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Induction of *pgp3* Expression and Reversion of the Multidrug Resistance Phenotype in 9-OH-Ellipticine-Resistant Chinese Hamster Lung Fibroblasts Transfected with the MYC Oncogene

Charlotte Delaporte, François Dautry† and Alain Jacquemin-Sablon*‡*

*Unité de Biochimie-enzymologie (CNRS URA 147), and †Laboratoire d'Oncologie Moléculaire (CNRS URA 1158), Institut Gustave Roussy, 94805 Villejuif, France

ABSTRACT. Chinese hamster lung cells resistant to the DNA topoisomerase II inhibitor 9-OH-ellipticine (DC-3F/9-OH-E) are cross resistant to various drugs through the expression of the MDR phenotype. The *myc* oncogene was approximately 10-fold amplified and 20-fold overexpressed in parental DC-3F cells as compared with DC-3F/9-HO-E cells. Transfection of the resistant cells with a mouse *c-myc* gene did not alter the resistance to topoisomerase II inhibitors and, in cells with a low multidrug (MDR) expression, reversed this phenotype. Northern and Western blot analyses revealed an increased expression of *pgp1* in the DC-3F/9-OH-E cells, which was not modified in the *myc*-transfected clones. However, *myc* expression in these clones resulted in an increased expression of *pgp3*, roughly in proportion to the level of *myc* expression. Transfection of the DC-3F/9-OH-E cells with the human *MDR3* gene, homologous to *pgp3*, also resulted in the reversion of the MDR phenotype. These results show that (1) expression of the transfected *myc* gene positively regulates *pgp3* expression but has no effect on *pgp1*; (2) when observed, reversion of the MDR phenotype is proportional to the levels of *myc* and *pgp3* expression; and (3) this reversion, resulting from *pgp3* expression, is associated with a decreased functional activity of the *pgp1* protein and might require an appropriate balance of *pgp1* and *pgp3* expression. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;1:59–66, 1997.

KEY WORDS. *myc*; *pgp3* expression; MDR reversion

Resistance to the DNA topoisomerase II inhibitor 9-OH-E§ in the Chinese hamster lung fibroblast cell line DC-3F was associated with several phenotypic changes. DC-3F/9-OH-E cells were cross resistant to DNA topoisomerase II inhibitors through an alteration of topoisomerase II activity and to other drugs such as actinomycin D, VCR, and taxol through the expression of the MDR phenotype. MDR expression differed from one subclone to another [1–3]. In addition, 9-OH-E-resistant cells displayed a markedly reduced tumorigenicity. When DC-3F/9-OH-E cells were grown for more than 18 months in the absence of a selecting agent, the resistance to topoisomerase II inhibitors remained unchanged [3], whereas the cells tended to lose their MDR phenotype and regain their tumorigenicity. These observations and those of others (reviewed in [4]) suggest that in some cases the same genes that control cell tumorigenicity might also regulate the expression of the MDR phenotype.

We previously showed [5] by Southern and Northern blot analyses that the *myc* oncogene was approximately 10-fold amplified and 20-fold overexpressed in parental DC-3F cells as compared with normal Chinese hamster lung fibroblasts. Both amplification and overexpression were markedly decreased in the drug-resistant cells. Transfection of a DC-3F/9-OH-E line expressing the MDR phenotype at a low level (approximately 4-fold resistance to VCR) with *c-myc* neither restored tumorigenicity nor sensitivity to 9-OH-E but reversed the MDR phenotype almost in proportion to exogenous *myc* gene expression [5]. Unexpectedly, an increased accumulation of MDR transcripts was observed in the *myc*-transfected clones, also in proportion to *myc* expression. This observation raised the issue of the mechanism of this reversion.

Mammalian *mdr* genes belong to a small family of highly homologous genes that encode membrane-bound glycoproteins [6]. On the basis of their functional properties, these proteins may be classified into two groups: class I includes the human *MDR1* gene, the mouse *mdr1* (or *mdr1b*) and *mdr3* (or *mdr1a*) genes and the Chinese hamster *pgp1* and *pgp2* genes, which encode proteins that act as ATP-

‡ Corresponding author: Dr. A. Jacquemin-Sablon, Unité de Biochimie-Enzymologie, Institut Gustave Roussy, 94805 Villejuif, France. TEL: 0142114552; FAX: 0142115276.

§ *Abbreviations:* 9-OH-E, 9-OH-ellipticine; MDR, multidrug resistance; VCR, vincristine; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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dependent pumps to lower the intracellular concentrations of certain cytotoxic drugs; class II includes the human *MDR3* (or *MDR2*), the mouse *mdr2* gene and the Chinese hamster *pgp3*, which are not involved in drug resistance. Recently, the mouse *mdr2* protein was shown to be a phosphatidylcholine transferase required for the secretion of phospholipids into bile [7, 8].

In this paper, we report that the MDR phenotype in the DC-3F/9-OH-E cells resulted from an increased expression of the *pgp1* gene. In the *myc*-transfected cells, *pgp1* expression was not modified, whereas the expression of *pgp3* was induced in proportion to *myc* expression. In cells expressing the *pgp1* gene at a low level, *myc* transfection was also associated with a reversion of the MDR phenotype. Furthermore, transfection of these cells with the human *MDR3* gene, which is homologous to *pgp3*, also resulted in the reversion of the MDR phenotype. This reversion, resulting from the functional inhibition of the pgp1 protein, was not observed in a DC-3F/9-OH-E line expressing *pgp1* at a high level (approximately 40-fold), thus suggesting that it requires the coexpression of the *pgp1* and *pgp3* genes in the same cell with an appropriate balance.

MATERIALS AND METHODS

Cell Cultures

The Chinese hamster cell line DC-3F and the 9-OH-E-resistant subline DC-3F/9-OH-E have been previously described, as have the media and growth conditions [1].

Selection of the *myc*-transfected clones has been previously described [5]. Briefly, the DC-3F/9-OH-E cells were cotransfected with two plasmids: pSVc-myc carried exons 2 and 3 of the mouse *c-myc* gene and pSVtk-neo-β conferred resistance to the antibiotic G418. The transfected clones, first selected for resistance to the antibiotic, were then assayed for the expression of the transfected *myc* gene by Northern blot analysis.

A similar cotransfection and selection procedure was used to introduce the human *MDR3* cDNA in the DC-3F/9-OH-E cells. The plasmid pJ3W-MDR3, which contained the *MDR3* coding sequence (3839 nt), was obtained from ATCC (Rockville, MD, USA).

Cellular accumulation of ^{3}H-vincristine was determined according to the method of Charcosset *et al.* [9].

RNA Blot Analysis

Polyadenylated RNAs from the different cell lines were purified from total RNA using the mRNA purification kit from Pharmacia LKB Biotechnology (Uppsala, Sweden) and by following the manufacturer's instructions. Three probes, A, B and C, which specifically recognize the mouse *mdr1a*, *mdr1b* and *mdr2* genes respectively [10], were kindly provided by Dr. S. Horwitz. Purified mRNAs (4.5 μg) were successively hybridized with probes A, B or C under the conditions described by Hsu *et al.* [10] except that prehybridization and hybridization were carried out at 52°C in 40% formamide. Under these conditions, probes A, B and C also specifically recognize the Chinese hamster *pgp1*, *pgp2* and *pgp3* sequences.

By following Dr. A. H. Schinkel's (Amsterdam) instructions, a human *MDR3*-specific probe [11] was isolated using Hind III and BamHI, which produced an approximately 1000-bp-specific 5′ MDR3 fragment.

Immunoblot Analysis of MDR Proteins

Plasma membrane proteins were prepared as described by Roy and Horwitz [12] and resolved on 7.5% SDS-PAGE gel. After electroblot transfer, nitrocellulose blots were probed with a rabbit polyclonal antibody (R3) whole serum raised against the mouse MDR protein (1:200 dilution), as described by Zeheb *et al.* [13]. The R3 antibody was kindly provided by Dr. S. Horwitz. After 3 hr of incubation at room temperature and rinsing, buffer A [13] containing the anti-rabbit Ig ^{125}-labeled whole antibody (14 μCi/μg; 10^5 cpm/mL), was added for 1.5 hr at room temperature. After rinsing, the membrane underwent autoradiography.

Immunoblot Analysis of the c-myc Protein

Cell lysate preparation, electroblotting onto nitrocellulose papers and probing with the antibody were carried out as described by Evan *et al.* [14]. The pan-myc (XM5FB) antibody, a rabbit polyclonal antibody raised against a synthetic peptide (APSEDIWKKFEL), kindly provided by Dr. G. Evan, was diluted 1:500. Detection with the anti-rabbit Ig ^{125}I-labeled antibody was carried out as above.

RESULTS

Characterization of myc *Expression and MDR phenotype in the* myc*-Transfected DC-3F/9-OH-E Cells*

As previously reported, the *myc* gene was first transfected in a DC-3F/9-OH-E line expressing the MDR phenotype at a low level [5]. Three clones with different transcription levels of the transfected *myc* gene (clone 7 > clone 5 > clone 13) and one clone (clone 1) transfected only with the G418-resistance plasmid were selected for further analysis.

We first examined the expression of the transfected *myc* gene at the protein level. Figure 1 (lane 1) shows that in the parental DC-3F cells the pan-myc antibody essentially recognized two proteins with mobilities corresponding to molecular weights of approximately 67 and 62 kDa. These proteins were virtually 3-fold less abundant in DC-3F/9-OH-E cells (lane 2) and in clone 1 (lane 3) than in DC-3F cells (lane 1). In the *myc*-transfected clones, a markedly increased expression of p62 was observed: for example, in clone 7 (lane 5), p62 expression was approximately 15-fold higher than in the untransfected cells and 10-fold higher than in the parental DC-3F cells. These results are consistent with our previous Northern blot experiments showing, in clone 7, a 30-fold increased *myc* transcription as compared with the DC-3F cells [5]. As expected from the pSVc-

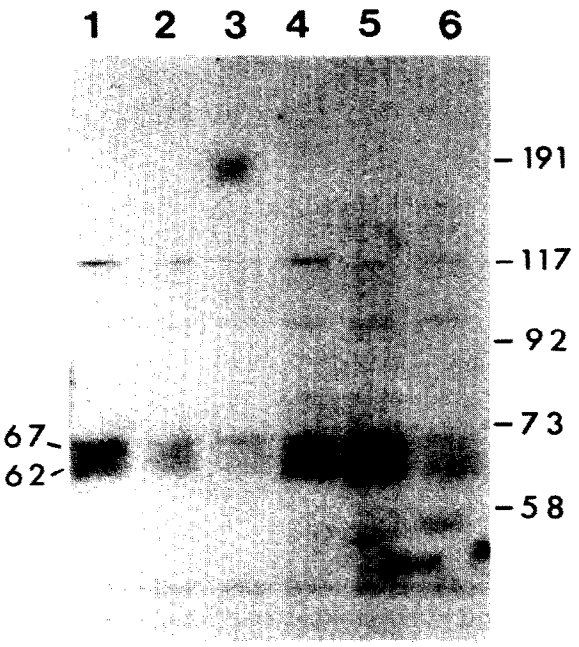

FIG. 1. Expression of the myc protein in the c-myc-transfected clones. Nuclear proteins (80 µg) were resolved on SDS-10% polyacrylamide gels, blotted to nitrocellulose membrane and probed with the pan-myc antibody XM5FB as described in Materials and Methods. Lane 1: DC-3F; lane 2: DC-3F/9-OH-E; lane 3: clone 1; lane 4: clone 5; lane 5: clone 7; lane 6: clone 13.

myc structure, which does not allow the expression of this protein, p67 was not significantly affected.

Determination of drug sensitivity in the *myc*-transfected clones indicated a reversion of the MDR phenotype [5]. To confirm this observation, the uptake of ^{3}H-VCR was compared in the different clones; Fig. 2 shows that the uptake of the drug was approximately 20% lower in the untransfected DC-3F/9-OH-E than in the parental DC-3F cells. Uptake of the drug in clone 1 was similar to that in the untransfected DC-3F/9-OH-E cells, whereas in clone 7 it was very close to that in the parental DC-3F cells. Because the lethal effect of a cytotoxic agent is an exponential function of the drug intracellular accumulation [15], a 20–25% decreased drug uptake is consistent with a 4-fold drug resistance.

These experiments show that the reversion of resistance to VCR in the *myc*-transfected DC-3F/9-OH-E cells was associated with an increased uptake of the drug, thus indicating an alteration of the MDR function related to the *myc* overexpression.

Expression of the pgp *Genes in* myc*-Transfected Cells*

In a previous work [5] using a 1.3-kb *Eco*RI–*Hind*III fragment from the mouse *mdr3* gene, which recognizes the three *pgp* genes as a probe, we observed an increased expression of these genes in DC-3F/9-OH-E cells as compared with parental DC-3F cells and a further increase in *myc*-transfected clones. We now examine the expression of the *pgp1*, *2*, and *3* genes by Northern blot analysis using three probes, A, B and C, previously described by Hsu *et al.* [10] that specifically recognize the mouse *mdr1a* (*mdr3*), *mdr1b* (*mdr1*) and *mdr2* genes, respectively. Under appropriate stringency conditions, these probes also specifically recognize Chinese hamster *pgp1*, *pgp2*, and *pgp3* genes, respectively. After fractionation by electrophoresis (1.2% agarose) and transfer to Hybond-N membrane, purified polyadenylated RNAs isolated from different cell types were successively hybridized with probes A, B and C.

Figure 3A shows that detection of *pgp1* transcripts with probe A reveals an increased expression of the *pgp1* gene in the DC-3F/9-OH-E cells (lane 2) as compared with the parental DC-3F cells (lane 1). In different experiments, an average 2-fold difference was observed, which is consistent with the low level of MDR resistance in this particular clone of DC-3F/9-OH-E cells. The *pgp1* expression in clone 1 (lane 3) was identical to that in the untransfected cells and remained unchanged in the *myc*-transfected clones (lanes 4–6). As a control, lane 7 shows a large overexpression of *pgp1* in the DC-3F/AD X variant, a cell line selected for a several-thousand-fold resistance to actinomycin D and that overexpresses the MDR protein. Figure 3B shows that probe B did not recognize any transcript in these cells, thus indicating that *pgp2* did not play a part in the MDR phenotype of the DC-3F/9-OH-E cells. In contrast, Fig. 3C shows that the *pgp3* gene was specifically expressed in the *myc*-transfected clones (lanes 4–6), roughly in proportion to the level of *myc* expression.

Western blot analysis of the MDR proteins (Fig. 4) confirms these results. The pgp1 protein, with a molecular weight of approximately 160 kDa, was present in all the tested cell lines. As discussed by Schinkel *et al.* [11], because of possible variations in the glycosylation of these

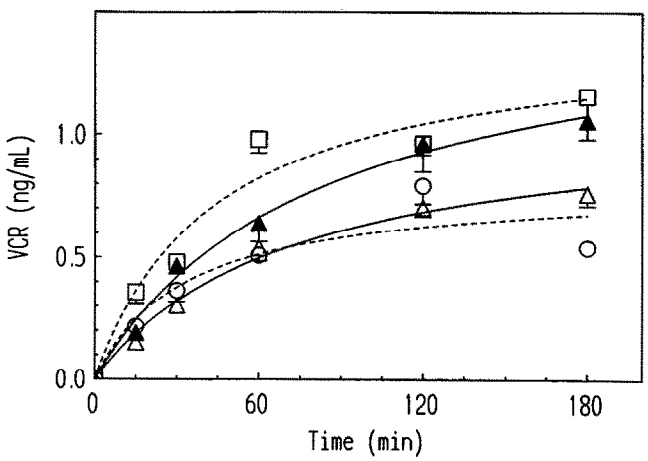

FIG. 2. Cellular accumulation of VCR. Cellular accumulation of ^{3}H-VCR (0.01 µg/mL) was determined as described elsewhere [8]. Each point represents the average of two independent experiments in which each determination was carried out in duplicate. The error bars correspond to the standard deviation on each point. Solid triangle, DC-3F; open triangle, DC-3F/9-OH-E; circle, clone 1; square, clone 7.

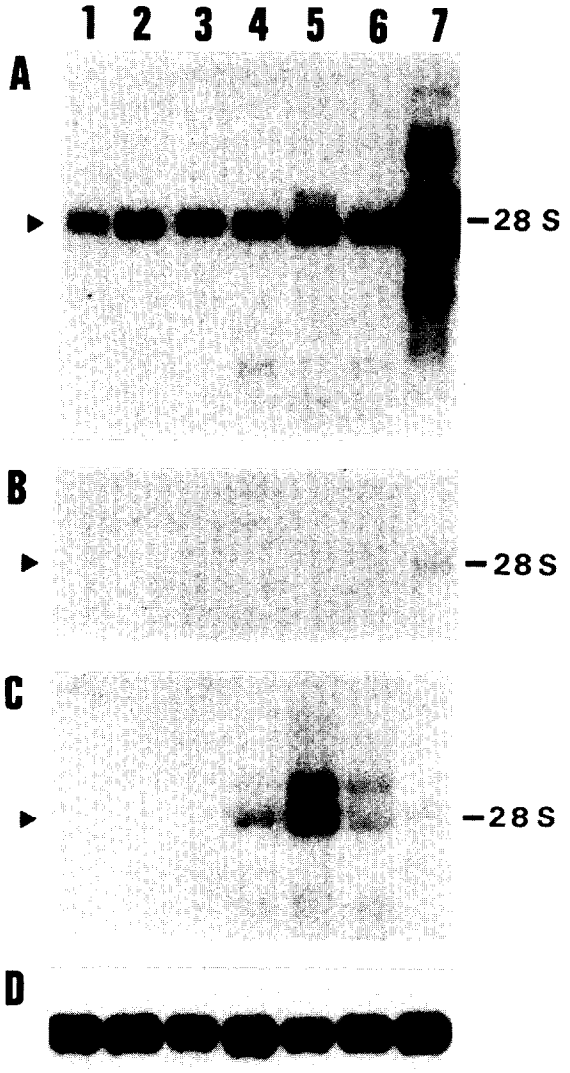

FIG. 3. Northern blot analysis of *pgp1*, *pgp2* and *pgp3* expression in *myc*-transfected cells. Purified polyA-RNAs (4.5 μg/lane) were size fractionated by agarose gel electrophoresis, transferred to Hybond membrane and successively hybridized with *pgp1* (A), *pgp2* (B), *pgp3* (C) and actin (D) probes. Details are given in Materials and Methods. Lane 1: DC-3F; lane 2: DC-3F/9-OH-E; lane 3: clone 1; lane 4: clone 5; lane 5: clone 7; lane 6: clone 13; lane 7: DC-3F/ADX. Arrowheads on the left indicate the expected positions of the *pgp* genes transcripts. The position of the 28S RNA is shown on the right.

proteins, the appearance of pgp bands was fuzzy. Therefore, weak differences, such as those expected to be associated with the low level of the MDR phenotype in the untransfected DC-3F/9-OH-E cells, are difficult to determine precisely. In most experiments, the intensity of this band was increased by approximately 1.5–2-fold in all the resistant lanes as compared with the sensitive cells in lane 1. This protein was the only MDR protein expressed in clone 1 (lane 3) and in the DC-3F/ADX variant (lane 8). In contrast, the three *myc*-transfected clones contain an additional MDR protein, with a molecular weight of about 145 kDa, which may correspond to the pgp3 protein. In agreement with the Northern blot analysis, this protein was most abundant in clone 7 (lane 5). A faint 145-kDa band was also detectable in the untransfected resistant cells. However, as shown in lane 9, this band was not detected in other experiments.

All these experiments indicate that the reversion of the MDR phenotype in the *myc*-transfected cells did not result from a decreased expression of *pgp1*. In contrast, *pgp3* expression appeared to be specifically induced by *myc* in a dose-dependent manner.

A relation between the expression level of the transfected *myc* gene and the induction of *pgp3* is further supported by the growth-phase dependence of *pgp3* expression in clone 10. In most of the *myc*-transfected clones, the transfected *myc* gene was expressed at about the same level during the exponential and plateau phases of growth, as expected for a gene under the control of an SV40 promoter. Similarly, *pgp3* expression in these clones was independent of the growth phase. For example, Figure 5 (lanes 5 and 6) shows that in clone 7 *myc* expression was even slightly increased at the plateau phase, as was *pgp3* expression. However, among the *myc* transfectants initially isolated, clone 10 displayed a different behavior: as shown in lanes 7 and 8, the *myc* expression level in clone 10 was decreased about 7-fold at the plateau phase as compared with the exponential phase (Fig. 5A), and a comparable decrease in the *pgp3* expression (Fig. 5B) was also observed.

Taken together, these results show that in the *myc*-

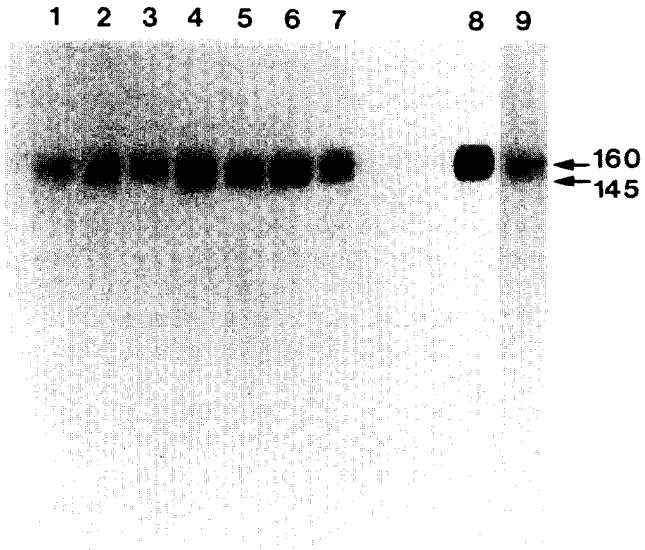

FIG. 4. Immunoblot analysis of *pgp* gene products in *myc*-transfected cells. Plasma membrane-enriched subcellular fractions (40 μg) were resolved on SDS–7% polyacrylamide gels, electroblotted to nitrocellulose filters and probed with the R3 antibody (see Materials and Methods). Lane 1: DC-3F; lane 2: DC-3F/9-OH-E; lane 3: clone 1; lane 4: clone 5; lane 5: clone 7; lane 6: clone 13; lane 7: clone 10; lane 8: DC-3F/ADX; lane 9: DC-3F/9-OH-E control from an independent experiment.

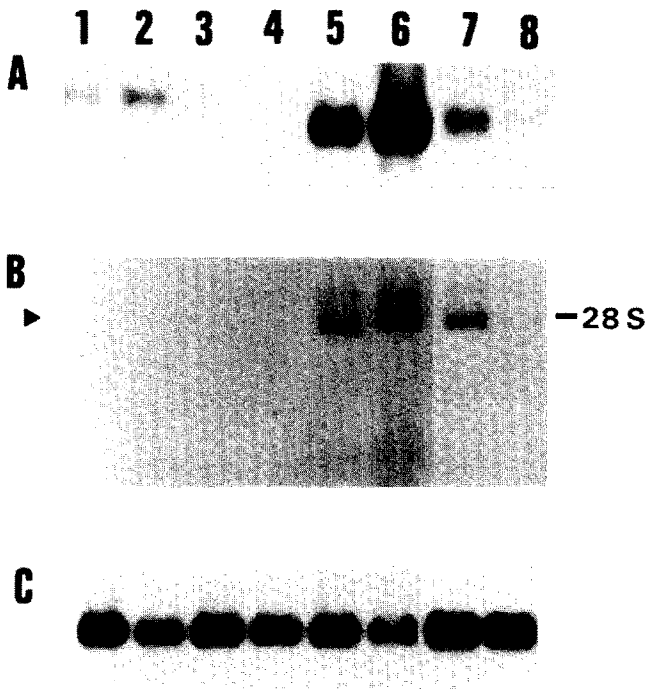

FIG. 5. Growth-phase dependence of *myc* and *pgp3* expression. Purified polyA-RNAs were prepared from the different cell lines either during the exponential phase of growth or at the plateau phase. After fractionation by agarose gel electrophoresis (4.5 μg/lane) and transfer to Hybond membrane, the RNAs were successively hybridized with *myc* (A), *pgp3* (B) and actin (C) probes. Experimental conditions are as described in Materials and Methods. Lanes 1–2: DC-3F, exponential and plateau; lanes 3–4: DC-3F/9-OH-E, exponential and plateau; lanes 5–6: clone 7, exponential and plateau; lanes 7–8; clone 10, exponential and plateau. The arrowhead on the left indicates the expected position of the pgp3 transcript.

transfected DC-3F/9-OH-E cells *pgp3* expression is positively regulated through the expression of the transfected *myc* gene.

Transfection of the Human MDR3 Gene in DC-3F/9-OH-E Cells

The absence of modification of *pgp1* expression in the *myc* transfectants associated with the induction of *pgp3* expression suggests that the coexpression of *pgp1* and *pgp3* could be responsible for the reversion of the MDR phenotype in these cells. This hypothesis could be directly tested by transfecting the *pgp3* gene in the DC-3F/9-OH-E cells. However, because hamster *pgp3* cDNA was not available, this experiment was carried out with its human homolog, *MDR3*. The plasmids pJ-W-MDR3 and pSVtk-neo-β were cotransfected in cells derived from the same DC-3F/9-OH-E subclone used in the previous experiments.

The transfected cells were selected for G418 resistance and then tested for *MDR3* expression. From approximately 40 tested clones, only 4 stable *MDR3*-expressing clones were obtained. A similar low proportion of expressing clones was previously reported for this gene [11]. A Northern blot analysis of *MDR3* and *pgp1* expression in these clones is presented in Fig. 6. A high level of *MDR3* expression was detected in one clone (cl 47), and a lower level was detected in the other clones, as shown for cl 58 in Fig. 6B. The *pgp1* expression was not modified in the transfected cells (Fig. 6C). The resistance to VCR was about 2-fold lower in cl 58 than in the untransfected cells and almost completely abolished in cl 47 (Fig. 7), thus showing that *MDR3* overexpression is associated with the reversion of

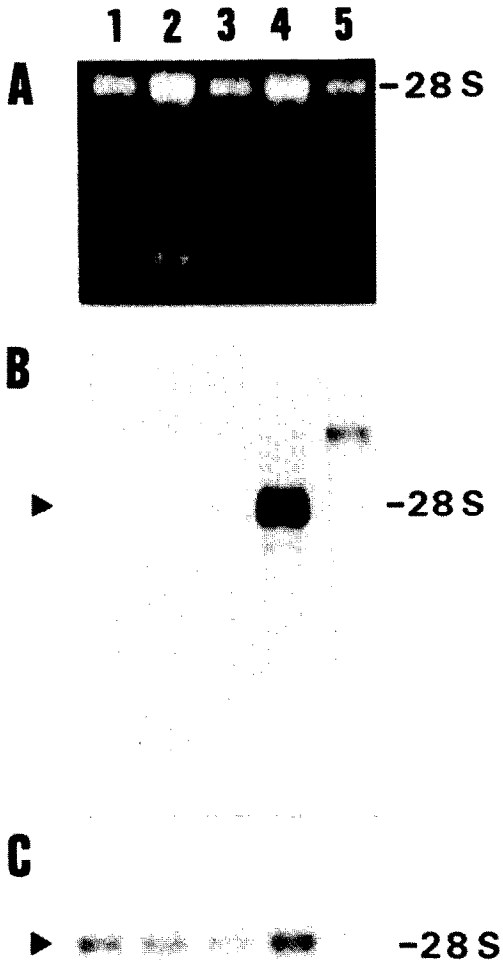

FIG. 6. Northern blot analysis of *MDR3* expression in pJ3-W-MDR3-transfected clones. After size fractionation by agarose gel electrophoresis and transfer to Hybond membrane, purified polyA-RNAs were successively hybridized with the *MDR3* (B) and *pgp1* (C) specific probes. (A) Ethidium bromide staining of the gel. Lane 1: DC-3F; lane 2: DC-3F/9-OH-E; lane 3: cl2 (transfected with pSVtk-neo-β); lane 4: cl47; lane 5: cl58. Arrowheads on the left indicate the expected positions of the *MDR3* (B) and *pgp1* (C) transcripts.

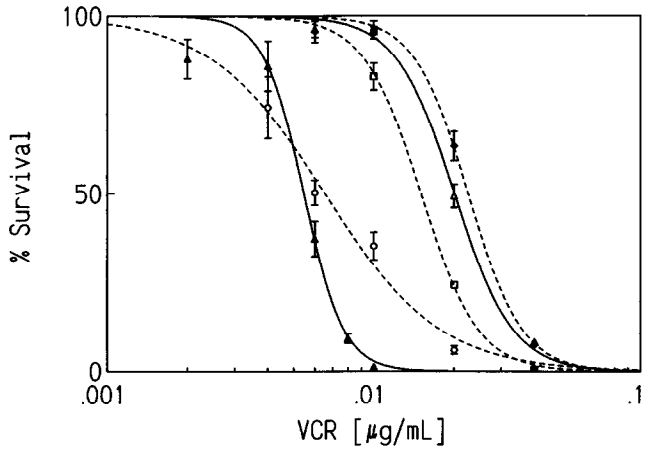

FIG. 7. Toxicity of VCR on DC-3F/9-OH-E cells transfected with pJ3-Ω-MDR3. Cloning efficiency was determined after 3 hr exposure to the indicated drug concentrations. Each point is the average of two independent experiments, each done in triplicate. Solid triangle DC-3F; open triangle, DC-3F/9-OH-E; diamond, cl2; circle, cL47; square cl58.

the MDR phenotype, again in relation to the expression level of the transfected cDNA.

These data indicate that the reversion of the MDR phenotype in the *myc*-transfected cells results from an alteration of *pgp3* expression relative to that of *pgp1*. To investigate this hypothesis further, we then examined the effect of *myc* overexpression in cells expressing *pgp1* at a different level.

Transfection of the myc *Oncogene in Cells with a High MDR Expression Level*

A DC-3F/9-OH-E clone, named variant A, with a *pgp1* amplification yielding an approximate 40-fold resistance to the MDR-sensitive drugs, was transfected with the pSVc-myc and pSVtk-neo-β plasmids. Twenty-five clones resistant to G418 and expressing high levels of the transfected *myc* gene were selected. In all, the resistance to VCR remained unchanged. Four of these clones with different *myc* expression levels (Fig. 8A, lanes 4–7) were further analyzed for *pgp1* and *pgp3* expression. Figure 8B shows that the expression of *pgp1* in variant A (lane 2) and in the myc transfectants, was about 20-fold higher than in the DC-3F cells (lane 1). Taking into account the loading differences measured from the hybridization signal with the actin probe shown in Fig. 8D, this experiment confirms that *myc* overexpression has no influence on the expression of *pgp1*. Figure 8C also shows that *pgp3* expression was induced in these clones, again roughly in proportion to the *myc* expression level. Therefore, despite a *pgp3* induction comparable to that observed in clone 7 (lane 8), there was no reversion of the MDR phenotype in these cells. If the previous hypothesis is correct, this means that MDR phenotype reversion in cells coexpressing *pgp1* and *pgp3* is only observed when both genes are expressed at comparable levels.

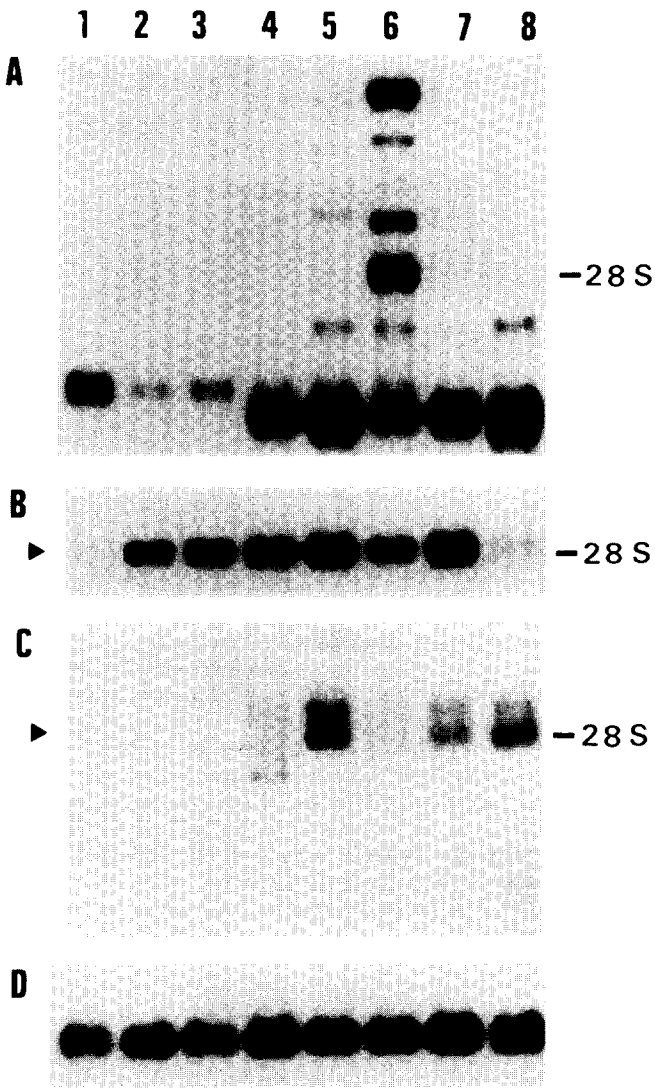

FIG. 8. Induction of *pgp3* expression after *myc* transfection in DC-3F/9-OH-E cells expressing a high level of *pgp1*. A DC-3F/9-OH-E subclone about 40-fold resistant to VCR (variant A) was cotransfected with the G418-resistance plasmid pSVtk-neo-β and pSVc-myc [5]. Purified polyA-RNAs (4.5 μg/lane) were size fractionated by agarose gel electrophoresis, transferred to Hybond membrane, and successively hybridized with *myc* (A), *pgp1* (B), *pgp3* (c) and actin (D) probes. Experimental conditions are as described in Materials and Methods. Lane 1: DC-3F; lane 2: untransfected variant A; lane 3: variant A transfected with the pSVtk-neo-β plasmid alone; lanes 4–7: clones A–D transfected with both plasmids; lane 8: clone 7. Although clone 7 RNA was hybridized on the same blot, the corresponding lane was cut to place it next to clone D and facilitate the comparison with the other *myc*-transfected clones. Arrowheads on the left indicate the expected positions of the *pgp1* (B) and *pgp3* (C) transcripts. Because of the high *pgp1* expression levels in variant A, the short exposure time of this autoradiography did not allow the visualization of the DC-3F and clone 7 bands.

DISCUSSION

The DC-3F/9-OH-E cells, selected for resistance to the topoisomerase II inhibitor 9-OH-E, were cross resistant to other drugs such as actinomycin D, adriamycine, VCR and taxol, which are sensitive to the MDR phenotype [1–3]. Expression of the MDR phenotype in the DC-3F/9-OH-E cells was demonstrated by (1) a decreased cellular accumulation of actinomycin D, adriamycine and VCR [2]; (2) the reversion of this cross-resistance pattern in the presence of verapamil, which restores a normal uptake of the different drugs [2]; and (3) an increased expression of the *pgp* genes [5]. However, from one subclone to another the expression of the MDR phenotype is variable, from approximately 4- to 40-fold [3].

Overexpression of a transfected *myc* gene in one DC-3F/9-OH-E subclone that expressed a low level MDR phenotype did not alter the resistance to topoisomerase II inhibitors but reversed the resistance to the MDR-sensitive drugs such as VCR, actinomycin D or taxol [5]. We show here that VCR accumulated at the same level in the parental sensitive DC-3F cells and in the *myc*-transfected clone 7, whereas drug accumulation was decreased by approximately 20% in the resistant DC-3F/9-OH-E cells. These data indicate that MDR phenotypic reversion in *myc*-transfected cells is associated with a decreased functional activity of the MDR protein, which may result from either a decreased synthesis of this protein or the inhibition of its activity.

Using specific probes, the transcripts corresponding to the three *pgp* genes were analyzed in two DC-3F/9-OH-E sublines: the 4-fold VCR-resistant subclone used in the *myc*-transfection experiments and a 40-fold VCR-resistant subclone. This study first revealed that the expression of the *pgp1* gene is increased in proportion to the resistance level, thus accounting for the MDR phenotype. Then, regardless of the initial *pgp1* expression level, *myc* overexpression in the transfected cells had no effect on *pgp1* expression. Western blot analysis of the *pgp1* protein confirmed that a decreased synthesis of this protein is not responsible for the reversion of the MDR phenotype. An inverse correlation between expression of the human *mdr1* gene and the N-*myc* oncogene has been previously described in human neuroblastomas [16] but was not observed in this system. Our data show that reversion of the MDR phenotype in the *myc*-transfected DC-3F/9-OH-E cells results from a functional alteration of the *pgp1* protein and not from the modulation of *pgp1* expression.

In the *myc*-transfected clones, *pgp3* expression was induced in proportion to the *myc* expression level. The positive regulatory effect of *myc* on *pgp3* was confirmed by the experiments on clone 10, where *pgp3* expression parallels the variation in *myc* expression during the cell growth phases. The molecular basis of this regulation is not presently understood. Two translational forms of the Myc protein have been identified in all species examined so far, and functional differences have been reported for these proteins [17]. In the *myc*-transfected clones, only the expression of the c-Myc 2 protein (p62) is increased. The lack of pgp3 induction in the parental cells suggests that it requires a certain threshold of c-Myc 2 synthesis and/or a qualitative alteration of myc regulation. Expression of the MDR genes is tissue specific [18, 19] and apparently controlled by multiple regulatory mechanisms (reviewed in [20]). The presence in the c-Myc protein of structural motifs seen in a number of transcription factors and the identification of genes directly upregulated by *c-myc* [21, 22] demonstrate the capacity of this gene to modulate gene expression. The c-Myc-specific DNA binding sequence CACGTG is not present in the *pgp1* promoter. If this motif were present in the sequence of the *pgp3* promoter, then a direct regulation of this gene by *myc* would be possible. Alternatively, *myc* probably modulates the activity of a number of genes, one of which might be a mediator in a regulation pathway of *pgp3*.

Transfection of the human *MDR3* cDNA in the DC-3F/9-OH-E cells also resulted in the reversion of the MDR phenotype, thus supporting a direct link between *pgp3* expression and MDR reversion in the *myc*-transfected cells. In DC-3F/9-OH-E cells expressing a low MDR, both the level of *pgp3* induction and MDR reversion correlated with the *myc* expression level. In cells expressing a high MDR, the reversion of this phenotype was not observed, thus suggesting that it requires an appropriate balance between the expression levels of both *pgp* genes. Recently, the mouse *mdr2* gene (homologous to *pgp3* and *MDR3*) was shown to act as a specific lipid flippase, which has no activity as a drug transporter [8]. However, some experimental data (reviewed in [6]) indicate that the functional transporter for the P-glycoprotein could be a multisubunit protein (dimer or even tetramer). The *pgp3* expression might then antagonize the activity of the *pgp1* protein by a direct interaction, leading to the formation of an inactive protein complex. However, the simultaneous expression of class I and II *MDR* genes, previously observed in some normal tissues [23, 24], suggests that an interaction between their products could only take place in cells presenting a deregulation of their expression, e.g. through an overexpression of *myc*.

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References

1. Salles B, Charcosset J-Y and Jacquemin-Sablon A, Isolation and properties of Chinese hamster lung cells resistant to ellipticine derivatives. *Cancer Treat Rep* **66:** 327–337, 1982.
2. Delaporte C, Charcosset J-Y and Jacquemin-Sablon A, Effects of verapamil on the cellular accumulations and toxicity of several antitumor drugs in 9-hydroxy-ellipticine resistant cells. *Biochem Pharmacol* **37:** 613–619, 1988.
3. Larsen AK and Jacquemin-Sablon A, Multiple resistance mechanisms in Chinese hamster cells resistant to 9-hydroxy-ellipticine. *Cancer Res* **49:** 7115–7119, 1989.
4. Chin KW, Ueda K, Pastan I and Gottesman KM, Modulation

of activity of the promoter of the human *MDR1* gene by *ras* and *p53*. *Science* **255:** 459–462, 1992.
5. Delaporte C, Larsen AK, Dautry F and Jacquemin-Sablon A, Influence of myc overexpression on the phenotypic properties of Chinese hamster lung cells resistant to antitumor agents. *Exp Cell Res* **197:** 176–182, 1991.
6. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 385–427, 1993.
7. Smit JJM, Schinkel AH, Oude Elferink RPJ, Groen AK, Wagenaar E, van Deemter L, Mol CAAM, Ottenhoff R, van der Lug NMT, van Roon MA, van der Valk MA, Offerhaus GJA, Berns AJM and Borst P, Homozygous disruption of the murine *mdr2* P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* **75:** 451–462, 1993.
8. Ruetz S and Gros P, Phosphatidylcholine translocase: a physiological role for the *mdr2* gene. *Cell* **77:** 1071–1081, 1994.
9. Charcosset J-Y, Salles B and Jacquemin-Sablon A, Uptake and cytofluorescence localization of ellipticine derivatives in sensitive and resistant Chinese hamster lung cells. *Biochem Pharmacol* **32:** 1037–1044, 1983.
10. Hsu SI, Lothstein L and Horwitz SB, Differential overexpression of three *mdr* gene family members in multidrug-resistant J774.2 mouse cells. *J Biol Chem* **264:** 12053–12062, 1989.
11. Schinkel AH, Roelofs MEM and Borst P, Characterization of the human MDR3 P-glycoprotein and its recognition by P-glycoprotein-specific monoclonal antibodies. *Cancer Res* **51:** 2628–2635, 1991.
12. Roy SN and Horwitz SB, A phosphoglycoprotein associated with taxol resistance in J774.2 cells. *Cancer Res* **45:** 3856–3863, 1985.
13. Zeheb R, Beittenmiler HF and Horwitz SB. Use of antibodies to probe membrane glycoproteins associated with drug-resistant J774.2 cells. *Biochem Biophys Res Commun* **143:** 732–739, 1987.
14. Evan GI, Lewis GK, Ramsay G and Bishop JM, Isolation and monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol* **5:** 3610–3616, 1985.
15. Le Pecq J-B, *Chimiothérapie anticancéreuse*. Hermann, Paris, 1978.
16. Nakagawara A, Kadomatsu K, Sato SS, Kohno K, Takano H, Akazawa K, Nose Y and Kuwano M, Inverse correlation between expression of multidrug resistance gene and N-myc oncogene in human neuroblastomas. *Cancer Res* **50:** 3043–3047, 1990.
17. Hann RS, Dixit M, Sears RC and Sealy L, The alternatively initiated c-Myc proteins differentially regulate transcription through a noncanonical DNA-binding site. *Genes Dev* **8:** 2441–2452, 1994.
18. Croop JM, Raymond M, Haber D, Devault A, Areci RJ, Gros P and Housman DE, The three mouse multidrug resistance (*mdr*) genes are expressed in a tissue-specific manner in normal mouse tissues. *Mol Cell Biol* **9:** 1346–1350, 1989.
19. Teeter LD, Becker FF, Chisari FV, Li D and Kuo MT, Overexpression of the multidrug resistance gene *mdr3* in spontaneous and chemically induced mouse hepatocellular carcinomas. *Mol Cell Biol* **10:** 5728–5735, 1990.
20. Teeter LD, Eckersberg T, Tsai Y and Kuo MT, Analysis of the Chinese hamster P-glycoprotein/multidrug resistant pgp1 reveals that the AP-1 site is essential for full promoter activity. *Cell Growth Differ* **2:** 429–437, 1991.
21. Benvenisty N, Leder A, Kuo A and Leder P, An embryonically expressed gene is a target for c-Myc regulation via the c-Myc-binding sequence. *Genes Dev* **6:** 2513–2523, 1992.
22. Bello-Fernandez C, Packham G and Cleveland JL, The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc Natl Acad Sci USA* **90:** 7804–7808, 1993.
23. Croop JM, Raymond M, Haber D, Devault A, Arceci RJ, Gros P and Housman DE, The three mouse multidrug resistance (*mdr*) genes are expressed in a tissue-specific manner in normal mouse tissues. *Mol Cell Biol* **9:** 1346–1350, 1989.
24. Buschman E, Arceci RJ, Croop JM, Che M, Arias IM, Housman DE and Gros P, Mdr2 encodes P-glycoprotein expressed in the bile canalicular membrane as determined by isoform-specific antibodies. *J Biol Chem* **267:** 18093–18099, 1992.