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Double Minute Chromosomes Carrying the Human Multidrug Resistance 1 and 2 Genes are Generated From the Dimerization of Submicroscopic Circular DNAs in Colchicine-Selected KB Carcinoma Cells

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This study characterizes amplified structures carrying the human multidrug resistance (*MDR*) genes in colchicine-selected multidrug resistant KB cell lines and strongly supports a model of gene amplification in which small circular extrachromosomal DNA elements generated from contiguous chromosomal DNA regions multimerize to form cytologically detectable double minute chromosomes (DMs). The human *MDR*1 gene encodes the 170-kDa P-glycoprotein, which is a plasma membrane pump for many structurally unrelated chemotherapeutic drugs. *MDR*1 and its homolog, *MDR*2, undergo amplification when KB cells are subjected to stepwise selection in increasing concentrations of colchicine. The structure of the amplification unit at each step of drug selection was characterized using both high-voltage gel electrophoresis and pulsed-field gel electrophoresis (PFGE) techniques. An 890-kb submicroscopic extrachromosomal circular DNA element carrying the *MDR*1 and *MDR*2 genes was detected in cell line KB-Ch^R-8-5-11, the earliest step in drug selection in which conventional Southern/hybridization analyses detected *MDR* gene amplification. When KB-Ch^R-8-5-11 was subjected to stepwise increases in colchicine, this circular DNA element dimerized as detected by PFGE with and without digestion with *Not* 1, which linearizes the 890-kb amplicon. This dimerization process, which also occurred at the next step of colchicine selection, resulted in the formation of cytologically detectable DMs revealed by analysis of Giemsa-stained metaphase spreads.

INTRODUCTION

Gene amplification increases the copy number of a gene and usually results in a higher level of its expression. Under certain growth conditions, the encoded gene product can provide cells with a selective growth advantage. To characterize the molecular events of gene amplification, many cultured cell lines selected for drug resistance, in which drug resistance genes are amplified, have been studied extensively (Hamlin *et al.*, 1984; Stark and Wahl, 1984; Stark, *et al.*, 1989; Schimke, 1988). Amplified DNA can vary in size from ~50 to 3000 kb (Hamlin *et al.*, 1984; Schimke, 1988; Stark and Wahl, 1984) and is usually located in either homogeneously staining regions (HSR) or in extrachromosomal elements such as double minute chromosomes (DMs) (Cowell, 1982; Biedler *et al.*, 1983; Hamlin *et al.*, 1984; Stark and Wahl, 1984). HSRs are amplified sequences that reside within a chromosome and can be detected cytologically because they fail to exhibit trypsin-Giemsa bands or show abnormal banding regions. HSRs that do not reside at the native locus of the amplified gene have been referred to as expanded chromosomal regions (ECRs) (reviewed in Schimke, 1988; Stark *et al.*, 1989).

In addition to the extrachromosomal DM structures, submicroscopic extrachromosomal circular DNAs harboring amplified genes have also been identified. These circular DNAs are too small to be detected by cytogenetic techniques (<1000 kb). Pulsed-field gradient or field-inversion gel electrophoresis combined with electron microscopy studies were first used to identify these

structures in HeLa cells that contained amplified copies of the dihydrofolate reductase gene (Maurer *et al.*, 1987). Since this initial study, submicroscopic circular DNAs have been detected in other mammalian cell lines containing amplified genes (Carroll *et al.*, 1987; Von Hoff *et al.*, 1988; Ruiz *et al.*, 1989). These circular DNAs have been termed episomes by Carroll *et al.* (1987) or amplisomes by Pauletti *et al.* (1990).

A variety of mechanisms appears to mediate the amplification of specific genes, even within a population of cells (reviewed by Schimke, 1988; Stark *et al.*, 1989; Smith *et al.*, 1990). The initial step(s) of gene amplification have been difficult to define. Several studies have used in situ hybridization/fluorescent techniques to identify very early amplification structures. These studies indicate that gene amplification can be mediated by either intra- or interchromosomal recombination events, such as sister chromatid exchange (Trask and Hamlin, 1989; Smith *et al.*, 1990), or by extrachromosomal circular DNA intermediates (Wahl, 1989; Ruiz and Wahl, 1990; Windle *et al.*, 1991).

Amplification mediated by circular DNA intermediates is of particular interest because a recent compilation of published cytogenetic studies has revealed that the extrachromosomal DM structures are the most common carrier of amplified genes in a large variety of tumors (Benner *et al.*, 1991; reviewed by Wahl, 1989). In addition, in vitro studies imply that many of the structural chromosomal abnormalities occurring in tumors and tumorigenic cell lines (Bishop, 1987; Tlsty *et al.*, 1989) may result from molecular mechanisms common to those mediating extrachromosomal gene amplification. For example, recent studies have provided firm evidence that some extrachromosomal elements have the potential to integrate into chromosomes, resulting in either HSRs, ECRs (Carroll *et al.*, 1988; Ruiz and Wahl, 1990; Von Hoff *et al.*, 1990; Windle *et al.*, 1991), or other chromosome abnormalities such as ring chromosomes (Windle *et al.*, 1991). These studies support earlier observations of gene amplification in hamster cell lines in which Biedler (1982) observed that the extrachromosomal circular DMs rapidly integrated into chromosomes, resulting in HSR structures (Biedler, 1982). Therefore, an understanding of DM formation and their extrachromosomal or intrachromosomal maintenance should provide insight into some of the biological processes involved in tumorigenicity and may provide opportunities to interfere with the progression of tumor cells, and some drug-resistant cells, in a variety of cancers.

DMs and the submicroscopic extrachromosomal circular elements replicate autonomously, approximately once per cell cycle (Barker *et al.*, 1980; Ruiz *et al.*, 1989), and segregate to daughter cells in a random fashion because of the absence of a centromere. Several studies of cultured cell lines have provided a molecular chronology of events in which submicroscopic circular DNAs precede the appearance of cytogenetically detectable DM structures (Carroll *et al.*, 1987, 1988; Von Hoff *et al.*, 1990). In addition, pulsed-field gel electrophoresis (PFGE) data from neuroblastoma biopsies imply that the multimerization of *MYCN* circles in vivo can generate DM structures (VanDevanter *et al.*, 1990). However, DM structures spanning several megabases of chromosomal DNA also appear to be formed directly from prematurely condensed chromosomes of replicating micronuclei (Sen *et al.*, 1989).

To further understand the amplification mechanism(s) that generate extrachromosomal DM structures in human cell lines, we analyzed the amplification events leading to the increased copy of the multidrug resistance (*MDR*1) gene in a series of colchicine-selected human KB carcinoma cell lines. Increased expression of the *MDR*1 gene results in increased production of the 170 000-Da P-glycoprotein that is localized in the membrane and acts as an efflux pump for a large variety of structurally unrelated neoplastic drugs, including the anthracyclines (doxorubicin, daunorubicin), vinca alkaloids (vinblastine, vincristine), epipodophyllotoxins (etoposide, teniposide), actinomycin D, colchicine, and taxol (reviewed in Gottesman and Pastan, 1988; Endicott and Ling, 1989). A human cDNA that was amplified in KB-C2.5, a high-level colchicine resistant line, was cloned from this cell line (Ueda *et al.*, 1987a,b). Sequence analysis of this clone revealed an open reading frame for a protein of 1280 amino acids that is organized in two homologous halves. Each half contains six transmembrane domains and an ATP-binding region (Chen *et al.*, 1986), which presumably provides energy for the transport process that expels the wide variety of drugs from the cell membrane (Cornwell *et al.*, 1987; Horio *et al.*, 1988). KB-C2.5 and the cell lines corresponding to each of the other steps of colchicine selection were stored in liquid nitrogen at the time of their derivation, providing us with a model system in which to study early and late events in gene amplification.

MATERIALS AND METHODS

Cell Lines and Cell Culture

In previous studies, the parent cell line, KB-3-1, was subcloned from a human KB epidermoid carcinoma cell line and the selections of the colchicine-resistant sublines were described (Akiyama *et al.*, 1985; Shen *et al.*, 1986; Figure 1, Selection 1). At the time of their isolation, the colchicine-resistant cell lines were frozen in tissue culture medium containing 7% dimethylsulfoxide and stored at −80°C. For our analysis, the cells were quick-thawed and passaged only one to two times before harvesting to isolate high-molecular-weight DNA (see below). In addition, some of the cell lines were continuously passaged at the appropriate colchicine concentration an additional 6 mo to 2 y and analyzed again to determine whether continued passage of these cell lines resulted in a change in the profile of the amplification structures (amplicons) harboring the *MDR*1 gene.

Eight additional KB cell lines were derived from single clones of KB-Ch^R-8-5-11 isolated at 100 ng/ml of colchicine with the use of steel cloning cylinders; these subclones were expanded to a population of 10^5 cells, which were passaged in increasing amounts of drug over

a period of 6 mo (Figure 1, Selection 2). These sublines consisted of slow-growing, loosely arranged cells when passaged at 1.0 μg/ml colchicine. These cell lines grew poorly at this drug concentration and were maintained at 0.75 μg/ml colchicine.

The cell line FEMX VMDRCl4A was constructed via transfection of the human melonoma cell line (FEMX) with the *MDR*1-containing retroviral vector, pHaMDR1/A (Pastan *et al.*, 1988), followed by stepwise colchicine selection to 0.45 μg/ml.

Cells were grown as monolayer cultures in a humidified incubator, 5% CO_2 at 37°C using Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, MD) supplemented with L-glutamine (GIBCO, Grand Island, NY), penicillin (50 units/ml (GIBCO), and streptomycin (50 μg/ml) (GIBCO). All cell lines were grown in 10% fetal calf serum (GIBCO), except the newly derived MDR cell populations KB-PC1-17, KB-PC1-3, KB-PC1-16, KB-PC1-1, KB-PC-1-9, KB-PC1-15, KB-PC1-18, and KB-PC1-2 (Figure 1, Selection 2), which required 15% fetal calf serum.

Cell Survival by Colony Formation

The dose-response curves of KB-3-1 and its colchicine-resistant sublines were determined as in previous studies (Akiyama *et al.*, 1985; Shen *et al.*, 1986a,b). Briefly, ~300 cells were plated in 60-mm dishes (Falcon, Lincoln Park, NJ) in the absence of drugs. The appropriate concentration of drugs was added 16 h subsequent to the seeding, and plates were incubated for 12–14 d. The colonies were stained (0.5% methylene blue in 50% ethanol) and counted. The D_{10} and D_{50} values are the concentrations of drug that reduced the cloning efficiency of the sublines to 10 and 50%, respectively, as compared with control platings without drugs. Relative resistance was determined by dividing the D_{10} value of the resistant sublines by the D_{10} value of the parental cells. Colchicine, adriamycin, and vinblastine were purchased from Sigma (St. Louis, MO) and prepared in dimethyl sulfoxide (Aldrich, Milwaukee, WI) at a stock concentration of 10 mg/ml and stored at −20°C.

Cytogenetic Analysis

As previously described (Fojo *et al.*, 1985), cells were grown for 24–36 h, at which time metaphase arrest was induced by the addition of colchicine. Concentrations ranging from 0.2 to 10 μg/ml were used depending on the colchicine resistance level of the cell line. Incubation was continued for 1–2 h. Cells were harvested, and chromosome spreads were prepared and analyzed by Giemsa stain.

High-Molecular-Weight DNA Preparation and Linearization of Circular Extrachromosomal DNAs

High-molecular-weight DNA was prepared as described by Smith *et al.* (1987). Using an agarose plug mold (Bio-Rad, Richmond, CA), $\sim 1.5 \times 10^7$ cells/ml in phosphate-buffered saline were embedded in molten agarose (InCert agarose, FMC, Rockland, ME). After 15 min at 4°C, the resulting agarose plugs, three to five plugs per cell line, were lysed and deproteinized in situ by incubation in 3.0 ml ESP lysis buffer (500 mM EDTA, pH 9.0, 1% sodium laruroylsarcosine, 2 mg/ml proteinase K [Boeringer Manheim, Indianapolis, IN]), for 48 h at 50°C. Plugs were stored in the ESP buffer for up to 1 y without loss of integrity of the high-molecular-weight DNA.

To linearize extrachromosomal circular DNA molecules, agarose plugs were placed in 2.0 ml of 0.5 M EDTA, pH 9.0, in 12-well culture dishes and exposed to 3000–15 000 cGy of irradiation from a 20-MeV linear accelerator (Varian NCI Radiation Oncology Branch). As seen in other studies on gamma irradiation (Goss and Harris, 1975; Jonasson and Harris, 1977; Van der Bliek *et al.*, 1988; Ruiz *et al.*, 1989), this range of irradiation introduces single-strand and double-strand breaks in high-molecular-weight DNA in a dose-dependent manner and, on the average, will linearize a significant number of circular DNA molecules by introducing a solitary double-strand break.

Alternatively, circular DNAs embedded in the agarose plugs were linearized by digestion with rare cutting restriction enzymes, *Not* 1 and *Sfi* 1 (Boehringer Manheim). To obtain complete restriction digests, the agarose plugs were washed thoroughly before restriction in the following manner. In polypropylene screw cap tubes, the ESP buffer was replaced with 10.0 ml of TE (10mM Tris-HCl, 1 mM EDTA pH 7.5) buffer, rotated for 15 min at room temperature (RT) and poured off. TE buffer 10.0 ml was added, and the tubes were gently shaken at 50°C for 30 min. The TE buffer was discarded and 10.0 ml of TE buffer, containing 150 μl of freshly prepared phenylmethylsulfonyl fluoride (17.5 mg to 1.0 ml EtOH) was added, and the tubes were rotated at RT for 4–6 h. This step was repeated, followed by two washes in 10.0 ml TE buffer. The plugs were stored in 2.0 ml of TE buffer. The restriction digestions were performed in 1.5-ml microcentrifuge tubes by incubating one-half of an agarose plug in 500 μl of restriction buffer with 10 units of enzyme overnight at the appropriate temperature. Before loading the digested plugs on the gel, the assay buffer was aspirated off and replaced with 1.0 ml of ES solution (ESP minus proteinase K). The inserts were incubated at 50°C for 2 h, the lysis solution was replaced by ESP, and the plugs were incubated an additional 2 h before loading.

Unidirectional High-Voltage Gel Electrophoresis and PFGE of High-Molecular-Weight DNA

To detect circular DNA molecules, DNA (~10.0 μg) corresponding to 1×10^6 cells (~1/4–1/5 of an agarose plug prepared using the Bio-Rad plug mold) were loaded directly onto 1% agarose gels (1× tris(hydroxymethyl)aminomethane-Borate buffer) and fractionated using high-voltage electrophoresis at 5.4 V/cm for 12–24 h at 4°C (Eckhardt, 1978; Carroll *et al.*, 1987; Ruiz *et al.*, 1989).

Alternatively, to detect circular DNA molecules that had been linearized in vitro either with irradiation or enzyme digestion (see above), ~10 μg of DNA were fractionated on 0.6–1% agarose gels (Seakem GTG agarose, FMC) with PFGE using the CHEF DR II system (Bio-Rad), which fractionates DNA under a contour-clamped homogeneous electric field (Cantor *et al.*, 1988). All gels for PFGE analysis were prepared and electrophoresed in 0.5× TBE (45 mM Tris base, 45 mM boric acid 1 mM EDTA, pH 8.3) buffer. A variety of voltage and ramping conditions (details provided in the figure legends) was employed to achieve optimal separation of large linear DNA fragments. For PFGE, molecular weight markers were the lambda ladder and the intact *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomes embedded in agarose plugs (purchased from FMC or Bio-Rad).

Transfer and Hybridization of DNA

The agarose gels containing the fractionated DNAs were stained for 30 min with 0.5 μg/ml ethidium bromide and nicked using a 254-nm UV transilluminator for an amount of time (20 s to 3 min) that resulted in ~90% transfer of DNA. After UV nicking, the gel was photographed using a 360-nm UV transilluminator, and the DNA was transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) or to zeta probe membrane (Bio-Rad) using capillary transfer in 20× SSC or in alkaline solution, respectively. After transfer, the blots were hybridized sequentially to several DNA probes labeled with ^{32}P by nick translation or random priming to a specific activity of $>2 \times 10^8$ dpm/μg of DNA. Membranes were stripped of hybridized probe before each subsequent hybridization. The following probes were used in these hybridizations: 1) probe pMDR5A, which corresponds to the middle of the *MDR*1 cDNA (Ueda *et al.*, 1987a,b); 2) probe pMDR2: PVUII, which corresponds to the 267 base pair *PVU* II fragment isolated from the *MDR*2 cDNA; 3) a probe specific for the repetitive alpha-satellite DNA in chromosome 7 (Oncor, Gaithersburg, MD) used in hybridization experiments to detect unresolved DNA sequences of varying sizes that migrate into compressed zones on PFGE gels; and 4) *AluK* and *Blur8* DNA (Kariya *et al.*, 1987; Deininger *et al.*, 1981,

respectively), repetitive probes used as controls for possible compressed zones on gels subjected to high-voltage gel electrophoresis.

RESULTS

Selection and Cytogenetic Analysis of the Colchicine-Resistant Sublines

The colchicine-resistant sublines KB-Ch^R-8, KB-Ch^R-8-5, KB-Ch^R-8-5-11, KB-C1, KB-C1.5, KB-C2.5, KB-C3.5, KB-C4, and KB-C6 were derived from a subclone of the HeLa carcinoma cell line KB-3-1 (Akiyama *et al.*, 1985; Shen *et al.*, 1986) (Figure 1, Selection 1). Frozen stocks (−80°C) of each of these cell lines were prepared at the time of their isolation. For this study, each of these cell lines were thawed, passaged in the appropriate concentration of colchicine, and harvested for subsequent analyses. Using conventional Southern/hybridization analysis, the copy number of the *MDR*1 and *MDR*2 genes in several of these sublines was determined and was in agreement with results from previous studies (Table 1). The *MDR*2 gene, a homolog of *MDR*1 that does not appear to play a role in MDR (Schinkel *et al.*, 1991), is immediately adjacent to *MDR*1 on chromosome 7. Amplification of the *MDR*1 and *MDR*2 genes was first detected at the third step of colchicine selection in cell line KB-Ch^R-8-5-11 but not in the cell lines KB-Ch^R-8 and KB-Ch^R-8-5, which were derived in the two preceding steps of the colchicine selection that shows increased expression of the *MDR*1 gene without increased gene copy (Shen *et al.*, 1986b).

In previous cytogenetic analyses, Fojo *et al.* (1985) showed the presence of DMs in the highly colchicine-resistant cell lines, e.g., KB-C2.5 (2.5 μg/ml colchicine). We repeated the cytogenetic analysis and confirmed the presence of DMs in the cell lines derived at the later steps of colchicine selection, e.g., KB-C1.5, KB-C2.5, KB-C4, and KB-C6 (Table 1). Because several recent studies have demonstrated that in cultured cell lines DMs are lost because of their apparent preference to integrate into chromosomes after continuous passage in culture (reviewed in Wahl, 1989), cytogenetic analysis was repeated on KB-C2.5 and KB-C4 after several months of continuous passaging in culture, and DMs were still detected in these cell line. Thus, in the presence of the drug, the DMs are stably maintained in the cell lines resistant to ≥1.5 μg/ml colchicine. In contrast, DMs were not detected in KB-C1 or KB-Ch^R-8-5-11, even though these cell lines harbor a five to ninefold increase in the number of *MDR*1 gene copies compared with the parent cell line KB-3-1 (Table 1). After 1 y of continuous passage, cytogenetic analysis of KB-C1.0 or KB-Ch^R-8-5-11 still did not detect DM structures. As expected, DMs were not present in cell lines that did not contain amplified copies of the *MDR*1 gene, e.g., KB-Ch^R-8-5, KB-Ch^{R}8, KB-3-1, or in the colchicine-revertant cell line KB-C-1-R1.

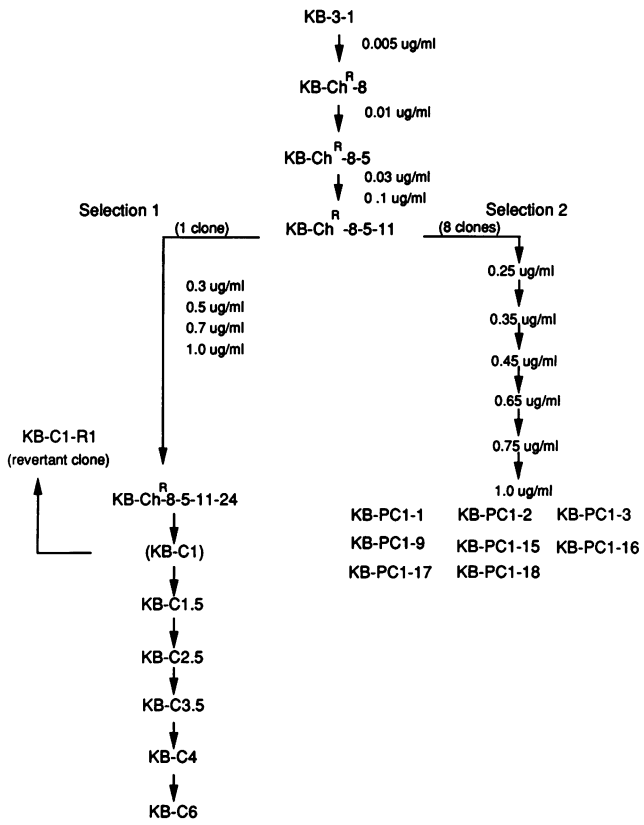

Figure 1. Derivation of the multidrug resistant KB cell lines used in this study. *Selection 1*, the human HeLa subline, KB-3-1, was selected in increasing amounts of colchicine (Akiyama *et al.*, 1985; Shen *et al.*, 1986a). Due to the difficulty of obtaining colchicine-resistant clones at the initial steps of drug selection, mutagenesis with ethyl methanesulfonate (EMS) was employed. However, once the subline KB-Ch^R-8-5-11 was isolated at 100 ng/ml colchicine, cell lines expressing increased colchicine resistance were easily obtained without mutagenesis. Independently isolated subclones, designated Ch^R (colchicine-resistant), or resistant populations, designated C, were isolated at a number of drug concentrations (the number immediately after the letter refers to the selecting concentrations of drug in μg/ml). KB-Ch^R-8-5-11-24 represents a cell line that was stored at −80°C immediately after adaptation to 1.0 μg/ml colchicine. KB-Ch^R-8-5-11-24 cells that continued to be passaged in culture were designated KB-C1. KB-C1-R1 is a revertant cell line subcloned from a KB-C1 population passaged in medium without colchicine for several months (Shen *et al.*, 1986a). *Selection 2*, the KB-PC1-sublines are colchicine-resistant populations selected from independent clones of KB-Ch^R-8-5-11; PC1 designates population passaged in colchicine at 1.0 μg/ml.

Amplified MDR1 and MDR2 Genes in Colchicine-Resistant Sublines at an Initial Step in Gene Amplification Reside on Submicroscopic Extrachromosomal Circular DNAs

To determine if the amplified copies of the *MDR*1 and *MDR*2 genes in KB-Ch^R-8-5-11 and KB-C1 are present on circular DNA molecules too small to be detected by cytogenetic techniques (i.e., <1000 bp), a gel electrophoretic analysis was employed. From each of the colchicine-resistant cell lines, high-molecular-weight DNA

Table 1. Amplification of MDR sequences in KB sublines and relative drug resistance

Cell line	Relative resistance to			Relative copy number *MDR1*	Double-minute structures
	Colchicine	Adriamycin	Vinblastine		
Colchicine selection 1					
KB-3-1	1	1	1	1	−
KB-Ch^R-8	2.1[a]	1.1[a]	1.2[a]	1	−
KB-Ch^R-8-5	5	4.2	6	1	−
KB-Ch^R-8-5-11	55.5	36	118	5	−
KB-C1	227	110	65.7	9	−
KB-C1.5	324[b]	ND	142[b]	15	+
KB-C2.5	487[b]	141[b]	206[b]	ND	+
KB-C4	1 750[b]	254[b]	159[b]	30[c]	+
Colchicine selection 2					
KB-PC1-1	136	168	355	26	+[d]
KB-PC1-3	144	174	302	27	+[d]
KB-PC1-16	105	195	276	32	+[d]
Vinblastine selection					
KB-V1	283	212	855	45	+[d]

The *MDR1* gene copy numbers are given relative to KB-3-1 cells. In all cell lines the *MDR2* copy number was determined to be approximately equivalent to that of *MDR1*, except in KB-V1 in which the *MDR2* gene copy is similar to the unamplified level in KB-3-1. KB-V1 was selected in multiple steps to resist vinblastine at 1 μg/ml (Shen et al., 1986a).

Relative resistance is expressed as the LD_{10} of the resistant line divided by the LD_{10} of the parental KB-3-1 cells. References for data obtained from other studies: [a]Akiyama et al. (1985); [b]Shen et al. (1986a); [c]Choi et al. (1988); [d]Cytogenetic studies were not performed on these cell lines but DM structures were present as determined by PFGE analyses (refer to Figs. 4 and 6). ND, not determined.

was prepared by in situ lysis and deproteinization. Approximately 10 μg of DNA was fractionated on a 1% agarose gel in a unidirectional high-voltage gradient (5.4 V/cm) at 4°C. Under these conditions, supercoiled circular molecules can be resolved in relation to the logarithm of their molecular weight, whereas fragmented chromosomal DNA migrates in a compressed (unresolved) zone near the dye front (Eckhardt, 1978; Ruiz *et al.*, 1989). Relaxed or nicked circular DNA, as well as large chromosomal fragments (>200 kb), remain trapped at the loading well. After electrophoresis, the fractionated DNAs were transferred from the gels to nitrocellulose membrane. These blots were sequentially hybridized to the *MDR1* probe, the *MDR2* probe, and the repetitive probes *AluK* and/or *Blur8*. All hybridization signals were stripped off the blots in between hybridizations. Circular supercoiled DNAs containing the *MDR1* and *MDR2* sequences were detected in DNAs isolated from cell lines KB-Ch^R-8-5-11 and in KB-C1 but not in DNAs isolated from KB-Ch^R-8, KB-Ch^R-8-5, KB-C1-R1, or KB-3-1 (Figure 2A). The circular amplicon in KB-Ch^R-8-5-11 (0.1 μg/ml colchicine) was not consistently detected using high-voltage gel electrophoresis. Therefore, to increase the copy number of the circular amplicons, sublines were derived from KB-Ch^R-8-5-11 by passaging 10^6 cells in 0.25, 0.35, or 0.45 μg/ml colchicine for 2–4 w. In these sublines, the circular *MDR1*/*MDR2*-containing amplicons were consistently detected. During these studies, two control cell lines were used: FEMX VMDRC14A, a cell line that contains approximately five to seven copies of the *MDR1* cDNA and KB-V1, a MDR cell line derived in our laboratory through a step-wise selection in vinblastine, in which Ruiz *et al.* (1989) have previously demonstrated the presence of *MDR1*-containing 600- and −750-kb submicroscopic supercoiled circular DNAs. High-molecular-weight DNA from these control cell lines was fractionated on gels in adjacent lanes (Figure 2, A and B). Supercoiled circular molecules containing the *MDR1* gene were not detected in FEMX VMDRC14A, indicating that the amplified copies of the *MDR1* cDNA were chromosomally integrated in this cell line. As expected, supercoiled *MDR1*-containing circular amplicons were detected in KB-V1. The intensity of the *MDR1*-hybridization signal was significantly higher in the KB-V1 cell line when compared with KB-C1, reflecting the higher copy number of the *MDR1* genes in this cell line (Table 1), most of which must reside on extrachromosomal circular amplicons. The *MDR2*-specific probe did not hybridize to the circular DNAs present in KB-V1 (Figure 2B). This result is in agreement with the fact that the *MDR2* gene is not amplified in the KB-V1 cell line (Table 1, see footnote). The trace amount of signal seen from the KB-V1 DNA after *MDR2* hybridization was due to the residual *MDR1* hybridization signal that was not completely stripped off the membrane before reprobing.

Hybridizations to the *AluK* and/or *Blur8* probes were used to determine that in all cell lines the *MDR1* and *MDR2* hybridization signals below the origin of the gels resulted from the presence of supercoiled circular DNAs

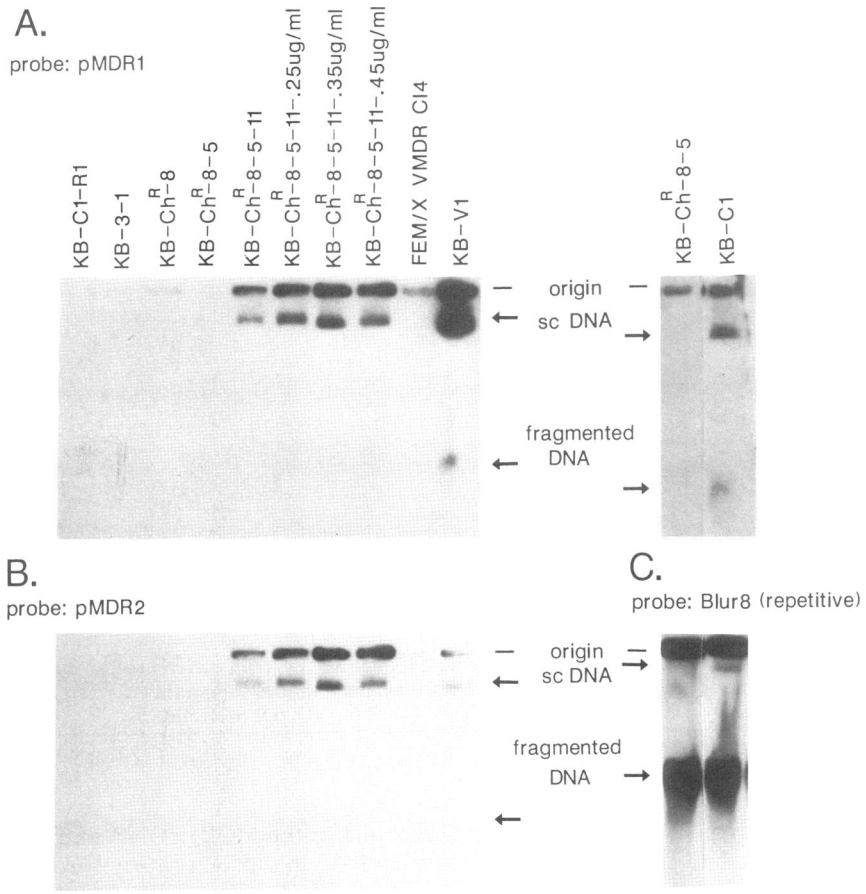

Figure 2. *MDR*1 and *MDR*2 sequences are present on supercoiled (sc) circular DNAs in colchicine-derived MDR sublines. After high-voltage gel electrophoresis, the fractionated DNA was transferred to nitrocellulose and hybridized with the following probes: (A) an *MDR*1 probe, (B) an *MDR*2-specific probe, and (C) an equal mix of the repetitive probes *AluK* and *Blur8*. Randomly fragmented DNAs (<200 kb) produced during the preparation of the high-molecular-weight DNA embedded in the agarose plugs migrated near the dye front.

and not from a possible compressed zone in which large fragmented chromosomal DNAs might be trapped. These probes, which represent highly repetitive sequences of mammalian cells, did not hybridize to this region of the gels in either the parental cell line KB-3-1, the revertant cell line KB-C1-R1, the colchicine-resistant cell lines KB-Ch^R-8 and KB-Ch^R-8-5 in which the *MDR*1 DNA is not amplified, or in FEMX VMDR C14. In contrast, these repetitive probes did hybridize to the circular DNAs in KB-Ch^R-8-5-11 and its sublines grown at 0.25, 0.35, and 0.45 μg/ml colchicine and in KB-C1 and KB-V1. One example of an *AluK/Blur8* control hybridization experiment is shown in Figure 2C.

Determination of the Size of the Circular MDR1 Extrachromosomal Molecules by Irradiation and PFGE

To determine the size of the circular DNA molecules that carry the amplified *MDR*1 and *MDR*2 genes in cell lines KB-Ch^R-8-5-11 and KB-C1, agarose plugs containing high-molecular-weight DNA from the colchicine-derived sublines were irradiated with 3000–5000 cGy. In control experiments, this range of radiation dose as compared with higher doses (e.g. 7000–15 000 cGy) linearized a significant percentage of the *MDR*1-containing circular DNA molecules in the KB-V1 cell line but produced the least degree of chromosomal breakdown as demonstrated by size-fractionation of DNA with PFGE performed at 14°C under contour-clamped homogeneous electric field conditions (Figure 3). The isolate of the KB-V1 cell line that we analyzed harbored only the 750-kb episome identified by Ruiz *et al.* (1989); the 600-kb episome was not present. Nonirradiated agarose plugs containing high-molecular-weight KB-V1 DNA did not show *MDR*1 hybridization to a specific band corresponding to a linear fragment of 750 kb (Figure 3, lane 3), unless the autoradiograms were overexposed. This small amount of the linearized circular *MDR*1 amplicon could result from mechanical shearing during the preparation and use of the agarose plugs. Alternatively, it may represent a small amount of the linearized form of this amplicon in vivo. During PFGE, intact circular DNAs remain trapped at the origin of the gel (in the loading well) or migrate into the zone of compression beneath the origin. Electrophoretic compressed zones, which are visible with EtBr-staining of the gels (Figure 3A), characteristically occur during PFGE due to the comigration of a large range of sizes of DNA fragments.

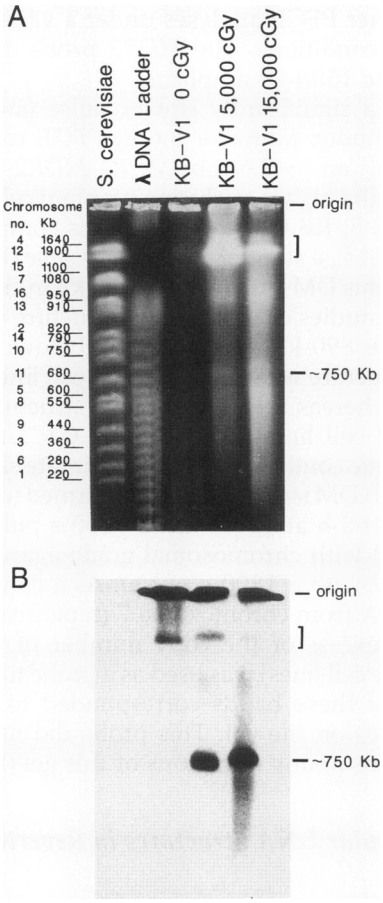

Figure 3. Irradiation of high-molecular-weight DNA linearizes *MDR*1 circular amplification structures in KB-V1. (A) An EtBr-stained 1% gel that was electrophoresed (PFGE) to resolve linear fragments between 200 and 1000 kb. The gel was run at 14°C at 200 V with a 60-s pulse for 15 h, followed by a 90-s pulse for 14 h. (B) Fractionated DNA was transferred to nitrocellulose and hybridized with a labeled *MDR*1 probe to detect linearized extrachromosomal *MDR*1 amplification structures, highlighted with arrows. An electrophoretic zone of compression into which linear fragments > 1000 kb in size migrate is bracketed. Lane 3 contains an agarose plug of high-molecular-weight KB-V1 DNA that was not irradiated.

When similar PFGE analysis was applied to the irradiated DNAs isolated from KB-Ch^R-8-5-11, its sublines grown at 0.25 and 0.35 μg/ml colchicine and KB-C1 (1.0 μg/ml colchicine), an 890-kb *MDR*1 containing linear DNA fragment was consistently detected (Figure 4, lane 3; Figure 6A, lanes 1 and 2). This linear DNA fragment was not detected in the parental line KB-3-1 or in the colchicine-resistant cell lines KB-Ch^R-8, KB-Ch^R-8-5, KB-C1.5, KB-C2.5, KB-C4, or KB-C6 (Figures 4–6). Hybridization studies demonstrated that sequences from the *MDR*2 gene were also present on this amplicon. Irradiation of the high-molecular-weight DNA was required to generate detectable amounts of the 890-kb *MDR*1/*MDR*2-containing amplicon. Thus, this DNA fragment results from the linearization of the supercoiled circular DNA molecules detected by high-voltage gel electrophoresis (see above; Figure 2). The 890-kb size of the circular DNA amplicon is consistent with the fact that these cell lines did not show DMs on cytogenetic analysis because the DNA size limit of resolution of light microscopy is ~1000 kb (Barker and Stubblefield, 1979).

In cell lines KB-C1.5, KB-C2.5, KB-C4, and KB-C6, irradiation of high-molecular-weight DNA resulted in linear DNA fragments of ~1.8 mb that hybridized to the *MDR*1 and *MDR*2 probes. We refer to this DM structure as 1780 kb in size (Figure 4) because subsequent studies confirmed this structure to be dimer of the 890-kb amplicon (see below; Figure 7). Larger DM structures were also detected that migrated to a major zone of compression immediately below the origin. The overexposure of several autoradiograms from independent experiments demonstrated trace amounts of the 1780-kb structure in KB-C1. Further, when the KB-C1 cell line was passaged in 1.5 μg/ml of colchicine for several weeks before preparing the DNA plug, PFGE demonstrated a reduction in the copy number of the 890-kb amplicon and easily detectable levels of the 1780-kb DM structures (Figure 4, lane 4). Thus, the

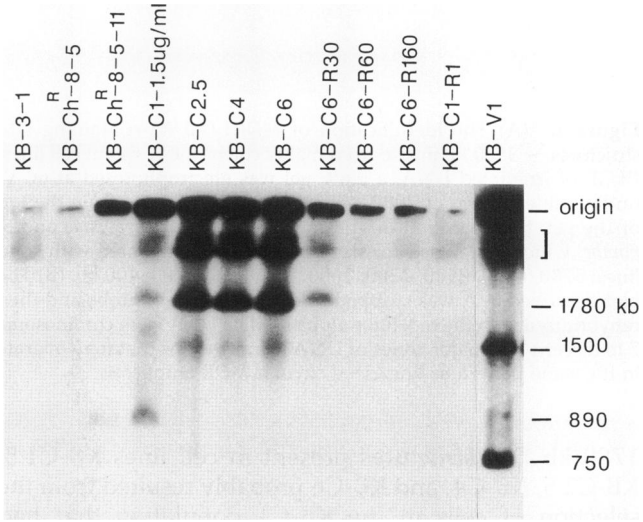

Figure 4. An 890-kb submicroscopic circular DNA as an early event in *MDR*1 gene amplification and the generation of unstable DM structures as a later amplification event. Irradiated DNA isolated from colchicine-derived cell lines and "revertant" lines was electrophoresed on a 1.2% gel to resolve DNA fragments ranging from 200 to 2000 kb under the following conditions: 150 V, with a 120-s pulse for 22 h, followed by a 240-s pulse for 30 h. DNA was transferred to a nitrocellulose membrane, which was hybridized to the *MDR*1 probe, stripped and reprobed with the *MDR*2-specific probe. Both probes gave the same hybridization signals except in the lane containing DNA from KB-V1; with the *MDR*2 probe only a very light signal, equivalent to single copy, was detected at the origin of the gel and in the zone of compression. The transfer of DNA to membrane, hybridization to the *MDR*1 probe followed by hybridization to the *MDR*2-specific probe were performed in all subsequent gel runs (Figures 5–9).

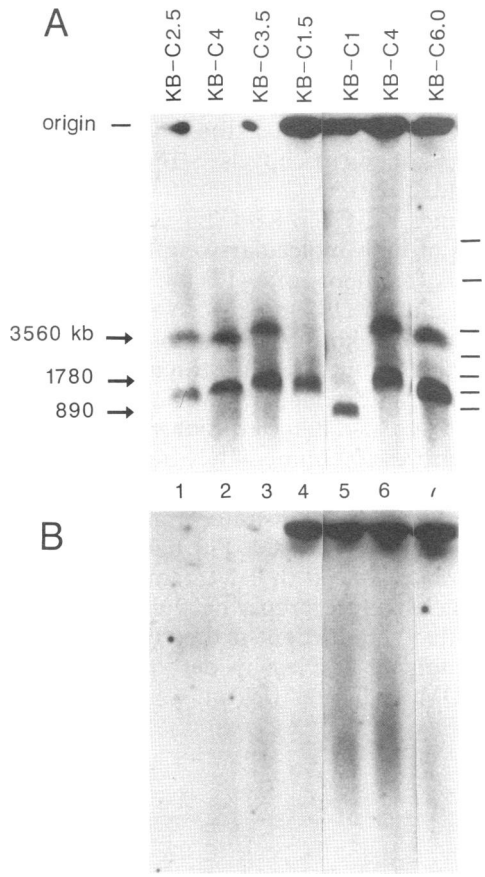

Figure 5. (A) The identification of *MDR1*/*MDR2*-containing DM structures ~ 3560 kb in size from highly colchicine-resistant cell lines. PFGE of irradiated DNA, a 0.6% gel was electrophoresed at 60 V, under a linear ramp of 2000–4000 s for 6.6 d. Starting from the top of the gel, the migration of chromosomes from *S. cerevisiae* and *S. pombe*, which were used for size standards, is designated with solid lines: 5700, 4600, 3500, 2200, 1600, 1125, and 1000–400 kb. (B) The blot described in A was stripped of hybridized *MDR* probe and then rehybridized with the repetitive alpha satellite DNA from chromosome 7 to discern any major zones of DNA compressions that may migrate in the same pattern as linearized circular *MDR* amplicons.

1780-kb DM structures present in cell lines KB-C1.5, KB-C2.5, KB-C4, and KB-C6 probably resulted from the selection of cells in the KB-C1 population that had formed a dimer of the 890-kb amplicon. In Figure 4, a diffuse *MDR1* hybridization signal in the colchicine-derived cell lines, KB-C2.5, KB-C4, and KB-C6, is present where fragments 1450–1500 kb migrate (lanes 5–7). This region represents a compressed zone in the gel, because a large amount of DNA was visualized in this region on EtBr-stained gels. Gels run under different pulse conditions and/or ramping conditions demonstrated that no specific linear *MDR1*- or *MDR2*-containing fragments of this size were present in these cell lines (see below; Figure 5). In contrast, the strong *MDR1*-hybridization signal to the KB-V1 DNA in this region represented DMs of 1500 kb that were consistently detected in other PFGE analyses under a variety of electrophoretic conditions. The *MDR2* probe did not hybridize to the 1500-kb amplicon.

By using a significantly slower pulse (switch time) and a continuous ramp for 6.6 d, PFGE resolved the larger DMs, an ~3.6-mb *MDR1*-*MDR2*-containing structure, in the highly colchicine-resistant cell lines (KB-C2.5, KB-C3.5, KB-C4, and KB-C6) as compared with chromosomal size standards from *S. pombe* (Figure 5A). We refer to this DM structure as 3560 kb in size because subsequent studies confirmed this structure to be a tetramer of the 890-kb amplicon (see below; Figure 7). This DM structure was not present in cell lines KB-C1.5 or KB-C1, whereas the 1780-kb DM structure was detected in all cell lines, except in KB-C1. Under other electrophoretic conditions, the approximate sizing of the larger 3.6-mb DM structures was confirmed (e.g., a 0.6% gel run for 160 h at 60 V with a 3600-s pulse; a 0.6% gel prepared with chromosomal grade agarose run for 72 h at 50 V with a 1800-s pulse). As a control, alpha satellite DNA from chromosome 7 (repetitive DNA sequences in excess of the copy number of the *MDR1* gene in these cell lines) was used as a probe to determine that none of these bands corresponded to any compressed zones on the gel. This probe did not produce discrete bands in any of regions of this gel (Figure 5B).

Loss of Circular DNA Structures in Revertant Cell Lines

By analyzing revertant cell lines, a decrease in *MDR1* gene copy was correlated directly with the loss of the 1780- and the 3560-kb DM structures detected with PFGE (Figure 4, lanes 8–11). In previous studies, partially revertant cell lines that showed sequential loss of *MDR1* message, *MDR1* gene copy and significant decreases in their colchicine resistance were isolated from KB-C6 after this cell line had been passaged in medium without colchicine for 30–160 d (Shen *et al.*, 1988). For this study, cell lines were passaged in medium without colchicine an additional 2–5 d until cells recovered from storage at −80°C were confluent, at which time high-molecular-weight DNA was prepared. Irradiation of DNA, followed by PFGE, demonstrated that the 1780-kb DMs and the 3560-kb DMs were significantly decreased in copy after 30 d in passage without drug (KB-C6-R30) and not detectable after 60 d without drug (KB-C6-R60) (Figure 4, lane 8 and 9, respectively). The hybridization signals in the loading lanes of irradiated DNA from cell lines KB-C6-R60 and KB-C6-R120 (120 d without drug) as compared with the parent KB-3-1 shows that some amplified structures are present in these cell lines, but they were not resolved by the irradiation procedure into linear structures that could enter the gel. Smaller extrachromosomal structures were not produced when selective pressure was withdrawn. Thus, the DMs that had been formed in the highly col-

chicine-resistant cells were lost as entire molecules in the absence of drug selection, most probably through random segregation at mitosis coupled to the selection of cells that have a faster growth rate. We have observed that all colchicine-resistant cell lines containing high copies of the DM structures have a significantly slower growth rate.

Independent Step-Wise Selections of KB-Ch^R-8-5-11 Consistently Result in the Evolution of DM Structures

To determine if DM structures, 1780 kb in size, were consistently formed when cell line KB-Ch^R-8-5-11 was subjected to increasing concentrations of colchicine, eight independent clones were isolated at 100 ng/ml colchicine and subjected to independent step-wise colchicine selections (Figure 1, selection 2). After ~6 mo, these sublines were passaged at 1.0 μg/ml colchicine but were not subcloned at any step of the selection. Therefore, each subline represented a population of amplification events. These sublines were designated as the KB-PC1-sublines. High-molecular-weight DNA isolated from these sublines was irradiated, followed by PFGE. Linearized DM structures 1780 and 3560 kb that hybridized to the *MDR*1- and *MDR*2-specific probes were present in all of these cell lines (Figure 6 and Table 1). In this gel, linear DNA fragments ≥ 2000 kb, including the 3560-kb *MDR*1-*MDR*2 containing DM structures, migrated at the zone of compression below the origin. The profile of linearized *MDR*1/*MDR*2-containing circular DNA structures in cell lines KB-PC1-2 and KB-PC1-18 were exactly like that of KB-PC1-15 and KB-PC1-3, respectively.

The frequent independent appearance of DMs whose size, 1780 kb, is an even multiple of the 890-kb amplicon present in KB-Ch^R-8-5-11 suggested that the most common mechanism generating DMs in these cell lines is the dimerization of the submicroscopic 890-kb amplicon. One exception was the subline, KB-PC1-16, that contained a DM structure ~ 1500 kb in size. This DM also hybridized to the *MDR*2-specific DNA probe and, therefore, represented a distinct DM structure from the 1500-kb DM structure in the KB-V1 cell line. The 1500-kb DM structure persisted in the KB-PC1-16 population after an additional 8.5 mo (430 d) of continuous passage, whereas the 1780-kb DM structure became less prevalent (Figure 6B, lane 3). The KB-PC1-16 subline was subcloned after 6 mo of passage; one of five subclones contained predominantly the 1500-kb DM structure (Figure 6B, lane 4), indicating that only some of the cells in the original population contained this DM structure that we presume arose as a deletion during the dimerization of the 890-kb amplicon.

Although the newly derived KB-PC1-sublines were never passaged at >1.0 μg/ml colchicine, the DNA copy number for the *MDR*1 and *MDR*2 genes was significantly higher than that in KB-C1 maintained at 1.0 μg/ml colchicine. Further, none of the KB-PC1-cell lines showed the pattern of preferential colchicine resistance with diminished vinblastine resistance characteristic of KB-C1 and its derivatives (Table 1). The sublines KB-PC1-2, KB-PC1-9, KB-PC1-17, KB-PC1-18, and KB-PC1-15 are not shown in Table 1 because clonal assays to determine their relative drug resistance were not performed. However, these cell lines were not preferentially colchicine resistant. This was determined by an assay in which the survival of 2×10^5 cells from each of these cell lines plated in medium containing various concentrations of vinblastine and colchicine was compared with the survival of 2×10^5 cells plated from the KB-C1 cell line. In these cell lines, the *MDR*1 (*MDR*2) copy number ranged from 15 (KB-PC1-17) to 27 (KB-PC1-2) compared with a relative copy number of 9 in the KB-C1 cell line. The increase in *MDR*1 gene copy in all of the newly derived cell lines appears to be required

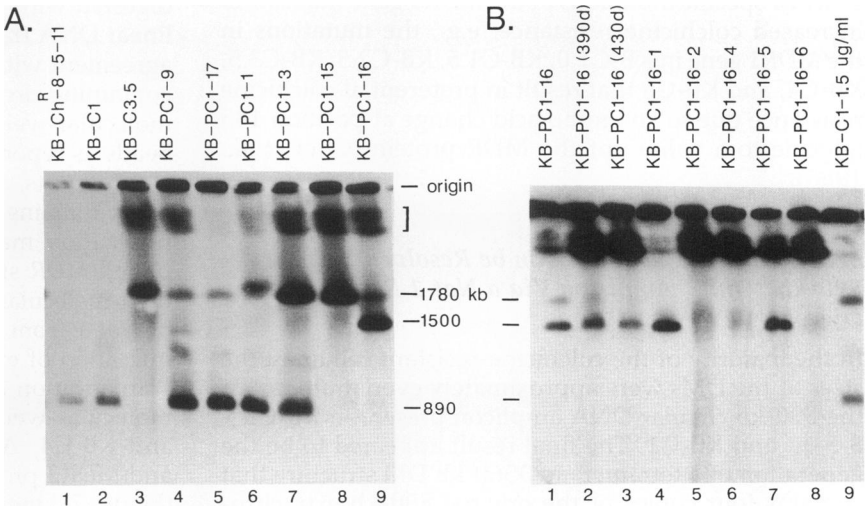

Figure 6. The high frequency of DM formation during step-wise colchicine selections of KB-Ch^R-8-5-11. (A) PFGE of irradiated DNA isolated from colchicine-resistant sublines. (B) PFGE analysis of clonal cell lines isolated from the KB-PC1-16 population. Conditions for gel electrophoresis were similar to those detailed in Figure 4, except that a 1% gel was used in this analysis.

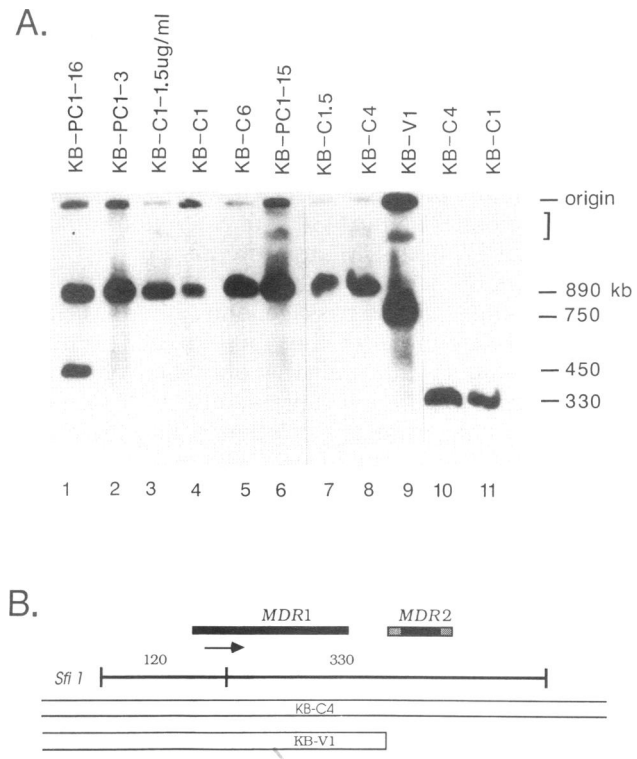

Figure 7. PFGE of *Not* 1-digested DNA demonstrates the resolution of *MDR1/MDR2*-containing DM structures into the 890-kb "unit" amplicon. (A) Lanes 1–9 contain high-molecular-weight DNA restricted with *Not* 1; lanes 10 and 11 contain DNA fractionated after restriction with *Sfi* 1. To fractionate the digested DNA, a 1% gel was run at 200 V with a pulse of 70 s for 15 h, followed by a 120-s pulse for 12 h. This gel would not resolve fragments > 1000 kb but would allow detection of fragments as small as 100 kb. (B) Map of the human *MDR1* locus (adapted from Chin *et al.*, 1989). The restriction fragments are shown in kilobase pairs. The known amplified sequences in KB-C4 and KB-V1 are boxed. The arrow denotes the direction of transcription of the *MDR1* gene.

by KB cells to survive 1.0 μg/ml colchicine in the absence of specific mutations in the *MDR1* gene that confer increased colchicine resistance, e.g., the mutations in the *MDR1* gene in KB-C1.0, KB-C1.5, KB-C2.5, KB-C3.5, KB-C4, and KB-C6 that result in preferential colchicine resistance due to an amino acid change at position 185 (glycine to a valine) of the MDR protein (Choi *et al.*, 1988).

MDR1-Containing DMs Can be Resolved into the 890-kb "Unit" Amplicon Via a Not 1 Restriction Digest

In the majority of the colchicine-resistant cell lines, the sizes of the DMs were approximately even multiples of the 890-kb circular DNA amplicon present in KB-Ch^R-8-5-11 and KB-C1. The final result appeared to be the generation of a tetramer, an 3560-kb DM structure that contains four copies of the original 890-kb extrachromosomal circular DNA. To provide firm evidence for this conclusion, a restriction enzyme that cleaved the 890-kb amplicon at a single recognition site should restrict the 1780- and 3560-kb DM structures at two and four sites, respectively, generating only linear amplicons of 890 kb. We identified *Not* 1 as an enzyme that linearizes the circular DNA amplicon in KB-Ch^R-8-5-11 into the 890-kb linear DNA fragment that was produced by the irradiation of high-molecular-weight DNA isolated from this cell line. Thus, *Not* 1 was used to restrict high-molecular-weight DNA from KB-C1.5, KB-C4, KB-C6, KB-PC1-3, KB-PC1-15, and KB-PC1-16. As controls, high-molecular-weight DNA from KB-C1 and the KB-C1 cell line that had been passaged for several weeks at 1.5 μg/ml colchicine was also restricted with *Not* 1. In all cases, with the exception of DNA from cell line KB-PC1-16, only a single *MDR1/MDR2*-containing 890-kb DNA fragment was generated in a gel run under electrophoretic conditions that size fractionated DNA fragments ≥ 50 and ≤1000 kb (Figure 7). In some of the cell lines, the *Not* 1 digestion of the high-molecular-weight DNA generated visible amounts of linear copies of the 890-kb DNA fragment in the EtBr-stained gel after PFGE. Similar results were found under electrophoretic conditions that resolved fragments ≥ 200 and ≤2000 kb. Because some of these cell lines contain only extrachromosomal elements larger in size than the 890-kb *MDR1/MDR2*-containing amplicon as determined by the irradiation studies, e.g., KB-C1.5, KB-C2.5, KB-C3.5, KB-C4, KB-C6 (Figure 4), and KB-PC1-15 (Figure 6), the results from the *Not* 1 digests provide direct evidence that the 890-kb *MDR1/MDR2*-containing DNA fragment is the "unit" amplicon from which the DM structures are comprised. In cell line KB-PC1-16, an additional band, ~450 kb, was generated (Figure 7, lane 1) and presumably results from digestion of the 1500-kb DM structure that the irradiation studies detected in this cell line (Figure 6, lane 9; see above).

High-molecular-weight DNA from KB-V1 was also digested with *Not* 1, and an *MDR1*-containing 750-kb linear DNA fragment was generated. This result was in agreement with the linearization of the 750-kb *MDR1*-containing circular DNA with a *Not* 1 digestion of high-molecular-weight KB-V1 DNA encapsulated in agarose beads as reported by Ruiz *et al.* (1989). In some of the *Not* 1 digests, a significant amount of *MDR1*-containing DNA remains trapped in the well. Although this hybridization may result from intrachromosomally amplified *MDR* structures, an incomplete *Not* 1 digest of high-molecular-weight DNA or the incomplete removal of *Not* 1 from the DNA after restriction could prevent migration of extrachromosomal structures into the gel.

In addition to the *Not* 1 digest, *Sfi* 1 digests of high-molecular-weight DNA were also performed on KB-C1 and KB-C4. After PFGE and hybridization to *MDR1* and *MDR2* probes, a 330-kb *Sfi* fragment was detected (Figure 7, lanes 10 and 11). Previous studies have dem-

onstrated that this *Sfi* fragment and a 120-kb *Sfi* fragment comprise the nonamplified native *MDR1/MDR2* locus and are present in the amplified copies of the *MDR1* and *MDR2* genes in KB-C4 (Chin *et al.*, 1989; Lincke *et al.*, 1991). Therefore, no gross DNA rearrangements occurred at the *MDR* locus during the early amplification events that generated the submicroscopic circular 890-kb DNA amplicon in cell lines KB-Ch^R-8-5-11 and KB-C1 nor during later amplification events that generated the DM structures comprised of covalently closed dimers and tetramers of the 890-kb "unit" amplicon in the colchicine-resistant cell lines KB-CB1.5, KB-C4, and KB-C6 (Figure 7B). In comparison, an *Sfi* 1 digest of KB-V1 DNA generated only a single copy level of the 330-kb DNA fragment; the amplified copies of the *MDR1* gene resided on at least two other *Sfi* 1 fragments, ~300 and 400 kb. These results are consistent with the absence of the *MDR2* gene on the amplified 750- and 1500-kb circular amplicons (see above; Figure 7B).

DISCUSSION

We have studied amplification of the *MDR1* locus in drug-resistant KB cells and found that DM structures were derived from the multimerization of submicroscopic circular DNAs. The unique aspects of this study are the size determinations of the DM structures, which are shown to be approximately even multiples of the size of the submicroscopic element, and the demonstration that restriction of the DM structures with *Not* 1 resolves the DM structures into linear copies of the submicroscopic circular DNA. The cell lines studied were derived from a subclone of KB-3-1 during a stepwise selection in colchicine (Figure 1, Selection 1). With cytogenetic analysis, DMs were observed in the cell lines that were adapted to the higher concentrations of colchicine, $\geq$1.5 μg/ml, but were not observed in cell lines derived at earlier steps in the selection, e.g., KB-Ch^R-8-5-11 and KB-C1 that also contained amplified copies of *MDR1* and *MDR2* (Table 1).

Formation of Submicroscopic MDR1/MDR2-Containing Circular DNAs

To identify the amplicon in the KB-Ch^R-8-5-11 and KB-C1, we used high-voltage gel electrophoresis and an irradiation protocol (Ruiz *et al.*, 1989). Both cell lines contained the amplified *MDR1* and *MDR2* genes on an 890-kb submicroscopic circular DNA (Figures 2, 4, and 6). This amplicon was stably maintained in the extrachromosomal state after continuous passage in the presence of drug for 1.5 y.

The formation of the *MDR1/MDR2*-containing 890-kb circular DNA was either the initial step in gene amplification at the *MDR* locus or a very early amplification event. This conclusion is based on two observations: 1) cell line KB-Ch^R-8-5-11, which harbors this extrachromosomal amplicon (Figures 4 and 6, lanes 3 and 1, respectively), is the first subline in which conventional Southern/hybridization methods detected *MDR1* and *MDR2* gene amplification (Table 1) (Shen *et al.*, 1986b) and 2) neither HSRs nor DM structures were detected in the initial cytogenetic studies performed on cell line KB-Ch^R-8-5-11-24 (Akiyama *et al.*, 1985; Whang-Peng, personal communication), referred to as KB-C1 in subsequent studies (Shen *et al.*, 1986b) (Figure 1). These observations are consistent with our identification of submicroscopic extrachromosomal amplicons containing the amplified *MDR1* and *MDR2* gene sequences in KB-Ch^R-8-5-11 and KB-Ch^R-8-5-11-24 (KB-C1). Because the *MDR2* gene does not appear to play a role in MDR (Schinkel *et al.*, 1991), we assume the *MDR2* locus has been passively coamplified as part of the 890-kb circular DNA amplicon in the colchicine-derived cell lines.

Characterization of the "Unit" Amplicon in the DM Structures

The 890-kb *MDR1*- and *MDR2*-circular DNA was not detected in the cell lines from later steps in the colchicine selection (KB-C1.5, KB-C2.5, KB-C4, and KB-C6) (Figures 4–6). The DM structures in these cell lines were determined to be ~1.8 and 3.6 mb. We refer to the specific size of these structures as 1780 and 3560 kb, respectively, because a *Not* 1 digest, which linearizes the 890-kb amplicon (Figure 7, lane 4), resolved these DMs into multiple linear copies of the 890-kb amplicon (Figure 7). Thus, these *Not* 1 restriction digests confirm that the DMs were composed of two or four copies of the 890-kb "unit" amplicon structure. During the colchicine selection, the 890-kb circular DNA dimerized to the 1780-kb DMs, which subsequently dimerized to generate the larger DM structures (3560 kb). During dimerization, no gross DNA rearrangements occurred within the *MDR1* or *MDR2* genes of the extrachromosomal amplicons as determined by *Sfi* 1 digests of the amplified and native locus (Figure 7B). The most probable mechanism through which the circular dimers were formed would be intramolecular homologous recombination in which one exchange or an odd number of exchanges occurred during or shortly after replication. This type of mechanism has been recently discussed in reference to the resolution of multimers of the *Escherichia coli* bacterial chromosome after replication (Kuempel *et al.*, 1991).

Although several past studies have demonstrated that on prolonged culture in drug DM structures will reintegrate into chromosomal loci to form ECRs, once the DMs were generated in the colchicine-resistant cell lines, they were stably maintained in the presence of drug in cell lines that were continuously passaged for 1 y (data not shown). In contrast, when the cell lines were pas-

saged in medium without drug, the copy number of the DMs quickly decreased, but the size of the DMs remained unaltered (Figure 4), indicating that homologous intramolecular recombination did not occur at any detectable level to resolve the DMs into their unit amplicon of 890 kb.

Analysis of eight independent cell lines selected by placing KB-Ch^R-8-5-11 in increasing concentrations of colchicine (Figure 1, Selection 2) confirmed that dimerization of the 890-kb amplicon was a common mechanism utilized by this cell line to generate DMs (Figures 6 and 7) and to increase *MDR*1 gene copy (Table 1). None of these sublines showed the preferential colchicine resistance of the KB-C1 cell line (Table 1) that results from a specific amino acid change in the *MDR*1 protein (Choi *et al.*, 1988). Therefore, during the initial colchicine selection, the mutations probably arose spontaneously on one of the extrachromosomal elements during the colchicine selection steps subsequent to the cloning of KB-Ch^R-8-5-11 but before the cloning of KB-Ch^R-8-5-11-24 (KB-C1) (Figure 1, Selection 1). With continued passage of KB-C1 in medium containing colchicine and when the KB-C1.5 cell line was established, the amplicon with the mutated *MDR*1 gene was easily selected because it confers increased colchicine resistance and resides on an extrachromosomal element that undergoes random segregation at mitosis.

Initial Amplification Event at the MDR Locus

One question that this study has not resolved is whether the 890-kb circular DNA in KB-Ch^R-8-5-11 was the first amplification structure that occurred at the *MDR* locus during the colchicine selection. After selection in 30 ng/ml colchicine (Figure 1), the surviving clones were not expanded but immediately reselected in medium containing 100 ng/ml colchicine. Therefore, the KB-Ch^R-8-5-11 cell line could represent one surviving cell from a clone whose initial amplification event was the formation of a larger unstable amplification structure, e.g., an HSR or DM. Resolution of this putative unstable amplicon into the 890-kb circular DNA would have had to be a high-frequency event, because the clones selected in 30 ng/ml colchicine (with ~1000 cells/clone) were not expanded before selection in 100 ng/ml colchicine. We cannot rule out this possible scenario because in a concurrent study of the vinblastine-selected KB cell lines, preliminary evidence demonstrates that initial amplification events generate megabase-length *MDR*1/*MDR*2-containing DM structures (Schoenlein, unpublished data). In subsequent vinblastine selections, these DMs appear to rearrange and "breakdown" to form the 750-kb submicroscopic *MDR*1-containing circular DNA present in KB-V1 (Figure 4).

Studies are in progress to determine if a deletion resides at one of the *MDR* chromosomal alleles in the colchicine-selected cell lines. Amplification of a region of DNA via an extrachromosomal element often results in a deletion at the corresponding chromosomal locus (Carroll *et al.*, 1988; Hunt *et al.*, 1990; Heard *et al.*, 1991). If a deletion is present, its respective size could provide information as to the size of the initial amplicon. We will also begin to investigate what other genes may reside on the 890-kb unit amplicon. A *Not* 1 digest of high-molecular-weight DNA from some of the colchicine-resistant cell lines results in large amounts of the linear 890-kb amplicon that are directly visible on EtBr-stained gels, providing an easy avenue for DNA cloning. Coamplification of genes located adjacent to *MDR*1 and *MDR*2 have been observed in MDR rodent cell lines (Van der Bliek *et al.*, 1986) and in a MDR human ovarian cell line (Van der Bliek *et al.*, 1988b).

Summary

In the colchicine-resistant cell lines analyzed in this study, amplification at a native mammalian locus occurred with the formation of a submicroscopic extrachromosomal circular DNA that eventually dimerized two times to generate visible DMs. These data provide direct evidence for an amplification model in which submicroscopic circular DNAs are precursors to DM structures (Wahl, 1989). Similar multimerization of circular DNAs appears to occur in vivo, e.g., in neuroblastomas containing amplified copies of *MYCN* (VanDevanter *et al.*, 1990). Therefore, the colchicine-selected cell lines may provide an ideal system in which to test therapeutic strategies to eliminate circular DNAs or prevent their multimerization.

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