DNA Amplification in Multidrug, Cross-resistant Chinese Hamster Ovary Cells: Molecular Characterization and Cytogenetic Localization of the Amplified DNA

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Abstract. Vincristine-resistant (VCR) Chinese hamster ovary (CHO) cells have been established by stepwise selection in increasing concentrations of vincristine. These cells exhibit multidrug cross-resistance to a number of drugs that have no structural or functional similarities. Cytogenetic analyses of resistant cells revealed the presence of double minutes and expanded chromosomal segments, thus implicating gene amplification as a possible mechanism of resistance. An amplified DNA segment isolated from other multidrug cross-resistant CHO cell lines (Roninson, I. B., H. T. Abelson, D. E. Housman, N. Howell, and A. Varshavsky, 1984, *Nature (Lond.)*, 309:626–628) is also amplified in our VCR lines. This DNA segment was used as a probe to screen a cosmid library of VCR genomic DNA, and overlapping clones were retrieved. All of these segments, totaling ~45 kilobases (kb), were amplified in VCR cells. Using in situ hybridization, we localized the amplification domain to the long arm of CHO chromosome 1 or Z1. Northern hybridization analysis revealed that a 4.3-kb mRNA was encoded by this amplified DNA domain and was overproduced in the VCR cells. Suggestions for the involvement of these amplified DNA segments in the acquisition of multidrug cross-resistance in animal cells are also presented.

A major obstacle to effective cancer chemotherapy is the development of drug resistance. The establishment of cells in culture resistant to the cytotoxicity of the tested drug remains a valuable approach in the study of mechanisms of clinical drug resistance. Multidrug drug resistance (MDR),[1] a phenomenon of cross-resistance to a variety of different drugs that have no similarities in their modes of action, has been observed in cultured animal cells primarily selected for resistance to various plant alkaloids or anthracyclines. A number of studies have been done to determine the mechanisms of MDR, and some of these have suggested that alterations in cytoplasmic membrane structure decrease intracellular accumulation of functionally diverse cytotoxic agents. Several investigators have reported such alterations in terms of overproduction of various cell surface glycoproteins (1, 2, 5, 13, 18) and accelerated drug efflux (11, 31) from resistant cells. Other reports describe overproduction of cytosolic proteins (19–21) or decreased amounts of cell surface glycoproteins (22) in various MDR cell lines. In many cases, resistance can be circumvented by adding calmodulin inhibitors or calcium channel blockers to the culture medium (32).

Additional reports demonstrated that abnormal structures such as double-minute chromosomes (DM) and homogenous staining region-like segments are present in MDR cell lines, suggesting that gene amplification may be involved (14, 15, 21). Recently, two reports have been published showing DNA amplification in Chinese hamster ovary (CHO) cells. Roninson et al. (24) used an elegant in-gel denaturation/renaturation method to show that DNA amplification had occurred in two MDR CHO cell lines. Fojo et al. (7) have recently applied the same technique in analysis of their human MDR cells lines, yielding similar results. Another report by Riordan et al. (23) demonstrated that the gene encoding a 175-kD membrane glycoprotein is amplified in an MDR CHO cell line that was established by selection in colchicine. In the present report, we show that gene amplification has also occurred in our independently established vincristine-resistant (VCR) CHO cells exhibiting MDR. Unique features in terms of cytogenetic manifestations and expression of the amplified gene in these MDR cell lines are also presented.

Materials and Methods

Cell Culture, Establishment of Drug-resistant Cell Lines, and Cytotoxicity Test of the Mutants

All cell lines were maintained as monolayer cultures at 37°C in 5% CO_2 in air in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY)

1. *Abbreviations used in this paper*: CHO, Chinese hamster ovary cells; 1× Denhardt's, 0.02% each of ficoll, polyvinylpyrollidone, and BSA; DM, double-minute chromosomes; ED_{50}, effective dose for 50% killing; MDR, multidrug resistance; 1× SSC: 0.15 M NaCl, 0.015 M Na citrate; 1× SSPE: 0.18 M NaCl, 10 mM $NaPO_4$ (pH 7.7), 1 mM EDTA; VCR, vincristine resistant.

supplemented with 10% fetal bovine serum (Hazelton, Denver, PA) and 0.1% neomycin (Pharma-Tek, Inc., Huntington, NY). Procedures for selection of VCR 1.5 and VCR 5 (designating survival in 1.5 μg/ml and 5 μg/ml vincristine, respectively) have been described (15). VCR 5 was originally referred to as VCR 6-7 (15). VCR 15 cells were established from VCR 5 by further selection in progressive increased vincristine concentrations, according to the following schedule: 10 μg/ml→12.5 μg/ml→15 μg/ml→17.5 μg/ml. Cells surviving at 17.5 μg/ml were routinely grown in 15 μg/ml of vincristine.

Dosage-response analysis was performed on cell lines by plating 500 cells in 3.5-cm dishes (Corning Glass Works, Corning Science Products, Corning, NY) in regular medium containing various concentrations of vincristine (Sigma Chemical Co., St. Louis, MO), Colcemid (Gibco), puromycin (Calbiochem-Behring Corp., La Jolla, CA), or ethidium bromide (Sigma Chemical Co.). After colonies developed (~10 d), the plates were stained with 5% crystal violet made in 5% ethanol and colonies were counted.

Cytogenetic Analysis

Cells were blocked in mitosis by adding Colcemid (0.06 μg/ml for CHO, 10 μg/ml for VCR 1.5 cells, and 20 μg/ml for VCR 5 and VCR 15 cells). Mitotic cells were harvested by gentle shake-off, swollen in 75 mM KCl for 15 min at room temperature, and fixed in three sequential changes of fixative (3:1 vol/vol, methanol/acetic acid). Air-dried slides were either stained by Giemsa (Gurr, Poole, England) (5% in 10 mM $NaPO_4$ [pH 6.8]) or ethidium bromide (50 μg/ml for 20 min at 25°C) and scored for DM chromosomes. Giemsa-trypsin (G-band) procedure was performed by the established method. CHO chromosomes were identified by the characteristic G-banding pattern described by Deaven and Petersen (6).

Preparation and Screening of Cosmid Library

High molecular weight DNA isolated from VCR 15 cells was partially digested by MboI (New England Biolabs, Beverly, MA) and the restriction fragments were fractionated by sucrose gradient centrifugation. DNA fragments of 30–45 kilobases (kb) were used to construct a cosmid library in pCV108. Detailed procedures were described by Ish-Horowicz and Burke (12) and modified by Lau and Kan (16). The cosmid DNA library was screened by the method described by Hanahan and Meselson (10) using the insert of pDR1.1 as a probe (24) (a gift from Dr. Igor Roninson, University of Illinois College of Medicine at Chicago).

Other Procedures

Procedures for isolation of DNA and RNA, and for in situ hybridization were those described previously (27). For Southern blot hybridization, DNA was digested with restriction enzyme to completion. After digestion, DNA was extracted with phenol/chloroform/isoamylalcohol and ethanol precipitated. An equal amount of DNA (10 μg) was loaded onto a 1% agarose gel, electrophoresed, and blotted onto nitrocellulose membrane. Hybridizations were carried out in 50% formamide, 5× SSPE, 1 Denhardts, and 100 μg/ml denatured salmon sperm DNA at 42°C. Northern hybridizations were in 50% formamide, 10% dextran sulfate, 1× Denhardt's, 0.05 M Tris-HCl (pH 7.5), 1 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, and 100 μg/ml salmon sperm DNA at 42°C. In each case, ~1 × 10^7 cpm denatured, nick-translated probe were added. Both hybridizations were washed under the following conditions: twice for 15 min in 2× SSC and 0.1% SDS at room temperature; twice for 30 min in 2× SSC and 1% SDS at 65-70°C; and twice for 30 min in 0.1× SSC and 0.1% SDS at room temperature.

Results

Table I shows the effective doses for 50% killing (ED_{50}) of wild-type CHO cells and for VCR CHO mutants grown in the presence of different concentrations of vincristine, colcemid, ethidium bromide, and puromycin. The ED_{50} to vincristine for CHO, VCR 1.5, VCR 5, and VCR 15 are 0.05, 2, 10, and 25 μg/ml, respectively. Therefore, mutants VCR 1.5, VCR 5, and VCR 15 were 40-, 200-, and 500-fold more resistant respectively, to the toxicity of vincristine than the wild type. These mutants were also cross-resistant to the cytotoxicities of the other three drugs tested. These drugs share no common structural or functional similarities with vincristine. The ED_{50} for CHO and VCR mutants shown in Table I demonstrated that the VCR mutant cell lines are MDR mutants. We noted that the levels of cross-resistance in these MDR mutants progressively increase as the steps of selection increase. It is also apparent that in VCR mutants, the level of cross-resistance to other drugs is always lower than resistance to vincristine.

Chromosomal Abnormalities in Vincristine-resistant Mutants

We investigated whether there are any chromosomal abnormalities in these VCR mutants. Chromosome spreads were prepared from wild-type CHO cells and from VCR mutants, stained by Giemsa, and observed under a light microscope. Abnormal chromosomal structures including dicentric chromosomes, ring chromosomes, endoreduplicated chromosomes, and chromosomes with breaks and gaps were frequently observed in ~5% of VCR 1.5 cells (not shown).

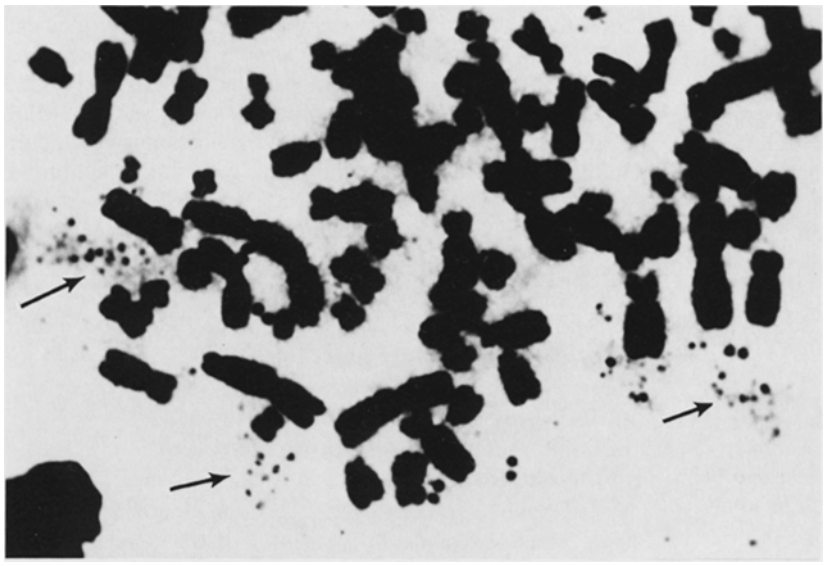

Figure 1. Giemsa-stained metaphase spreads of VCR 1.5 cells. *Arrows*, the double minutes.

Such unusual chromosomal structures were very rarely observed in the wild-type CHO lines (<0.1%).

The most often observed abnormality in metaphase spreads of VCR 1.5 cells were DM (Fig. 1). About 50% of VCR 1.5 cells contained DM; the number of DM per cell ranged from 0 to ~50. Usually higher number of DM were found in polyploid cells (Fig. 1) than in near diploid cells. DM were not found in wild-type CHO cells.

The described abnormal chromosomal structures were not frequently found (<2% of the cells) in cell lines with high levels of VCR, i.e., VCR 5 and VCR 15 cells. Instead, the most significant chromosomal abnormality associated with VCR 5 and VCR 15 cells was the presence of an additional chromosomal segment. Fig. 2 shows a composite karyotype of the CHO parental line, and MDR mutant lines, VCR 5, and VCR 15, analyzed after Giemsa-trypsin banding. Over 20 complete karyotypes were analyzed and each chromosome Z7 in the mutant cells possessed an addition to the telomere of the long arm. The additional chromosomal segments on Z7 in these VCR mutants showed discernible banding patterns rather than a homogenous staining pattern that are present in a number of drug-resistant cell lines (4). In addition, there appears a constricted region on the long arm of Z1, exhibiting an abnormally banding region. Abnormally banding regions containing a low level of gene amplification have been reported in other drug-resistant cells (17).

Gene Amplification and Overexpression of mRNA in VCR Cells

The presence of DM and an additional chromosomal segment in the VCR mutants suggests that gene amplification may have occurred in these mutants. We therefore used a cloned DNA sequence (pDR1.1) that was amplified in two MDR CHO cell lines independently selected for resistance to adriamycin and colchicine to determine whether the same DNA sequence is also amplified in our VCR cell lines.

Southern hybridization of the pDR1.1 probe to genomic DNAs from CHO and VCR cells demonstrated amplification of this DNA segment in resistant cells (data not shown). Because the sequence that hybridized with the insert of pDR1.1 was not transcribed in the VCR mutants (not shown), we screened a 500,000-clone cosmid library prepared from VCR 15 DNA with the insert of pDR1.1 to search for flanking, transcribed sequences. Two cosmid DNA clones, designated pDR6 and pDR7, were isolated. Fig. 3 shows the restriction enzyme cleavage sites of BglI, EcoRI, PstI, and Bam HI on pDR6 and pDR7, with the location of pDR1.1 indicated. The restriction enzyme map for BamHI generally agrees with that recently published by Gros et al. (8).

We used pDR6 as a probe to determine whether the sequences other than that in the pDR1.1 were also amplified in these VCR cell lines. Fig. 4 *a* shows an autoradiogram of a Southern blot hybridization of EcoRI-digested genomic DNAs from CHO, VCR 1.5, VCR 5, and VCR 15 cells hybridized with ^{32}P-labeled pDR6 DNA. The same amount of DNA (10 μg) was loaded on each lane and autoradiogram was exposed for the same length of time (24 h). Hybridization signals corresponding to EcoRI restriction fragments of 8, 6, 4, 2.8, and 2 kb were shown in the VCR samples but not visible in the CHO sample. This indicated that all the sequences in these fragments were amplified in these VCR cell lines. We noted that no significant further amplification of these DNA sequences occurred during the selection of VCR 15 cells from VCR 1.5 (judged from the band intensities of the VCR 15 sample and VCR 1.5 sample as shown in the autoradiogram; Fig. 4 *a*, lanes *2–4*). Consistent hybridization signals were seen for the VCR 1.5, VCR 5, and VCR 15 samples and no new hybridization bands were seen in the DNA samples from more resistant cell lines. Furthermore, all the EcoRI fragments as detected by Southern blot hybridization shown in Fig. 4 *a* can be located from the restriction map for the pDR6, as Fig. 3 shows. These results suggest that no major DNA rearrangement occurred in this amplified DNA locus. Using the entire pDR6 as a hybridization probe, we have consistently failed to detect a clear hybridization signal from the CHO sample. The reason for this is probably the presence of repetitive DNA sequences in the pDR6 insert. In fact, such repetitive sequences may also have caused the high hybridization background Fig. 4 *a* shows.

To address this problem, we sought to determine the locations of repeated DNA sequences in the cosmids, pDR6, and

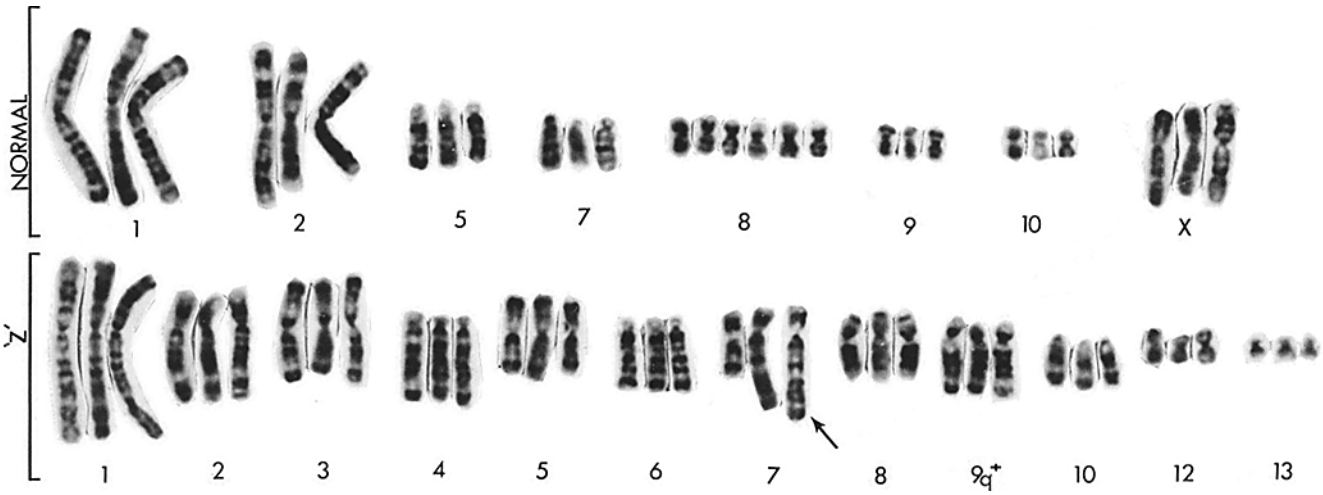

Figure 2. Giemsa-trypsin banding of CHO parental line and VCR mutants. Chromosomal arrangement is according to that published by Deaven and Peterson (6). In each case, the CHO chromosomes are shown on the left, VCR 5 in the middle, and VCR 15 on the right. *Arrow*, the additional chromosome segment.

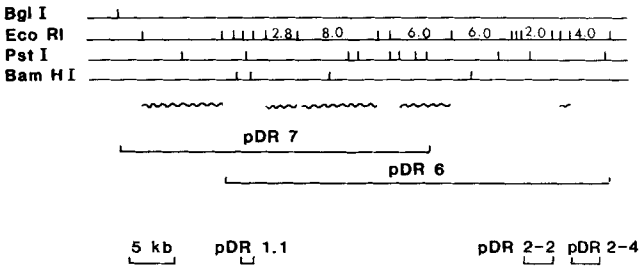

Figure 3. Restriction enzyme cleavage sites of the cosmid clones, pDR6 and pDR7, plasmid clone pDR1.1., and plasmid subclones, pDR2-2 and pDR2-4. Only the inserts in these clones are shown. The wavy lines indicate restriction fragments shown to contain repetitive sequences. The numbers represent the sizes (in kilobases) of EcoRI fragments detected using pDR6 insert DNA as a probe to Southern blot hybridization with genomic VCR DNA digested with EcoRI (Fig. 4 *a*).

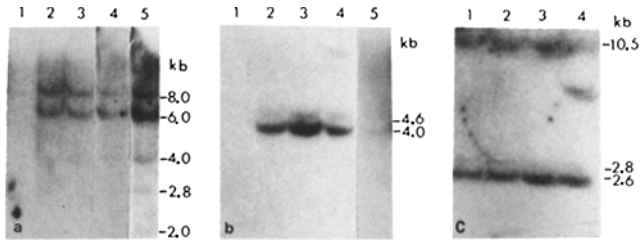

Figure 4. Southern blot hybridization analysis of genomic DNAs from wild-type CHO and VCR mutants. (*a*) Hybridization pattern when the cosmid pDR6 was used as a probe to hybridize with 10 μg of EcoRI-digested CHO-, VCR 1.5-, VCR 5-, and VCR 15-blotted DNAs (lanes *1–4*, respectively). (Lanes *1–4*) 24-h exposure, lane *5* is a 48-h exposure of the VCR 15 lane. (*b* and *c*) Hybridization analyses of pDR2-4 and CHO β-tubulin cDNA, respectively, hybridized to duplicate blots containing 10 μg EcoRI-digested CHO, VCR 1.5, VCR 5, and VCR 15 DNAs (lanes *1–4*, respectively). Exposure times were 3 h (*b*) and 24 h (*c*). Lane *b5* shows extended exposure (24 h) of the CHO lane (lane *b1*).

pDR7. pDR6 and pDR7 DNAs were digested with EcoRI, the restricted fragments were separated by gel electrophoresis, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled total CHO DNA. This hybridization favors the annealing of repeated DNA sequences. Therefore, the results of this experiment allowed us to localize several restriction fragments that contain repetitive DNA sequences as Fig. 3 shows (wavy lines). The repeat-free EcoRI fragments in pDR6 were subcloned into pBR322, designated pDR2-2 and pDR2-4. Fig. 3 shows the locations for the inserts of these two subclones.

We used the insert of pDR2-4 as probe to hybridize in a Southern blot with 10 μg of CHO, VCR 1.5, VCR 5, and VCR 15 genomic DNAs digested by EcoRI. Fig. 4 *b* shows the results of this hybridization. In this case, duplicate filters were made; one was used to hybridize with the pDR2-4 (Fig. 4 *b*) probe and the other to the CHO β-tubulin cDNA (Fig. 4 *c*). The CHO β-tubulin cDNA, a gift of F. Cabrel (University of Texas Medical School at Houston) was obtained by screening CHO cDNA library with human β-tubulin cDNA. Details for the cDNA isolation will be published elsewhere. CHO β-tubulin cDNA probe was used in the hybridization experiments to estimate the levels of amplified pDR2-4 DNA sequences in these cells in reference to the β-tubulin gene, which is not amplified in these cells (15).

Fig. 4 *c* shows a Southern blot hybridization of CHO, VCR 1.5, VCR 5, and VCR 15 with β-tubulin cDNA. The hybridization signals for these DNA samples were similar. We used densitometric methods to quantitatively analyze the hybridization signal corresponding to the 2.6-kb fragment (Fig. 4 *c*), and the results showed that the variations fall within ±4% of those of the CHO DNA. This indicated that similar amounts of DNA were loaded onto each lane. When the duplicate filter was used for hybridization with probe pDR2-4, the hybridization signals in the VCR DNA samples, but not the CHO sample, can be readily detected. After a prolonged exposure time, however, we can see the hybridization signal in the CHO sample, indicating amplification of pDR2-4 DNA sequences in the VCR cell lines. We used densitometric methods to analyze the levels of DNA amplification in these MDR cells. About 40-, 45-, and 40-fold amplifications exist in the VCR 1.5, VCR 5, and VCR 15 cell, respectively (Table I). We note that the increase of DNA amplification between VCR 1.5 and VCR 15 cells is very small, if any. However, the level of resistance to vincristine increase 12.5-fold between these two cell lines.

We note that two hybridization bands corresponding to 4.6- and 4-kb EcoRI fragments were detected with the pDR2-4 probe. This suggests that two DNA sequences homologous to the insert of pDR2-4 were coamplified in these VCR mutants. It is obvious however that the sequence containing the 4-kb EcoRI fragment is amplified several folds more than the one with the 4.6-kb fragment.

In an attempt to analyze whether any messenger RNA was transcribed from the amplified DNA sequences, we used pDR2-4 as a probe in Northern blot hybridization analysis of 5 μg blotted poly $(A)^+$ RNAs from wild-type and VCR 1.5 cells. A hybridization signal corresponding to a 4.3-kb mRNA can be clearly observed in the samples derived from VCR mutants, but only barely detectable in the sensitive CHO sample (Fig. 5, lane *1*). Prolonged exposure of the autoradiographic film clearly showed that the 4.3-kb mRNA were also expressed in the sensitive CHO cells (Fig. 5, lane *9*). The hybridized probe was denatured by boiling it in distilled H_2O for 5 min. The poly $(A)^+$ RNA on the same filter was rehybridized with ^{32}P-labeled CHO β-tubulin cDNA probe. Similar intensities of hybridization signals corresponding to 1.8-kb and 2.6-kb β-tubulin mRNA were detected (Fig. 5 lanes *3* and *4*), suggesting that similar amounts of poly $(A)^+$ RNA was loaded in this experiment. Again, densitometric methods in combination with use of autoradiographic films that were exposed for different lengths of time (for CHO sample) were used to estimate the level of mRNA production in VCR 1.5 cells. The abundance of the 4.3-kb mRNA is ~40 times higher in VCR 1.5 cells than that in the CHO cells, a level roughly consistent with that of DNA amplification in this cell line.

Fig. 5 also shows the results of a Northern blot of total cellular RNA isolated from CHO, VCR 1.5, VCR 5, and VCR 15 (lanes *5–8*) hybridized with pDR2-4 plus β-tubulin cDNA probes. We used total RNA instead of poly $(A)^+$ RNA in this experiment to avoid the problem of differing levels of rRNA contamination in respective samples, which would make a quantitative estimate of the levels of overproduction of mRNA more difficult. We have found that rRNA contents in different preparations of poly $(A)^+$ RNA samples vary

Table I. Dosage Response Analysis and Levels of DNA Amplification and mRNA Expression in CHO and VCR Mutant Cells

Cell line	*ED_{50}				Level of DNA amplification	Level of 4.3-kb mRNA production
	Vincristine	Colcemid	Ethidium bromide	Puromycin		
CHO	0.05 (1)§	0.15 (1)	2.0 (1)	10 (1)	1	1
VCR 1.5	2.0 (40)	2.0 (20)	30.0 (15)	400 (40)	40	40
VCR 5.0	10.0 (200)	8.0 (80)	200.0 (100)	1000 (100)	45	40
VCR 15.0	25.0 (500)	ND	ND	1500 (150)	40	40

ND, not determined.
* Dose of drug that kills 50% of cells. (ED_{50}, μg/ml).
§ Numbers indicated in parenthesis correspond to level of resistance, when wild-type CHO is normalized to 1. The levels of DNA amplification and overexpression of mRNA in the mutant cells are also expressed in relative values considering the basal level in the parental CHO cells as 1. These values were estimated from the densitometric scans of the corresponding autoradiographs.

(not shown). The hybridization signals corresponding to 2.6-kb and 1.8-kb given by the β-tubulin cDNA probe, was not significantly different in VCR RNA samples, thus indicating that similar amounts of mRNA were loaded. The hybridization signals corresponding to 4.3 kb, given by the pDR2-4 probe, were not significantly different among the VCR 1.5, VCR 5, and VCR 15 samples. Our densitometric estimates showed that the levels of 4.3 kb in these VCR RNA samples varied by at most ±2%. These results suggest that the levels of steady-state mRNA production are not different, and certainly not correlated to the levels of drug resistance in these stepwise selected MDR mutants.

pDR2-2 was similarly used as a probe for Northern hybridization. The results demonstrate that this flanking restriction fragment does not hybridize with the 4.3-kb mRNA (data not shown).

Localization of the Amplified DNA Sequences in the VCR Mutants

We performed in situ hybridization to localize the amplified DNA sequences in the VCR lines to determine whether the amplified DNA sequences are localized in the additional chromosomal region on the Z7 marker chromosome. Fig. 6, *a* and *b* shows the results of using an entire pDR6 as a probe in hybridization of VCR 1.5 and VCR 15 chromosomes, respectively. In each case, >25 metaphase plates were analyzed, the silver grains were consistently clustered on the long arm of one of the two longest chromosomes. Even without using banding analysis, this chromosome can be readily identified as either chromosome 1 or Z1. The chromosome that contains the amplified DNA sequences is markedly different from the Z7 marker chromosome.

Similar results were obtained when repeat-free DNA was used as a probe in in situ hybridization experiments. Fig. 6, *c* and *d* show the results of using insert pDR2-4 as a probe in hybridizations to VCR 1.5 and VCR 15 cells, respectively. An analysis of >50 metaphase plates in each cell line shows that the distribution of silver grains is concentrated on the region proximal to the telomere of chromosome lq or Zlq (Fig. 6, *c*, *d*, and inserts).

Discussion

Gene amplification in drug-resistant animal cells selected by stepwise increases of drug concentrations has been reported in a number of systems (for review, see references 25, 29). We report here that amplification of a specific gene can also take place in VCR CHO cells that exhibit multidrug cross resistance.

The characteristics of gene amplification manifested in our VCR cell lines are as follows:

(*a*) Neither the level of gene amplification nor the level of the 4.3-kb transcript in our VCR mutants strictly correlate with the level of drug resistance. Our VCR 1.5 cells (40-fold drug resistance) contained ~40-fold amplification of the gene. Further selection to ~500-fold resistance (VCR 15) did not result in additional amplification of the gene or increase in the accumulation of the 4.3-kb RNA transcript. Our failure to detect increased amplification of the DNA sequences in either VCR 5 or VCR 15 cells suggests that either additional genetic loci may be involved or a posttranscriptional regulation of the 4.3-kb mRNA is responsible for increased resistance of vincristine.

We have additional evidence showing that this observation is also applicable in another independently established MDR CHO cell line. VBR 2, an MDR CHO line established by selection in vinblastine, is 10-fold less resistant to vincristine than VCR 15 cells; yet the level of DNA amplification in VBR 2.0 cells is about the same as in VCR 15 cells (Sen, S., L. D. Teeter, and T. Kuo, manuscript in preparation).

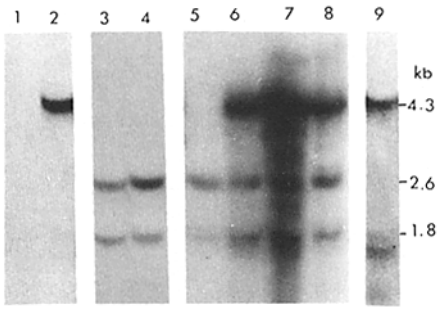

Figure 5. Northern blot hybridization analysis of RNAs from the wild-type CHO and VCR mutants to pDR2-4 and/or CHO β-tubulin cDNA. 5 μg poly $(A)^+$ lanes (*1–4*) or 10 μg total RNA (lanes *5–8*) were separated by glyoxal gel electrophoresis and blotted onto Gene Screen membrane (New England Nuclear, Boston, MA). (Lanes *1* and *2*) An autoradiogram of poly $(A)^+$ RNAs from CHO and VCR 1.5, respectively, hybridized with pDR2–4. (Lanes *3* and *4*) The same blot hybridized with β-tubulin cDNA. In lanes *5–8*, total RNAs from CHO, VCR 1.5, VCR 5, and VCR 15, respectively, were hybridized with both pDR2-4 and β-tubulin. Exposure times were 3 and 6 h for lanes *1–4* and *5–8*, respectively. (Lane *9*) A 65-h exposure time of the CHO sample in lane *1*, a low basal level of the 4.3-kb transcript is evident.

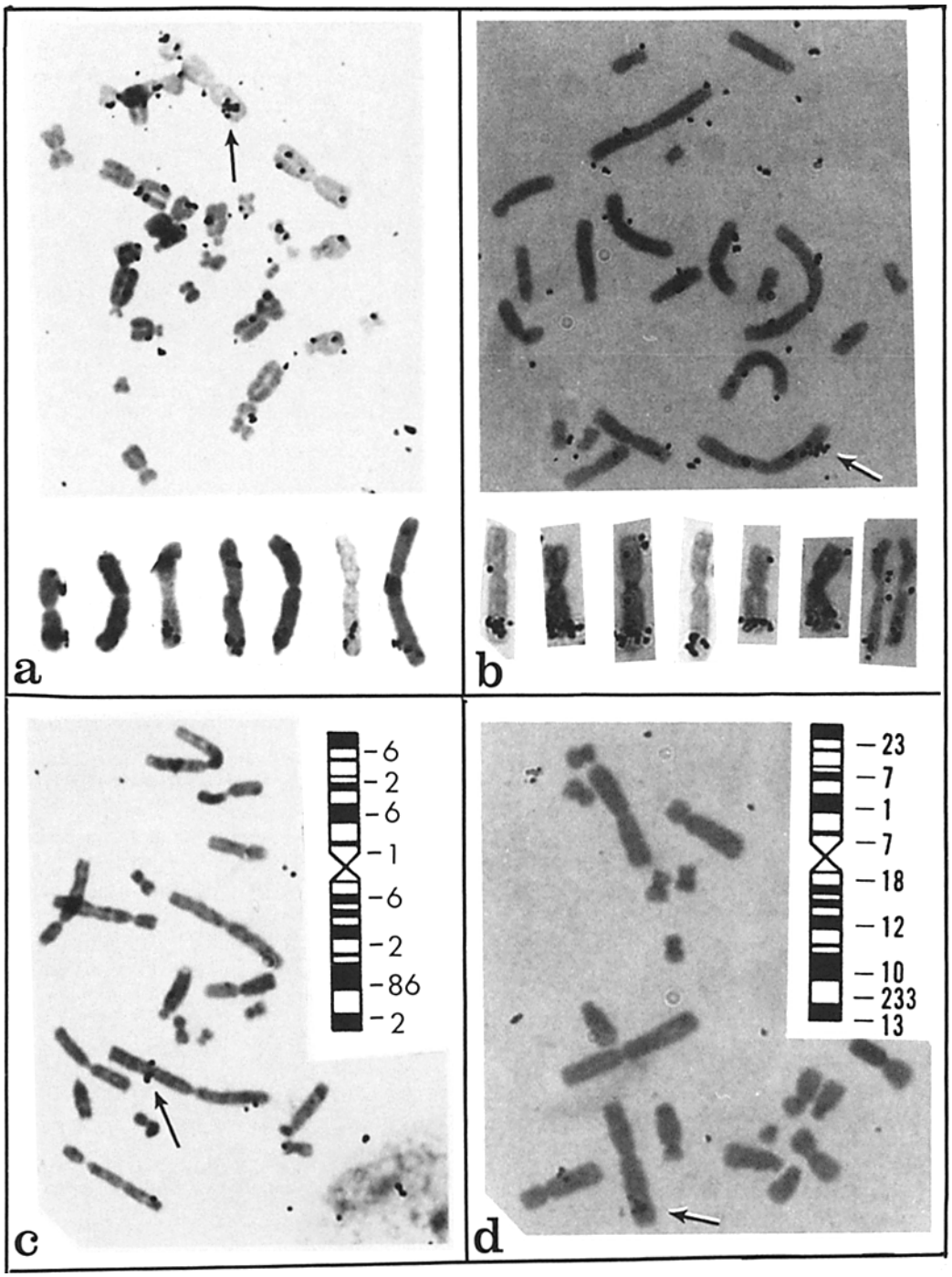

Figure 6. In situ hybridization localization of the amplified DNA sequences in the VCR 1.5 cells (*a* and *c*) and VCR 15 cells (*b* and *d*) using the probes pDR6 (*a* and *b*), containing a 40-kb-long sequence, and pDR2-4 (*c* and *d*) a repeat-free sequence of ~4 kb derived from pDR6. (*a* and *b*) Metaphase spreads. Note that only one of the two longest chromosomes contain the silver grains on its long arm. The lower part in the same panel shows cut-out chromosomes that contain clusters of silver grains. (*c* and *d*) Metaphase spreads, each with the grain clusters localized on the long arm of one number 1 chromosomes. (*Inset*) A composite distribution of labeled sites on number 1 chromosomes for >50 cells analyzed. *a* and *b* exposed 6 d; *c* and *d*, 12 d.

(*b*) The amplified genes in the drug-resistant mutants that have been published in the literature are usually located in abnormal chromosomal structures, either DM or homogenous staining region. The amplified gene in our VCR mutant is not located at the abnormal additional chromosomal regions on Z7. An explanation why the amplified DNA in chromosome 1 (or Z1) in our VCR cells show no homogenous staining region–like structure that was described in well known methotrexate-resistant CHO cells (4), may be because the level of amplification is too low and/or the amplification DNA domain is too small. Fougere-Deschatrette et al. (9) have shown that in methotrexate-resistant rat hepatoma cell lines with 40–50-fold amplification of DNA segments hybridizing to dihydrofolate reductase, substantial

variation in karyological manifestations were observed. Interestingly, in one clone, no chromosomal modification was observed.

The fact that only ~50% of VCR 1.5 cells contain DM in a clonally selected MDR mutant suggests that the DNA sequences in DM may not be solely responsible for MDR. Our in situ hybridization data showed that the amplified DNA sequences are located on the long aim of chromosome Z1 (or 1) in this cell line. We do not know whether the same amplified DNA sequences are also present in DM. Owing to the difficulty that DM were unidentifiable under autoradiographic film after metaphase plates were treated with HCl/NaOH or heat for hybridization (not shown), in situ hybridization failed to provide unambiguous evidence that the silver grains were not distributed on the DM. Fractionation of DM from VCR 1.5 cells chromosomes to implicate their involvement in amplification is technically difficult, due to the problem of chromosome fragmentations that may be copurified with DM.

Despite a number of studies, the mechanisms of MDR in animal cells have not been elucidated. In this report, we show that the DNA sequences amplified in the cell lines used by Roninson et al. (24) are also amplified in our VCR cell lines. We also have preliminary evidence that the same DNA sequences are amplified in other MDR CHO mutants independently selected for resistance to vinblastine and adriamycin (Sen, S., L. D. Teeter, and T. Kuo, manuscript in preparation). Amplification of similar genes has recently been detected in other independently established cell lines (26, 28, 33). These results, together with those reported by Roninson et al. (24), indicate that the same DNA sequences are commonly amplified in several MDR CHO cells. Additionally, several lines of evidence suggest that this gene may be related to the P-glycoprotein gene reported by Ling and his associates (23). (*a*) The P-glycoprotein gene is also amplified in a number of cell lines exhibiting the MDR phenotype (23, 33), including the VCR 5 cell line described here (23). (*b*) The P-glycoprotein gene also encodes a transcript of about the same size. (*c*) The P-glycoprotein gene has been in situ localized to Chinese hamster chromosome 1q 3–8 (30). (*d*) The restriction fragments that are hybridized with the P-glycoprotein gene probe are very similar to those in Fig. 4 *a*. (*e*) Both Ling's probe and the pDR2-4 probe described here are highly conserved, i.e., both are cross-hybridizable to human DNA under relative stringent hybridization conditions (23, and our unpublished observations).

Whether the P-glycoprotein gene is responsible for MDR in animal cells requires further investigation by DNA-mediated gene transfer of a full length cDNA inserted into an expressible vector. However, we have another type of functional evidence linking chromosomes 1 and Z1 in our VCR cells with the maintenance of the PDR phenotype. In somatic cell hybrids prepared from a fusion of VCR CHO and sensitive mouse cells, the loss of CHO chromosomes 1 and Z1 is concomittant with loss of resistance in segregants (Teeter, L. D., J. A. Sanford, S. Sen, R. L. Stallings, M. J. Siciliano, and M. T. Kuo, manuscript submitted for publication).

An important aspect of drug resistance in cancer chemotherapy is knowing how often MDR occurs in clinical samples. Ling and his associates (3) have detected overproduction of the P-glycoprotein in some human solid tumor biopsies using a monoclonal antibody. With the available cloned amplified DNA described here, it will be of significance to test whether gene amplification and overexpression of amplified gene have occurred in tumor cells, especially those derived from patients for whom chemotherapy has failed. We are currently using the insert of pDR2-4 as a probe to isolate the homologous human gene for this purpose.

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