, independent of the mode of action of the drug.

Up-regulation of MDR1 mRNA Is Not Due to Gene Amplification, Gene Rearrangement, or Transcriptional Activation—Gene amplification is a common mechanism for the up-regulation of *MDR1* mRNA in cell lines (4) and is normally accompanied by rearrangements and deletions in the amplified locus. Genomic DNA from drug-resistant (KC40 and KD30) cells had the same *MDR1* gene copy number as the parental line K562, thus no detectable amplification or rearrangements of the locus had occurred (Fig. 2A).

To determine whether *MDR1* mRNA up-regulation was due to transcriptional activation (40), we used two different approaches. We initially used a gene reporter assay in which an *MDR1* promoter fragment was placed upstream of the luciferase reporter gene (34). This reporter plasmid has been used previously to demonstrate transcriptional activation of *MDR1* in, among others, human colon carcinoma SW620 cells (34). Luciferase expression was equivalent in naïve (not drug-treated) and all of the drug-resistant K562 lines (Fig. 2B), suggesting that there is no promoter up-regulation. As AP-1 plays a role in the transcriptional activation of *MDR1* (41), and because K562 cells resistant to etoposide (another P-glycoprotein substrate) have up-regulated levels of *c-jun* and *c-fos* mRNAs and increased AP-1 binding activity (42, 43), it was necessary to exclude the possibility that the AP-1 pathway had been inactivated in these cells. To do this we used a reporter plasmid, *pAPI-luc*, in which the luciferase gene expression was driven by four human collagenase TPA-responsive elements

and the minimal rat prolactin promoter (35). This TPA-responsive element has been used in numerous studies because of its high affinity for the AP-1 complex, and, as expected, transient transfection in Jurkat T-cells results in an increase of luciferase activity upon TPA activation (35), which increases *MDR1* gene expression in several cell lines, including K562 cells (44). Naïve and drug-resistant cells transiently transfected with *pAPI-luc* produced similar levels of luciferase activity, both before and after TPA activation (Fig. 2C). Thus, the pathway responsible of activating AP-1 is equally functional in both naïve and drug-resistant K562 cells, and the lack of transcriptional activation of the *MDR1* promoter in drug-resistant cells cannot be due to a lack of AP-1 functionality.

To confirm that transcriptional activation of the *MDR1* promoter is not responsible for *MDR1* mRNA up-regulation we also studied the *MDR1* promoter in its native chromosomal context by nuclear run-on experiments. Despite the increase in steady-state *MDR1* mRNA levels, transcription initiated from the *MDR1* promoter was low compared with transcription from other control genes (*e.g.* *GAPDH*, *β-actin*, or *ε-globin*) and did not increase significantly in drug-resistant or drug-induced cells (data not shown). Thus, transcriptional activation does not appear to be responsible for the up-regulation of *MDR1* mRNA in K562 cells following drug induction or upon selection for drug resistance.

Up-regulation of MDR1 Is Due to mRNA Stabilization—Because the increase in steady-state *MDR1* mRNA was not due to *de novo* mRNA synthesis, we asked whether changes in the rates of mRNA decay might be involved. To determine the half-lives of *MDR1* mRNA in naïve, drug-induced and drug-resistant cells, we treated cells with actinomycin D to inhibit transcription (see “Experimental Procedures”). *MDR1* mRNA from naïve K562 cells had a very short half-life (approximately

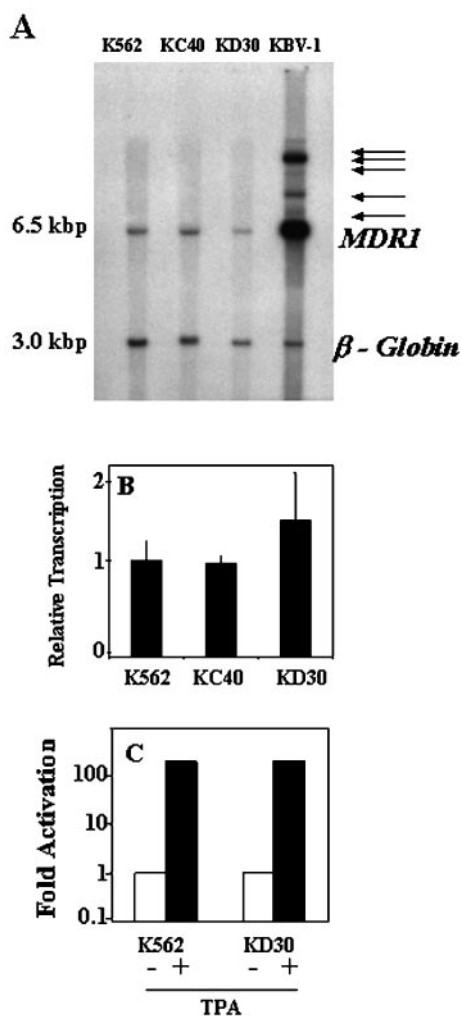


FIG. 2. Increased *MDR1* mRNA levels are not due to gene amplification or transcriptional activation. A, Southern blot analysis of the *MDR1* and β -globin loci of naïve (untreated) and drug-resistant (KC40 and KD30) K562 cells. The KB-V1 cell line was used as a positive control for amplification and rearrangement of the *MDR1* locus; arrows show the positions of rearranged *MDR1* fragments in this cell line. B, transient expression of a luciferase reporter gene driven by the *MDR1* promoter was measured after cotransformation of naïve and drug-resistant (KC40 and KD30) cells with p*MDR1*(-1202) and a bacterial β -galactosidase reporter gene driven by the constitutive *EF1 α* promoter (p*EFlacZ*). Luciferase activity was normalized to β -galactosidase activity, and the data are shown as transcription relative to naïve K562 cells 2 days after transfection. Data show the average and S.D. from three independent experiments. C, AP-1 is not involved in the up-regulation of *MDR1* mRNA in drug-resistant (KD30) cells. The AP-1-dependent luciferase reporter plasmid (p*AP1-luc*), containing four tandem copies of the TPA-responsive element coupled to the rat prolactin minimal promoter, was cotransfected with p*EFlacZ* into K562 and KD30 cells (clear bars). Cells were stimulated with 20 ng/ml phorbol 12-myristate 13-acetate during the last 12 h, and luciferase and β -galactosidase expression were measured as above (filled bars).

1 h) determined by real-time RT-PCR (Fig. 3A). In contrast, *MDR1* mRNA half-life values for doxorubicin- and colchicine-resistant K562 cells were 12–16 h (Fig. 3A). This was confirmed by Northern analysis (Fig. 3B) and is in good agreement with the 14-h half-life reported for another independently derived doxorubicin-resistant K562 line (K562/ADR) (45). Drug-induced K562 cells also had a long *MDR1* mRNA half-life (Fig. 3A). The half-lives of other short-lived messages, such as *Id2* and *RAR- α* , were confirmed to be 1–2 h as reported previously (29, 30) in both naïve and drug-resistant K562 cells (Fig. 3C). Similarly, the half-life of a long-lived message (*GAPDH*) was also unchanged in drug-resistant cells (20–24 h in both naïve

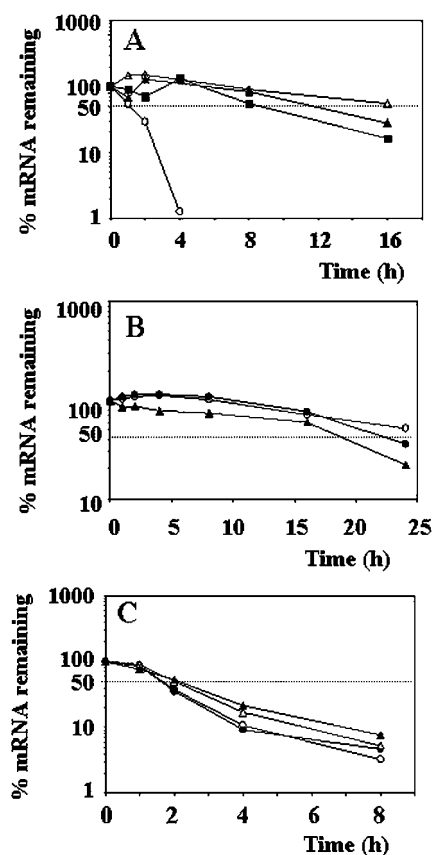


FIG. 3. Increased *MDR1* mRNA levels are due to an increase in mRNA half-life. A, *MDR1* mRNA decay pattern after actinomycin D addition to naïve (white circle), drug-induced (black square), and drug-resistant (white triangle, KC40; black triangle, KD30) K562 cells obtained by RT and real-time quantitative PCR. A representative drug induction (with vinblastine) is shown. *MDR1* mRNA half-lives were 1 h for the naïve and greater than 10 h for drug-induced and -resistant cells. B, mRNA decay pattern of K562 (white symbol) and KD30 (black symbols) cells was followed by Northern analysis (using ten μ g of total RNA) and hybridization with *GAPDH* (circles)- and *MDR1* (triangles)-specific probes. The *GAPDH* mRNA half-life was 20–24 h in both naïve and drug-resistant cells, and the half-life of *MDR1* mRNA was 15–20 h in KD30 cells. Similar half-lives were obtained for colchicine-resistant KC40 cells (data not shown). *MDR1* message was undetectable by Northern hybridization in naïve K562 cells. C, mRNA decay analyzed by Northern analysis and hybridization to probes for other short-lived messages (*circles*, *Id2*; *triangles*, *RAR- α*) in naïve K562 (white symbols) and doxorubicin-resistant KD30 (black symbols) cells. The half-lives of the transcripts (1–2 h) were similar in each cell type. Results from KC40 cells were essentially the same (data not shown).

K562 and KD30 cells) (Fig. 3B). Thus, the stabilization of the *MDR1* mRNA is specific and not a general phenomenon affecting other short-lived mRNAs. In conclusion, up-regulation of *MDR1* mRNA levels following exposure to drugs, after either a transient induction or drug selection, is primarily due to a specific increase in mRNA stability.

Drug Induction of *MDR1* mRNA Does Not Lead to P-glycoprotein Expression—Transient exposure to cytotoxic drugs (drug induction) led to an increase in *MDR1* mRNA levels through mRNA stability. This observation led us to ask whether *MDR1* mRNA up-regulation was accompanied by an increase in P-glycoprotein expression. Cell surface expression of P-glycoprotein was measured by flow cytometry using the P-glycoprotein-specific monoclonal antibody UIC2 (36). There was no significant increase in UIC2-positive cells following drug induction at any stage during the 4-day incubation period compared with naïve K562 cells. In contrast, cells selected for resistance to the P-glycoprotein substrates colchicine (KC40

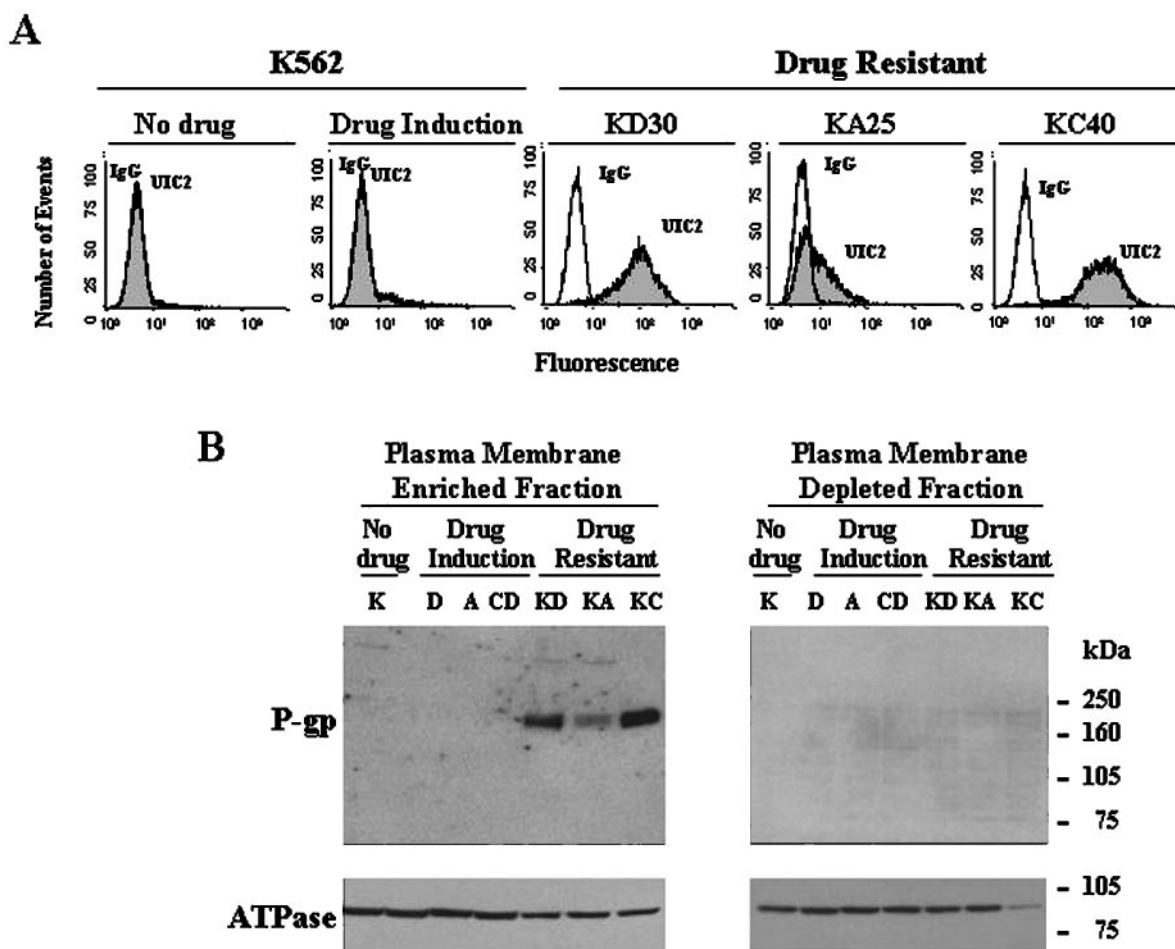


FIG. 4. Up-regulation of *MDR1* mRNA in drug-induced K562 cells does not lead to expression of P-glycoprotein. *A*, flow cytometric analysis of surface P-glycoprotein expression using the UIC2-phycoerythrin-conjugated antibody (filled peak) and the corresponding IgG isotype control (clear peak). For naïve and drug-induced cells, the IgG and UIC2 peaks overlap, showing that active P-glycoprotein is not present at the cell surface. A representative drug-induction experiment with colchicine after 3 days is shown. Qualitatively similar data were obtained with other drugs (vinblastine, colcemid, doxorubicin, or cytarabine) or if measured 1, 2, or 4 days after drug induction (data not shown). For the drug-resistant cell lines KC40 and KD30, UIC2 fluorescence was higher than the IgG control, indicating expression of P-glycoprotein on the surface of the cells. Approximately 40% of cytarabine-resistant (KA25) cells showed limited P-glycoprotein expression. *B*, Western blot analysis of P-glycoprotein (*P-gp*) in plasma membrane-enriched and -depleted fractions (200 μ g protein) from K562 naïve (*K*), drug-induced (*D*, doxorubicin; *A*, cytarabine; *CD*, colcemid), or drug-resistant cells (*KD*, KD30; *KA*, KA25; *KC*, KC40). An anti- Na^+/K^+ -ATPase antibody was used as loading control. Drug inductions were carried out for 3 days.

cells) or doxorubicin (KD30 cells) showed a large (10- to 100-fold) increase in UIC2 reactivity, indicating the presence of active P-glycoprotein on the cell surface. Cells selected for resistance to cytarabine (KA25 cells), which is not transported by P-glycoprotein, showed much lower levels of surface P-glycoprotein expression than the KC40 and KD30 cells, despite having similar *MDR1* mRNA levels. Only 40% of cytarabine-resistant (KA25) cells expressed surface P-glycoprotein, consistent with the fact that resistance to cytarabine is known to be mediated by other P-glycoprotein-independent mechanisms (11) (Fig. 4A).

The Absence of P-glycoprotein at the Cell Surface Is Not Due to a Defect in Protein Trafficking—Because the assay above detects only active P-glycoprotein in the plasma membrane, it was necessary to exclude the possibility that P-glycoprotein was expressed in drug-induced cells, but either inserted in the plasma membrane in an inactive form or accumulated intracellularly. Cell membranes from naïve, drug-induced, and drug-resistant K562 cells were analyzed for P-glycoprotein expression by immunoblotting using the monoclonal antibody C219. A band of \sim 190 kDa, corresponding to mature P-glycoprotein, was detected in the plasma membrane-enriched fraction from drug-resistant lines but not from the naïve or drug-

induced cells (Fig. 4B). P-glycoprotein was also absent from plasma membrane-depleted fractions (*i.e.* nuclear and rough endoplasmic reticulum) of these cells, indicating that the protein was not retained intracellularly (Fig. 4B). The intensity of the C219-specific band was consistent with the level of UIC2 binding (Fig. 4A). Thus, although drug-induced and drug-resistant cells express *MDR1* mRNA, only drug-resistant cells are able to express P-glycoprotein from *MDR1* mRNA.

***MDR1* mRNA-positive Cells That Cycle Normally Can Lack P-glycoprotein**—Because transient treatment with cytotoxic drugs led to cell cycle arrest, it was necessary to exclude the possibility that inhibition of cell cycle progression was responsible for the failure to express P-glycoprotein. The colchicine analogue colcemid was used, because its microtubule depolymerizing activity can be relieved following extensive washes of the cells (46). K562 cells were induced for 4 days with colcemid, which completely arrested the cell cycle and up-regulated *MDR1* mRNA (Fig. 5, A and B). The washed cells were transferred to drug-free medium, where they remained quiescent for a further 2–3 days before proliferating at the same rate as untreated cells (Fig. 5A). The colcemid-induced increase in *MDR1* mRNA persisted for at least 19 days after drug withdrawal (Fig. 5B), yet this did not lead to the synthesis of

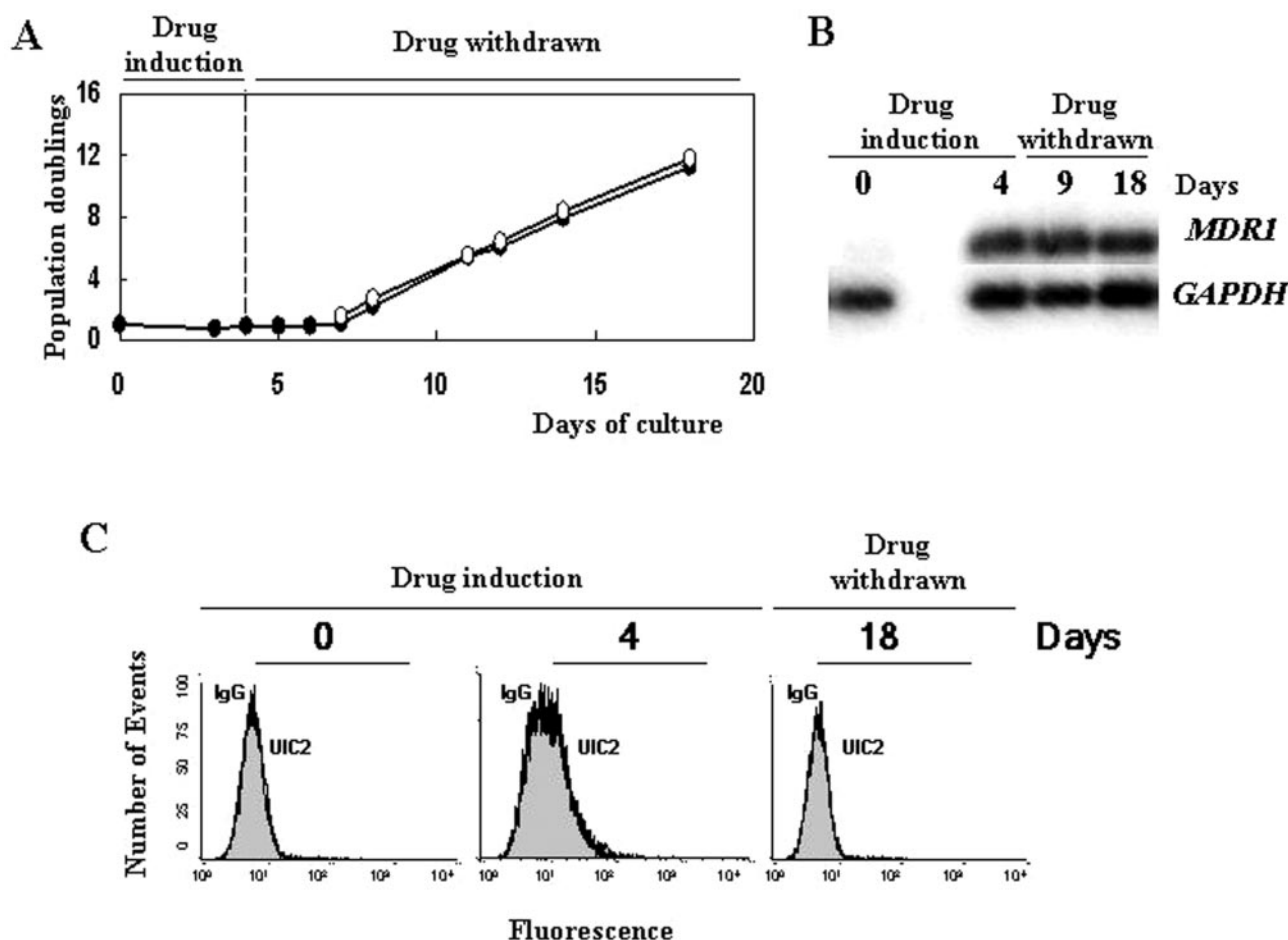


FIG. 5. Lack of P-glycoprotein expression by drug-induced K562 cells is not due to cell cycle arrest. *A*, K562 cells were treated with colcemid for 4 days and then grown drug-free for a further 14 days (filled circles). The growth curves of naïve (untreated) K562 cells (open circles) are comparable. *B*, *MDR1* and *GAPDH* transcripts from the cells were amplified by RT-PCR and detected by Southern blot hybridization using oligonucleotide probes. *C*, flow cytometric analysis of surface P-glycoprotein expression in the cells using the UIC2-phycoerythrin-conjugated antibody (filled peak) and the corresponding IgG isotype control (clear peak). IgG and UIC2 fluorescence signals overlapped, indicating no P-glycoprotein in the surface of the cells after colcemid withdrawal, despite the presence of *MDR1* mRNA.

P-glycoprotein (Fig. 5C). Therefore, mitotic arrest is not responsible for the lack of P-glycoprotein expression in drug-induced cells.

This was further confirmed by studying single-cell-derived clones from KA25 cells (selected as resistant to the non-P-glycoprotein substrate cytarabine). Clones from both P-glycoprotein-expressing (UIC2-positive) and non-expressing (UIC2-negative) subpopulations (Fig. 4A) (data not shown) were obtained by flow cytometry sorting. RNA analysis indicated that the *MDR1* transcript was up-regulated in both types of cells (data not shown). Thus, cell selected for resistance to cytarabine and growing normally can also express *MDR1* mRNA but still fail to express P-glycoprotein. Therefore, cells that cycle normally, either after drug withdrawal or after selection with a non-P-glycoprotein substrate, can up-regulate *MDR1* mRNA but fail to express P-glycoprotein.

Increased Levels of *MDR1* mRNA Do Not Correlate with P-glycoprotein Levels in Other Cell Types—To test whether the absence of P-glycoprotein expression in *MDR1* mRNA-positive cells was a phenomenon particular to K562 cells, we studied the acute lymphocytic leukemia-derived cell line CCRF-CEM. In contrast to K562 cells, naïve CCRF-CEM cells were found to have detectable *MDR1* mRNA levels, which increased upon drug induction. No P-glycoprotein was detected in the induced cells (data not shown). We also studied a non-leukemic-derived cell line, the EBV-transformed B cell line MANN, in which the

naïve untreated cells express significant levels of *MDR1* mRNA but no P-glycoprotein (binding to UIC2 monoclonal antibody; data not shown). These cells are also unable to efflux rhodamine 123, a fluorescent P-glycoprotein substrate (47). The lack of correlation between steady-state *MDR1* mRNA and P-glycoprotein levels in these cell lines suggests that the mechanism regulating P-glycoprotein expression is not restricted to K562 cells.

***MDR1* mRNA Is Not Associated with Translating Polyribosomes in Cells Failing to Express P-glycoprotein**—To test whether the drug-induced *MDR1* mRNA had undergone RNA editing or processing that rendered it untranslatable, the full *MDR1* cDNA from colcemid-induced cells was sequenced and compared with the sequence deposited in the databases (GenBank™ accession number M14758). When polymorphisms were found, they were always confirmed to exist in the *MDR1* cDNA sequence from a drug-resistant K562 cell expressing P-glycoprotein (KC40). No differences between the mRNA sequences were found, showing that the *MDR1* mRNA does not undergo editing or processing during short-term drug exposition or drug selection, which could account for lack of P-glycoprotein.

Because stress can have a general negative effect on protein synthesis (48–50) we also measured the rate of incorporation of [³⁵S]Met into nascent proteins in naïve and drug-induced K562 cells. There was approximately an 8% decrease in the rate of overall protein synthesis after 24 h in the presence of cytotoxic

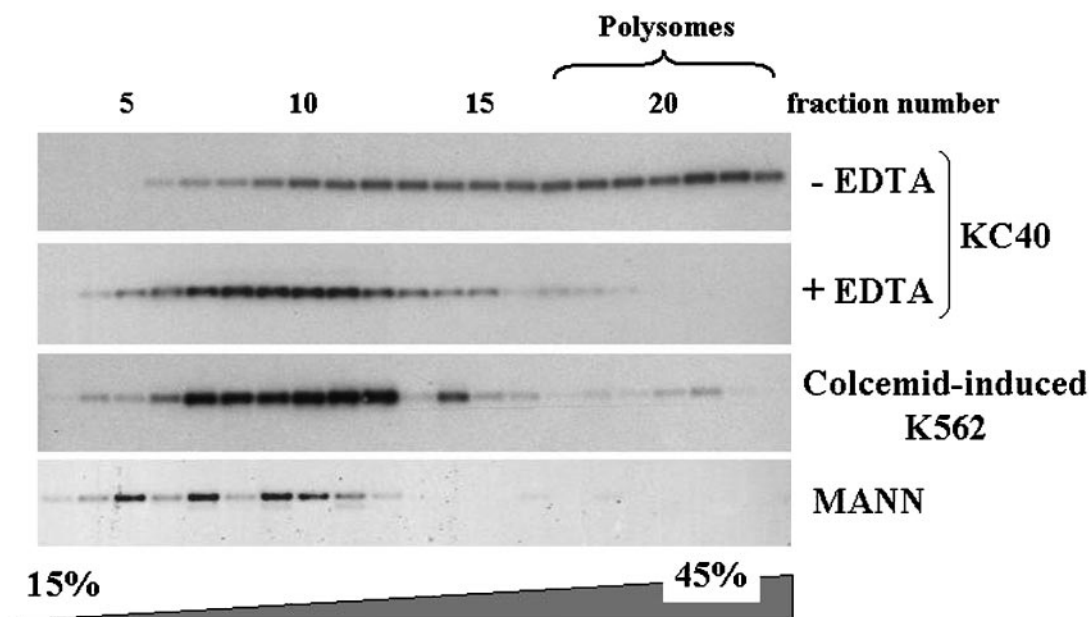


FIG. 6. *MDR1* mRNA is not associated with translating polyribosomes in cells failing to express P-glycoprotein. Detection of *MDR1* mRNA in fractions from a 15–45% sucrose gradient of cell extracts by semi-quantitative RT-PCR is shown. Fractions (0.5 ml) were collected from the top of the gradient, and RNA was isolated. Where indicated, ribosomes were dissociated by the inclusion of EDTA in both the extraction and gradient buffers. A representative induction with colcemid for 3 days is shown. Similar results were obtained after doxorubicin induction. In drug-resistant cells expressing P-glycoprotein *MDR1* mRNA migrates with the heavy (containing polyribosomes) fractions. In both drug-induced K562 and MANN (EBV-transformed human B lymphocytes) cells, which do not express P-glycoprotein, *MDR1* mRNA migrates with the light fractions lacking polyribosomes, indicating that it is not being translated.

drug, which decreased further to 40% after 72 h (data not shown). Thus, upon treatment with cytotoxic drugs there is a general decrease in the rate of protein synthesis. However, although *de novo* translation is decreased by the drug treatment, this effect is insufficient to account for the complete lack of expression of P-glycoprotein. To confirm that the absence of P-glycoprotein expression in drug-induced cells was due to a failure to translate *MDR1* mRNA, we examined *MDR1* mRNA association with polyribosomes. Cell lysates were fractionated by sucrose gradient ultracentrifugation, and the amount of *MDR1* mRNA in individual fractions was determined by semi-quantitative PCR (51). In drug-resistant cells, a substantial portion of *MDR1* mRNA was associated with high molecular weight polyribosomes (toward the bottom of the gradient) indicating that the *MDR1* mRNA was efficiently translated. When ribosomes were dissociated by including EDTA in the extraction buffer and sucrose gradients, the position of the *MDR1* mRNA shifted toward the lighter (non-polysome) fractions of the gradient, as expected. In contrast, for drug-induced K562 cells *MDR1* mRNA migrated at approximately the same position as for EDTA-dissociated KC40 cells (top of the gradient), indicating that in these cells *MDR1* mRNA is not associated with polyribosomes and is not being translated (Fig. 6). To exclude the possibility that the absence of association between *MDR1* mRNA and polyribosomes is due to a general reduction in the rate of protein synthesis, caused by the cytotoxic drugs, the association of *MDR1* mRNA with ribosomes was also examined in P-glycoprotein-negative EBV-transformed B lymphocytes (MANN cell line). In these cells, which had not been exposed to cytotoxic drugs, *MDR1* mRNA was not associated with polyribosomes (Fig. 6). The translational block is, therefore, not due to the effect of cytotoxic drugs and represents a novel mechanism for regulating P-glycoprotein expression.

DISCUSSION

Expression of *MDR1*/P-glycoprotein in blasts from leukemia patients has been adversely correlated with the clinical re-

sponse to chemotherapy (2, 3, 52). We have used a K562 leukemic cell model system to study the mechanisms regulating *MDR1* gene expression involved in the acquisition of MDR. Unexpectedly, we found that in these cells, up-regulation of P-glycoprotein expression was a two-step process, mRNA stabilization and relief from a translational block.

Three drug-resistant K562 cell lines were developed, resistant to the P-glycoprotein substrates, doxorubicin and colchicine, or to the non-P-glycoprotein substrate, cytarabine. In contrast to many previous studies, we developed these lines by just a single-step selection to very low doses of cytotoxic drug, rather than the traditional stepwise selection at increasing drug concentrations. This enabled us to work at drug concentrations and levels of drug resistance closer to those found in the clinic and avoided amplification and rearrangements of the *MDR1* locus frequently found in highly resistant cell lines but never documented *in vivo* (Fig. 2A) (1).

As expected, drug-resistant K562 lines, as well as naïve K562 cells transiently exposed to a range of cytotoxic agents, up-regulated steady-state *MDR1* mRNA levels (Fig. 1A). This effect was independent of the mode of action of the drug. Similarly, it has been reported that heat shock, UV radiation, arsenate, or sodium butyrate also induces *MDR1* gene expression in a variety of systems (40). Transcriptional activation is generally assumed to be the principal mechanism for up-regulating *MDR1* gene expression, and several regulatory elements (AP-1, heat shock element, Sp1, Y-box, CAAT box) have been identified and characterized in the *MDR1* promoter region (40). In this study we found that in K562 leukemic cells transiently exposed to drugs or further selected for drug resistance the increased *MDR1* mRNA levels could not be accounted for by transcriptional activation of the *MDR1* promoter (Fig. 2B). Furthermore, the AP-1-dependent activation of the *MDR1* promoter, observed in other cell lines, was not apparent in K562 cells despite the fact that the AP-1 activation pathway by protein kinase C was shown to be functional in both naïve and

drug-resistant K562 cells (Fig. 2C). Because K562 cells do not express the tumor suppressor gene *p53* (53), the absence of transcriptional activation in the cells could, at least in part, be due to a lack of functional p53, which is known to activate the *MDR1* promoter (54).

In contrast to transcriptional activation, we found that up-regulation of *MDR1* mRNA in K562 cells is due to an increase in mRNA stability (Fig. 3, A and B). This was specific to the *MDR1* mRNA as the stability of other short-lived mRNA species remained unaffected (Fig. 3C). Interestingly, in naïve K562 cells, *MDR1* mRNA has a very short half-life (around 1 h), which, in addition to the low rate of transcription from the *MDR1* gene, can account for the low steady-state levels found in naïve cells (Fig. 1A). The *cis*-determinants involved in mRNA stabilization are usually, but not exclusively, located in the 3'-untranslated region of the transcripts (55). The *MDR1* 3'-untranslated region is short (378 nucleotides) and contains several potential AU-binding protein recognition sites that could regulate rapid mRNA decay (55). The short half-life of *MDR1* mRNA in naïve K562 cells contrasts with the few studies that report a longer half-life, *i.e.* 8 h in the human hepatoma cell line HepG2 (56). The *MDR1* mRNA half-life increased 10-fold upon transient exposure of cells to cytotoxic drugs and in the drug-resistant lines (KD30 and KC40) (Fig. 3). Similar long half-lives have also been described in a K562 line resistant to doxorubicin (45). The stabilized *MDR1* mRNA from drug-induced K562 cells persisted for several days after the recovery from the cytotoxic stress but eventually returned to the basal level of naïve K562 cells (Fig. 5B), consistent with dilution of a *trans*-acting stabilizing factor upon cell division to levels below a functional concentration. One interpretation of the data is that this putative factor(s) is activated upon drug exposure.

In contrast to other studies using different systems (16, 24), following transient drug exposure (drug induction) the increase in steady-state *MDR1* mRNA was not accompanied by a parallel increase in P-glycoprotein expression in K562 cells (Fig. 4). This effect could, in part, be due to a decrease in the rate of global protein synthesis due to cytotoxic stress caused by the drug (48–50). *MDR1* mRNA, but no P-glycoprotein, was also demonstrated in K562 cells after colcemid withdrawal (Fig. 5B) and in K562 cells resistant to the non-P-glycoprotein substrate cytarabine (data not shown). In addition, CCRF-CEM cells and EBV-transformed human lymphocytes do not express P-glycoprotein despite being *MDR1* mRNA-positive (data not shown). A lack of correlation between *MDR1* expression and P-glycoprotein function has also been reported previously in acute myeloid leukemia cell lines (57) and human bone marrow lymphoid cells (58). There is, therefore, a clear lack of correlation between the presence of *MDR1* mRNA and P-glycoprotein in several lymphocyte cell models, showing it is not specific to K562 cells or the drug treatment regime.

In this study we show that translational regulation plays an important role in determining the levels of P-glycoprotein expression. Translational control most likely operates at the level of initiation, because *MDR1* mRNA does not migrate with the polysome fractions in P-glycoprotein-negative cells. As the sequence of *MDR1* mRNA from P-glycoprotein-expressing and non-expressing cells is identical, regulation must involve *trans*-acting factors (59, 60). Upon drug selection this translational block is overcome, enabling the cells to grow in the presence of the cytotoxic drug.

In summary, the data show that in K562 cells up-regulation of *MDR1* gene expression occurs at two distinctive steps, mRNA stabilization and translational initiation. That these two steps are distinct is also illustrated by data obtained with cytarabine, a cytotoxic drug not transported by P-glycoprotein.

Cytarabine was as effective as the P-glycoprotein substrates in inducing *MDR1* mRNA stabilization, suggesting that this is a general response to drug-induced stress. Upon selection for cytarabine resistance, P-glycoprotein was expressed in a subpopulation of cells, in contrast to selection for resistance to P-glycoprotein substrates where all cells expressed P-glycoprotein (Fig. 2A). *MDR1* mRNA was up-regulated in clones derived from both the P-glycoprotein-positive and P-glycoprotein-negative cells (data not shown) indicating that in these cells the first step in the acquisition of MDR has occurred. The second step, relief from the translational block, had also occurred in a subpopulation of cells. As cytarabine induces apoptosis (61), and caspase-dependent apoptotic pathways are inhibited in P-glycoprotein-overexpressing K562 and CCRF-CEM cells (62), the expression of P-glycoprotein in this subpopulation of cytarabine-resistant cells may indicate that P-glycoprotein confers a selective growth advantage, perhaps through inactivation of caspase-dependent processes.

These findings have implications for attempts to circumvent or overcome drug resistance in the clinic, at least for leukemia. First, the demonstration that *MDR1* mRNA levels do not necessarily correlate with P-glycoprotein expression show that measuring *MDR1* mRNA as a clinical surrogate for P-glycoprotein-mediated drug resistance is inappropriate. Second, as drugs themselves can induce stabilization of *MDR1* mRNA, the first necessary step in acquiring P-glycoprotein-mediated drug resistance, drug treatment regimes should be developed to minimize this occurrence. Finally, as induction of drug resistance involves two specific steps, stabilization of *MDR1* mRNA and subsequent overcoming of a translational block, each of these steps provides novel and potentially specific targets for circumventing drug resistance in the clinic.

Acknowledgments—We thank Kathleen Scotto and Mercedes Rincon for the gift of p*MDR1* and p*P1-luc*, respectively, Katie Smith for assistance with flow cytometry, and Nic Jones and Anne Willis for helpful discussions.

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