Characterization of Gene Rearrangements Leading to Activation of MDR-1*†‡§

Lyn M. Huff†, Jong-Seok Lee§, Robert W. Robey‡, and Tito Fojo†‡

From the †Medical Oncology Branch, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, Maryland 20892 and §Seoul National University, Bandung Hospital, Seoul 151-742, South Korea

Expression of the MDR-1/P-glycoprotein gene confers drug resistance both in vitro and in vivo. We previously reported that gene rearrangements resulting in a hybrid MDR-1 transcript represent a common mechanism for acquired activation of MDR-1/P-glycoprotein. We have identified hybrid MDR-1 transcripts in nine MDR-1-overexpressing cell lines and two patients with relapsed ALL. We characterize these rearrangements as follows. 1) Non-MDR-1 sequences in the hybrid MDR-1 transcripts are expressed in unselected cell lines, showing that these sequences are constitutively expressed. 2) The rearrangements occur randomly and involve partner genes (sequences) on chromosome 7 and on chromosomes other than 7. Breakpoints have been characterized in six cell lines. In one, the rearrangement occurred within intron 2 of MDR-1; in the other five, the rearrangement occurred to >96 kb 5′ of the normal start of transcription of MDR-1. In one cell line, homologous recombination involving an Alu repeat was observed. However, in the remaining five cell lines, nonhomologous recombination was observed. 3) The rearrangements arise during drug selection. The acquired rearrangements are not detected in parental cells. 4) Five of the six active promoters that captured MDR-1 controlled MDR-1 from a distance of 29 to more than 110 kb 5′ to MDR-1. Transcription was initiated in an antegrade or retrograde direction. We conclude that drug selection with natural products targeting DNA or microtubules leads to DNA damage, nonhomologous recombination, and acquired drug resistance, wherein MDR-1 expression is driven by a random but constitutively active promoter.

Overcoming clinical drug resistance, both intrinsic and acquired, remains a challenge for the oncologist in the treatment of human malignancy, even in the current era of novel therapies (1, 2). The hallmark of clinical drug resistance is cross-resistance to multiple structurally unrelated compounds used in the treatment of cancer, such as the vinca alkaloids, the anthracyclines, the epipodophyllotoxins, the taxanes, and actinomycin D. The ABC transporters are the most extensively studied mechanisms of drug resistance both in vitro and in vivo. Of the 48 members of this super family of proteins, four (MDR-1/ABCB1, MRPI/ABCC1, MRP2/ABCC2, and BCRP/MXR/ABCG2) have been shown to confer drug resistance in human malignancy (3–6). The first ABC transporter described in drug resistance was MDR-1/ABCB1, located on chromosome 7q21.1. MDR-1 encodes a cell surface 170-kDa P-glycoprotein (Pgp), composed of 12 transmembrane domains and two nucleotide binding sites. P-glycoprotein requires the hydrolysis of ATP to efflux cytotoxic substrates across the cell’s plasma membrane. Increased expression of Pgp on the tumor cell causes increased drug efflux and decreased intracellular accumulation of the chemotherapy drug. The clinical outcome is a drug-resistant tumor.

Much effort has gone into studying the regulation of MDR-1 expression in drug-selected cancer cell lines and in patient samples. Transcription factors that bind to the MDR-1 promoter and increase transcription (7, 8), epigenetic changes due to methylation and acetylation of the MDR-1 promoter (7, 9–11), and gene rearrangements 5′ to the MDR-1 promoter that result in a hybrid MDR-1 transcript with a constitutively active gene promoter (12) have all been shown to affect MDR-1 gene expression.

Previously, we reported that when screening for point mutations in the MDR-1 gene, a mismatch was detected by RNase protection analysis in a series of adriamycin-selected human colon carcinoma cell lines, designated S48-3s/ADR. Further studies demonstrated a deletion of the first 68 residues of the coding sequence and 23 residues from the 5′-UTR in the first step of the selection. Using 5′-RACE, the MDR-1 mRNA of S48-3s/ADR cells was shown to be a hybrid message of non-MDR-1 and MDR-1 sequences. Somatic cell hybrids localized these 5′ partner gene sequences to chromosome 4, and fluorescence in situ hybridization provided clear evidence of a translocation between chromosomes 4 and 7 (12). Additional observations established that this translocation was an acquired phenotype that occurred in the first step of the selection and led to the overexpression of MDR-1. Activation occurred because endogenous expression of the chromosome 4 gene was higher than MDR-1, and co-amplification of the chromosome 4 sequences and MDR-1 was shown in cell lines selected for

*This work was supported by the Intramural Research Program of NCI, National Institutes of Health, Center for Cancer Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
†The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–3.
‡A Commissioned Officer in the United States Public Health Service. To whom correspondence should be addressed: NCI, National Institutes of Health, Center for Cancer Research, Medical Oncology Branch, Bldg. 10, Rm. 12N226, 9000 Rockville Pike, Bethesda, MD 20892. Tel.: 301-402-1357; Fax: 301-402-1608; E-mail: tfojo@helix.nih.gov.
§The abbreviations used are: Pgp, P-glycoprotein; UTR, untranslated region; RACE, rapid amplification of cDNA ends; NHEJ, nonhomologous end joining; DSB, double strand break; HR, homologous recombination.
Gene Rearrangements Lead to Overexpression of MDR-1

higher levels of resistance. With the sequencing of the human genome, these chromosome 4 sequences were identified as arising from the 5′-UTR of the SET 7 gene, a histone 3 lysine 4 methyl transferase, located on 4q28 (13). This translocation provided a model for activation of MDR-1.

We thus sought additional examples in other drug-selected ce line. We recognized such rearrangements would preferentially occur in the 5′-UTR, since this would avoid significant disruption of protein structure. Previous studies had concluded the MDR-1 gene had both a “downstream” promoter responsible for transcription from the normal start site and an “upstream” promoter (14). We proposed that transcripts arising from an “upstream” promoter might in some cases be hybrid mRNAs composed of 5′ partner gene sequences fused to MDR-1 at residue −194. We have identified gene rearrangements in nine drug-resistant cell lines and two patient samples, one with acute lymphocytic leukemia who failed induction chemotherapy and one with drug-refractory acute lymphocytic leukemia (Table 1) (12, 15). In the present study, we report further characterization of these cell lines and describe the rearrangements in six of them. The result presented herein establishes the changes as an acquired phenotype that occurs primarily following nonhomologous end joining (NHEJ) and that are very diverse, reflecting the pliability available to cancer cells needing to become drug-resistant.

EXPERIMENTAL PROCEDURES

Cell Lines—The drug-resistant cell lines used in these experiments were derived from the following parental cancer cell lines: S48-3s (colon), MCF-7, ZR-75B, MDA-MB-231 (breast), EW36 (Burkitt’s lymphoma), and 8226 (multiple myeloma) (12, 15, 16). The drug-resistant sublines were grown in the following concentration of MDR-1 substrate: S48-3s ADR10 (10 μg/ml adriamycin), S48-3s ACT 0.2 (200 ng/ml actinomycin-D), MCF-7 TX400 (400 ng/ml paclitaxel), MCF-7 AD500 (500 ng/ml adriamycin), ZR-75B TX320 (320 ng/ml paclitaxel), ZR-75B AD600 (600 ng/ml adriamycin), MDA-MB-231 VB100 (100 ng/ml vinblastine), EW36 VCR120 (120 ng/ml vincristine), and 8226 DOX40 (400 nm doxorubicin). The colon cancer cell line, S48-3s, and its drug resistant sublines, S48-3s ADR 10 and S48-3s ACT 0.2 (12, 17), were grown in a 1:1 mixture of I scove’s modified Eagle’s medium/ Ham’s F-12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. All other cell lines were grown in Iscove’s modified Eagle’s medium supplemented as described above.

RNA Probe Synthesis and RNase Protection Analysis—Hybrid RNA probes used in the RNase protection assays were derived by reverse transcription-PCR as previously described (12). RNA from each drug-resistant cell line was used as the template in the reverse transcription reaction to generate a hybrid RNA probe unique to each drug-resistant cell line. A 3′ primer specific for MDR-1 exon 1 (−127ATTCCCTAGAAAATCGGAA−146) was used to generate a CDNA from each drug-resistant cell line using Meloney murine leukemia virus reverse transcriptase (Invitrogen) as previously described (12). A PCR product representing the hybrid RNA transcript was then generated using a 5′ primer specific for each 5′ partner sequence in the hybrid MDR-1 transcript and the MDR-1-specific 3′ primer used in the reverse transcription reaction. The resulting PCR product was thus unique to each drug-resistant cell line and was cloned into the pCR1 cloning vector (Invitrogen) by TA cloning (18). Radioactive hybrid RNA probes were then synthesized from these plasmids containing hybrid sequences by transcribing 1 μg of plasmid DNA with either T7 or SP6 polymerase and [32P]UTP (3000 Ci/mmol) (Amersham Biosciences) as previously described (15, 19).

Solution hybridization was then performed using 20 μg of mRNA from parental and drug-resistant cells and 1.5 × 10⁵ cpm of the radiolabeled hybrid RNA probe unique to that drug-resistant cell line. Hybridization conditions and gel electrophoresis were carried out as previously described (20).

DNA Probe Synthesis and Southern Analysis—DNA probes used in Southern blot analysis were generated by PCR using genomic KB 3-1 DNA as a template and the primer sequences listed in supplemental Table 1. The Expand Long PCR kit (Roche Applied Science) was used to generate long PCR probes (P1, P2, and P4). All other DNA probes (1A–1D and 2A–2E) were generated by standard PCR technology using TaqDNA polymerase (Invitrogen) All probes were synthesized by TA cloning into the pCR1 cloning vector (Invitrogen) as described above. The plasmid was then cut with restriction enzymes and electrophoresed on a 1% agarose gel to isolate the insert DNA. The insert was excised from the gel and purified by Spin-X columns (Costar Inc., Corning, NY). Radioactive DNA probes were then synthesized using the Rediprime RmT labeling kit and [32P]dCTP (3000 Ci/mmol) from GE Healthcare (Piscataway, NJ).

Genomic DNA was isolated from parental and drug-resistant cell lines using the Wizard Genomic DNA purification Kit (Promega, Madison, WI) and digested with HindIII. Then 10 μg of digested DNA was loaded per lane on a 1% agarose gel. After electrophoresis, the gel was then transferred to nitrocellulose and hybridized with the radiolabeled DNA probe at a final concentration of 1.0 × 10⁶ cpm/ml Hybridsol (Intergen, Purchase, NY) (21).

Probes P-2, 2A, and P-4 have internal HindIII sites. Fragments protected by probe P-2 yielded 11.5, 6.5, and 1.9 kb bands. Probe 2A yielded 1.4 and 1.1 kb bands, and probe P-4 yielded 4.9 and 4.7 kb bands, which appear as one band on autoradiography.

Luciferase Constructs, Cloning, and Transfections—Initial searches in GenBank™ mapped identical partner sequences in the EW36 VCR120 and MDA-MB-231 VB100 cell lines 275 kb 5′ to the MDR-1 start site near several expressed sequence tags subsequently identified as the 5′-UTR of the MCFP gene. Before they were identified as the MCFP gene, we thought that these partner sequences might be the 5′-UTR of some unknown gene and took advantage of the promoter data base, Proscan, to search a 1-kb region surrounding these sequences to look for promoter activity (22). Results from the search allowed us to design the following primers to construct luciferase plasmids. Restriction enzyme sites (HindIII and Xhol sites are highlighted in boldface type) were introduced into the primers to control the direction of each fragment: 1F′ (134951gattcgagccggcgccggctcgac) and 1F (134951actaaagcttgctacacagacgctcgac) and 2F5′ (154839tgcctcggagtctgggccccggctcgac) and 2F (154839gaccgctacagcagcgctcgac).
Gene Rearrangements Lead to Overexpression of MDR-1

TABLE 1
Cell lines and refractory leukemia samples with hybrid MDR-1 transcripts

| Cell line/Patient | Tissue of origin | Drug selection | Non-MDR-1 gene/chromosome | Length of non-MDR-1 sequence cloned (bp) | Breakpoint location (nucleotide) |
|-------------------|------------------|----------------|--------------------------|---------------------------------------|---------------------------------|
| S48-3s ADR10      | Colon            | Adriamycin (10 μg/ml) | SET 7/4q28                | 250                                    | 4,941 of AC112326 (SET 7); 35,422 of AC002457 (MDR) |
| S48-3s ACT 0.2    | Colon            | Actinomycin D (200 ng/ml) | HSCARG/16p13.3; HKOX-2/16p13.3 | 67                                    | 148,476 of AC005096 (CASP); 120,304 of AC002457 (MDR) |
| MCF-7 TX400       | Breast           | Paclitaxel (400 ng/ml) | CASP-7/4q22               | 97                                    | 23,394 of AC005065 (MLLS); 85,826 of AC002457 (MDR) |
| MCF-7 AD*500      | Breast           | Adriamycin (500 ng/ml) | MLLS/7q22                 | 352                                    | 150,288 of AC03083 (MCFP); 60,784 of AC002457 (MDR) |
| ZR-75B TX320      | Breast           | Paclitaxel (320 ng/ml) | Chromosome 7              | 259                                    | 122,806 of AC03083 (MCFP); 58,972 of AC002457 (MDR) |
| ZR-75B AD600      | Breast           | Adriamycin (600 ng/ml) | Chromosome 7              | 259                                    | 4,840 of AL354815 (Chr 13); 127,038 of AC002457 (MDR) |
| MDA-MB-231 VB100  | Breast           | Vinblastine (100 ng/ml) | MCFP/7q21.13             | 198                                    |                                                |
| EW36 VCR120       | Lymphoma         | Vincristine (120 ng/ml) | MCFP/7q21.13             | 252                                    |                                                |
| 8226 DOX40        | Myeloma          | Doxorubicin (400 nm)  | Chromosome 13             | 221                                    |                                                |
| ALL no. 1         | Relapsed ALL     | Induction failure²  | NRB-1/7q31                | 70                                    |                                                |
| ALL no. 2         | Relapsed ALL     | Multiply relapsed²  | Chromosome 1q31.3-32.1    | 177                                    |                                                |

* Patient never achieved remission during induction therapy. MDR-1 substrates received during induction were as follows: vincristine, daunorubicin, and VM-26 (teniposide).

² Patient relapsed while on maintenance therapy and had seven subsequent relapses before dying 45 months after diagnosis. MDR-1 substrates received were as follows: vincristine, daunorubicin, doxorubicin, and VM-26 (teniposide).

RESULTS
We had previously reported that in four drug-resistant cell lines and two patient samples, a hybrid MDR-1 mRNA could be detected using 5' RACE. Furthermore, we demonstrated that the hybrid message identified in a given drug-resistant cell was detected exclusively in that cell line or patient sample. These findings were consistent with the existence of rearrangements that were unique to each resistant cell line or patient sample (12, 15). Our present study extends our original reports with five additional drug-resistant cell lines in which hybrid MDR-1 transcripts were identified. All cell lines and patient samples in which these hybrid MDR-1 mRNAs were identified are summarized in Table 1. Also summarized in Table 1 is the concentration of Pgp substrate in which the cells were grown and the name or chromosome location of the 5' partner gene identified in each hybrid MDR-1 mRNA. The partner sequences cloned from the 5' RACE products of 11 hybrid transcripts vary in length from 67 to 352 base pairs. To establish that the hybrid MDR-1 mRNAs were the predominant or exclusive MDR-1 transcripts, the RNase protection experiments shown in Fig. 1 were performed. Results are shown for four cell lines. In each instance, the sequence comprising the junction of the hybrid message has been subcloned and utilized as the template for the RNA probe. We had previously shown that the 5’ partner sequences in the hybrid mRNAs were expressed in the un-
selected parental cells and, in a majority of cases, could be detected in many different cells, consistent with a housekeeping function (12). These observations were confirmed by RNase protection. As shown in the figure, in a majority of cases, RNA isolated from the parental cells was able to protect the non-MDR-1 portion of the probe (252, 198, 221, or 223 bp; for the probe hybridized with mRNA from the MDA-MB231 cells and its resistant subline, the 198-bp fragment detected on the original autoradiogram is not reproduced in the figure). In no case was the full-length probe representing the hybrid MDR-1 mRNA protected by parental cell RNA, consistent with the results of the more sensitive PCR assay (discussed below) that suggested these hybrid transcripts were acquired during the process of drug selection. In contrast, RNA isolated from the resistant cells protects the full-length probe (319, 265, 288, or 365 bp), indicating that hybrid resistant cells protects the full-length probe (319, 265, 288, or 365 bp), indicating that hybrid MDR-1 mRNAs (67 bp of MDR-1 exon 1) are present therein. In addition, protection of the non-MDR-1 portion of the probe is also seen, and in every case the level appears higher than in the parental cells. Possible explanations for this observation are discussed below.

As a next step, we set out to determine in a subset of the drug-resistant cell lines the site and nature of the rearrangement. This portion of the work was confined to the cell lines, because sufficient quantities of RNA and DNA were not available from the patient samples. Instead of constructing a genomic library for each resistant cell line and probing with MDR-1 probes and then with probes containing the 5′ partner sequences, we adopted an alternate strategy (see Fig. 2). We knew the cell lines had amplified MDR-1 to varying degrees, and by extension the area of the rearrangement. We had also previously shown that with the exception of the S48-3s ADR cell line, in every other case, the partner sequence in the hybrid message was “fused” to MDR-1 at a residue that is 194 base pairs 5′ of the normal start site of MDR-1. The result of this is that in all cases, except for the S48-3s ADR cell line, the sequence is a hybrid MDR-1 transcript but not a hybrid protein. In all cases, this results in a P-glycoprotein this is expressed at high levels and is fully functional (see supplemental Fig. 1).

Therefore, as a first step, we concentrated on narrowing down the site of rearrangement on the “MDR-1 side” of the breakpoint. In order to do this, we began by subcloning three segments of DNA (designated P1, P2, and P4 in Fig. 2) located in the proximal 15–95 kb 5′ of the normal MDR-1 start site. These three fragments, as well as all other fragments subsequently used to examine for amplification, were chosen to be free of repetitive sequences, which, as discussed below, are very prevalent in the 5′ region of MDR-1. By using these fragments as probes in a Southern blot analysis of HindIII-digested genomic DNA, we were able to narrow the site of the breakpoint relative to these probes. We then performed additional Southern analyses using probes (1A–1D and 2A–2E) located between the initial three probes (P-1, P-2, and P-4) to identify fragments that were amplified and fragments that were present at “single copy level.” These fragments used as probes in the second screen were in closer proximity to each other. In some cases, HindIII fragments of unexpected length were detected, indicating that a rearrangement had occurred in close proximity to or within the genome sequences encompassed by the fragment used as the probe. Two examples of this strategy and the results obtained are shown in Fig. 2. In the 8226 DOX40 cell line, the initial screen showed that fragment P2 was amplified, whereas fragment P4 was single copy. On subsequent blots, amplification of fragment 2E was also shown, and with the knowledge that P4 was single copy and the appearance of an additional 6.2 kb band, we narrowed the site of the breakpoint to the region between 2E and P4. In addition, a 7.0 kb band was detected in the DOX40 DNA hybridized with P4, indicating that the rearrangement introduced a new HindIII site. In MCF-7 Adint500, fragment 1D was amplified, whereas P2 was single copy, localizing the breakpoint in this cell line between these two fragments. An additional 3.2 kb band was detected in the Adint500 DNA hybridized with P2 also indicating a new HindIII fragment due to the rearrangement.

We obtained the sequence for the breakpoints by isolating a PCR product spanning the breakpoint. An example of this strategy for the MCF-7 Adint500 cell line is shown in supplemental Fig. 2. Fig. 3 summarizes the site of the breakpoints between the “capturing genes” and MDR-1 in six of the cell lines, including two that have been previously published (25). With the exception of one cell line, S48-ADR10, all breakpoints occurred 5′ of the start of transcription of the MDR-1 gene. In the S48-ADR10 cell line, the breakpoint occurred between exons 2 and 3 of the MDR-1 gene (12). In the S48-ADR10 cell line, the distance from the breakpoint in intron 2 to exon 3 was 3.9 kb. In the six cell lines with breakpoints 5′ of the start of transcription, the distance to the breakpoint ranged from 22 to 91 kb. On the non-MDR-1 side of the breakpoint, the distances between the sequences in the hybrid mRNA and the breakpoint in the genomic sequence were smaller, ranging from 1.9 to 26 kb.
Because the 140-kb region proximal to the start of \textit{MDR-1} transcription contains a large number of Alu repeats and other retrotransposable elements (LINE, L1, MIR, MCFP, MaLR, MCFP, MIR, LTR, and ERV), we anticipated that rearrangements involving these repetitive elements would be common. However, as the schematic in Fig. 4 shows, although Alu or other repeats were commonly found near the breakpoints, with the exception of MCF TX400 cells, they did not contribute to the breakpoint sequences. Alu repeats were only identified in the breakpoint of MCF TX400 cells, the only cell line for which evidence of homologous recombination was found (25). In the other six cell lines, nonhomologous recombination appears to have occurred at the site of the rearrangement.

The results in two resistant cell lines deserve attention: EW VCR120 and MDA-MB-231 VB100. We had previously noted that these two cell lines contained the same hybrid \textit{MDR-1} transcript (15) (data not shown). We were confident that this did not represent contamination because the cloning was performed in two separate laboratories, and one of the cell lines did not represent contamination because the cloning was performed in two separate laboratories, and one of the cell lines did not represent contamination because the cloning was performed in two separate laboratories. (Supplemental Fig. 3). A product was not obtained using DNA from parental cells even when the PCR was carried out to 50 cycles, making it unlikely that even a single cell in the original seeding of 10^6–10^7 cells contained the rearrangement.

By examining the sequence data available for the human genome (available on the World Wide Web at www.ncbi.nlm.nih.gov/), we were able to identify the 5' partner gene in seven of the 11 cell lines and patient samples in which we have documented rearrangements. In the cases where the partner gene could be identified (SET 7, HMOX-2, HSCARG, CASP, MCFP, ML55, MCFP, ML55, MCFP, ML55, and MCFP, ML55, and MCFP, ML55, and MCFP, ML55, and MCFP, ML55, and MCFP, ML55, and MCFP, ML55), where the genomic organization was known, the sequences of the partner gene contained in the hybrid transcripts were from the 5'-untranslated region of the partner gene. This observation is consistent with control of expression of the hybrid \textit{MDR-1} transcript by the promoter of the 5' partner gene. One example of note is that observed in the EW VCR120 and MDA-MB-231 VB100 cell lines, which as noted above contain identical hybrid transcripts but unique breakpoints. When the GenBank database was examined, the genomic organization shown in Fig. 5 was discerned. The partner sequences contained in the hybrid \textit{MDR-1} transcripts are 275 kb 5' to \textit{MDR-1} exon 1. When initially identified, this sequence was seen to be adjacent to a cluster of expressed sequence tags that belonged to an unidentified gene. This gene has since been identified as \textit{MCFP} (mitochondrial carrier fam-

---

\*\*3 L. M. Huff and T. Fojo, unpublished observations.\*\*
Gene Rearrangements Lead to Overexpression of MDR-1

FIGURE 5. MCFP captured MDR-1 in two different cell lines (EW VCR120 and MDA-MB-231 VB100). These cell lines contain identical hybrid transcripts but unique breakpoints. A, the MCFP sequences contained in the hybrid MDR-1 transcripts were located 275 kb 5' to MDR-1 exon 1 and share 120 bp sequence with a second gene, ASK, that transcribes in a direction opposite of MCFP as seen in the schematic. Before MCFP was identified, this region upstream from MDR-1 was identified as several expressed sequence tags. B, to determine if this region had promoter activity, six reporter constructs (1F, 1R, 2F, 2R, 3F, and 3R) spanning the expressed sequence tags were engineered by cloning the three fragments shown in 8 into a luciferase vector (pGLb) in both orientations. The locations of the six constructs are shown in relation to MCFP exon 1. C, the promoter activities of the six vectors are summarized graphically. Constructs 1F and 1R show promoter activity 30 and 10% that of the cytomegalovirus promoter, respectively, and both show significantly higher activity than the thymidine kinase promoter (TK) and the empty vector negative control, pGLb. This suggested that a region composed of the sequences contained within or near fragments 1F and 1R were probably functioning as the promoter for the hybrid MDR-1 transcript in EW VCR120 and MDA-MB-VB100 cells.

FIGURE 6. Sequencing of 5'-RACE products from S48-Act-D 0.1 cells yielded two hybrid RNAs. Non-MDR-1 sequences that comprised these two transcripts were identified as two genes (HMOX-2 and HSCARG) on chromosome 16p13.3, separated by 1500 bp and transcribed in opposite directions as indicated by the long arrows at the top of A. B shows a schematic of the hybrid transcripts detected by reverse transcription PCR shown in C when using S48-3s ACT 0.1 RNA as template. Primers used in the PCR are indicated by the small horizontal arrows. The HSCARG sequences that comprised the hybrid MDR-1 transcript were transcribed in a retrograde direction, suggesting that sequences proximal to exon 1 of HSCARG regulate MDR-1 transcription by starting in exon 1 of HSCARG and transcribing retrograde direction to that of normal HSCARG transcription.

Gene Rearrangements Lead to Overexpression of MDR-1
cally, six reporter constructs (1F, 1R, 2F, 2R, 3F, 3R) were engineered by cloning the three fragments shown in Fig. 5b into the luciferase vector, pGLb, in both orientations. The promoter activities of the six vectors are summarized in Fig. 5c. As can be seen, several of the constructs had detectable promoter activity, with the highest observed in the construct with fragment 1F in the MDR-1 orientation. Indeed, the promoter of this construct was substantially higher than that of the thymidine kinase control and nearly 3% of the cytomegalovirus control. This suggested that a region composed of the sequences contained within fragment 1F or its vicinity were probably functioning as the promoter for the hybrid MDR-1 transcript in EW VCR60 and MDA-MB-VB100. Note that this region also has substantial promoter activity in the opposite orientation (1R). Although neither the promoter region of the ASK gene nor the MCFP gene has yet been characterized, they are probably included in the constructs evaluated. Most likely the promoter of the MCFP gene is responsible for the hybrid MDR-1 transcript or less likely the ASK promoter transcribes in a direction retrograde to its normal transcription.

A retrograde direction is a possibility, as shown by the results obtained with S48-Act 0.1 cells shown in Fig. 6. Sequencing of the 5'-RACE products from S48-Act 0.1 cells yielded two hybrid RNAs. The partner sequences isolated were from the 5'-untranslated regions of two different genes on chromosome 16p13.3 that are separated by 1500 bp of genomic sequence. These two genes, HMOX-2 and HSCARG, are transcribed in opposite directions, as indicated by the arrows in the upper portion of the figure. HMOX-2 sequences in the hybrid mRNAs are transcribed in their normal direction of transcription. However, the HSCARG sequences that contribute to the other hybrid mRNAs are transcribed retrograde to the normal direction of HSCARG transcription. This suggests that a region in proximity to exon 1 of HSCARG regulates MDR-1 by transcrib-
ing through HSCRG exon 1 in a retrograde manner. To date, we have unsuccessfully cloned the breakpoint in this cell line.

**DISCUSSION**

Since its original description 30 years ago, increased expression of Pgp has been frequently observed in cell culture models of multidrug resistance and in clinical samples obtained from patients with tumors that are refractory to chemotherapy. Although progress has been made, the regulation of Pgp expression is not fully understood, with most of the data examining expression controlled by the normal MDR-1 promoter (7). Is MDR-1/Pgp expression in drug-selected cells and refractory tumors under similar regulatory control as that in normal tissues or drug-sensitive cells? Our results suggest that the answer is no. We have previously reported that in drug-resistant cells, gene rearrangements lead to the capture of MDR-1 by an unrelated promoter that can control MDR-1 transcription. However, although we previously concluded that “gene rearrangements at or near Alu sequences” were probably involved in the transcriptional activation of MDR-1 (25), the evidence presented herein indicates that Alu repeats are not involved in the majority of such rearrangements. Furthermore, we report that the active promoter can control MDR-1 transcription proceeding in an antegrade or a retrograde direction and show that the distance from the capturing promoter to the start of MDR-1 transcription can be substantial even after the rearrangement, underscoring the cancer cell’s flexibility to achieve the desired phenotype.

Historically rearrangements in cancer cells, most notably those that are important in many leukemias and lymphomas, have predominantly involved similar partner genes or molecular rearrangements (26–29). In our models, we found different “capturing genes” with the exception of the MCFP gene that captured MDR-1 in two cell lines (EW VCR60 and MDA-MB-231 VBI00). We note that the latter is the closest gene to MDR-1 that is transcribed in the same direction as MDR-1, and the region surrounding its 5’ UTR has very high promoter activity. All of the other capturing genes were unique. We expected that similarities would be found in the molecular aspects of the rearrangements and hoped therein to find some guidance for averting the emergence of drug resistance. However, the data clearly indicate that at the molecular level, a similar diversity exists. This diversity occurs because the principal requirement for the “capturing” gene is a promoter that is sufficiently active to result in overexpression of MDR-1 a property shared by many genes including those commonly viewed as housekeeping genes. The diversity is also a result of the likely etiology: drug-induced chromosomal damage. Unlike rearrangements involving the immunoglobulins, such as are found in some lymphomas, the site of the chromosomal break would be expected to be random (30). Instead of the recurrent pattern of rearrangements that we expected based on our preliminary findings, we found a very diverse profile. These observations are similar in some respects to others reported in the literature in aneurismal bone cysts, a disease where USP6 (ubiquitin-specific protease 6), an oncogene with limited expression in normal cells is activated following rearrangements (31). In aneurismal bone cysts, the evidence has shown fusion of noncoding promoter regions of highly expressed genes to the USP6 oncogene found on chromosome 17p13. The noncoding partners (TRAP150, ZNF9, OMD, CDH11, and COL1A1) that “capture” USP6 have been found on chromosomes 1, 3, 9, 16, and 17, respectively. As in our MDR-1 studies, the 17p13 chromosomal translocations fuse a noncoding promoter region of a highly expressed gene to its USP6 partner, resulting in increased USP6 transcription without altering the USP6 protein structure. The results of the rearrangements were structurally similar fusion transcripts as described herein for the hybrid MDR-1 transcripts (31). Although the breakpoints for the aneurismal bone cyst rearrangements have not been cloned, we predict that they would reveal a very diverse molecular pattern. Our results are also similar to the activation of the proto-oncogene BCL6, a transcriptional repressor gene located on chromosome 3p27 that has been reported in diffuse large B-cell lymphomas (32). In a study of 98 patients with diffuse large B-cell lymphomas, BCL6 gene rearrangements were detected in 14 patients, with the predominant translocation partner gene being the IgH genes. However, in five patients, either the promoter and the first exon or both the first and second exon of other partner genes (pim-1, CIITA, TFRR, elfAII, and ikaros located on chromosomes 6, 16, 3, 3, and 7, respectively) replaced the endogenous promoter and first exon of BCL6, leading to expression of a partner/BCL6 fusion transcript. Although similar in this respect, we note that unlike the BCL6 gene rearrangements, where the breakpoints involving the five capturing genes clustered within a 2.2-kb region in the first intron of BCL6 (32), our observations demonstrate a more diverse pattern, again probably a result of the randomness of drug-induced chromosomal damage. This diversity also underscores the flexibility cancer cells possess to achieve a needed goal, in this case a drug-resistant phenotype.

It is interesting to note that, with the exception of S48k-ADR cells, the promoter region of the capturing genes that control MDR-1 expression in the resistant cells are 29–110 kb 5’ to the MDR-1 transcription start site. This ability of a promoter to regulate expression from a long distance has some precedence although not as an acquired property as described herein. One example where a normal tissue-specific promoter can regulate gene expression 350 kb 5’ to the start of translation has been described for the thyroid hormone receptor β gene (THRB) (33). Additional genes whose tissue-specific expression can be regulated by promoter sequences located long distances 5’ to start of translation include CYP19 (P450 aromatase; 79 kb 5’) (34), HNF-4a (hepatic nuclear factor 4a; 46 kb 5’) (35), and DMD (dystrophin; >90 kb 5’) (36). To this list we would add MDR-1 as a gene whose expression can be regulated long distance by a non-MDR-1 gene promoter. However, unlike all other previous reports, this long distance control occurs only after drug selection and is not an endogenous property of MDR-1. Whether the final mRNA product is a result of splicing remains to be determined, but it would represent an enormous waste, unless the nucleosome structure allows for some “jumping” as transcription is occurring.

DNA is under constant attack from endogenous and exogenous toxins, and cells have developed multiple mechanisms to ensure DNA integrity. Each DNA repair mechanism corrects a
different subset of lesions. DNA damage resulting in double strand breaks (DSBs) is particularly genotoxic, because both strands are damaged, precluding the use of the opposite strand as a template for the repair process. There is extensive evidence that DSBs are responsible for chromosomal instability and rearrangements (37). Among chemotherapeutic agents in common use that are Pgp/MDR-1 substrates, Adriamycin and etoposide are known to induce DSBs (38–40). Furthermore, it is likely that actinomycin-D and at least paclitaxel among the microtubule active agents (and possibly vincristine and vinblastine) can also cause DSBs (41–45). Thus, it is possible that the rearrangements reported herein occurred as a result of a drug-induced DNA break, a hypothesis that we feel is favored by the evidence of the rearrangement as an acquired phenotype. We do recognize that chemicals are not required to induce DSBs. They can also arise when replication of a chromosome that contains one break converts the single strand break to a DSB on one of the sister chromatids as the replication fork collapses. Indeed, DNA replication has an intrinsic probability of DSB formation that results in about 10 DSBs per cell cycle, and these DSBs can be efficiently repaired (46). Although most repaired DSBs, whether drug-induced or spontaneous, are inconsequential, a repaired DSB that brings an active promoter in close proximity to the MDR-1 gene is likely to have a selective advantage for that cell, if the population in which it is found is undergoing selection for drug resistance, so that instead of being induced by drug, the rearrangements characterized in the present study could have occurred randomly and been propagated as a result of the drug selection pressure. However, we would argue that the latter possibility is less likely given the evidence presented herein examining genomic DNA in parental cells that indicates the rearrangements were not preexisting but instead were acquired after drug exposure.

In human cells, DSBs such as the ones that occurred in our drug-resistant sublines can be repaired by either homology-directed (homologous recombination; HR) or nonhomologous (NHEJ) repair pathways (47–50). HR repairs DSBs using the undamaged sister chromatid as a template, removing damage in an error-free process. By contrast with HR, NHEJ requires minimal or no homology to couple the DNA ends generated in DSBs. Because NHEJ simply attaches ends together without a template, it is more error-prone and more likely to result in chromosomal damage. Our preliminary results had indicated that Alu-mediated homology-directed recombination might be important. However, this expectation proved incorrect, since with one exception, the repair that brought the non-MDR-1 gene in close proximity to MDR-1 did not involve homology. Homology was observed in paclitaxel-selected MCF-7 TX400 cells, and this involved an Alu repeat 84 kb upstream of the MDR-1 gene and an Alu repeat 26 kb’ to the non-MDR-1 (CASP) gene. A 79-bp intra-Alu deletion flanked by χ-like sequences and a 26-bp core Alu consensus sequence was found at the rearrangement junction (25). This was the only example where homology was identified and was confined to the Alu repeat. By comparison, NHEJ occurs between sites with microhomologies and may require the presence of cleavage-associated motifs, such as χ-like sequences, translin binding sites, alternating purine-pyrimidine tracts, curved or bent DNA, and recognition sites for topoisomerase I and II (51). The results presented herein favor NHEJ as the repair mechanism, and interestingly, alternating purine-pyrimidine AT tracts were found on either side of the breakpoints in the drug-resistant cell lines characterized in this study (data not shown). Although, as shown in Fig. 5, Alu or other repeat sequences (LINE-1, MIR, MaLR) were detected near the breakpoint sequences, this is not surprising, since the 112 kb of genomic sequence 5’ of MDR-1 has a high density of Alu and other retrotransposable elements (51). We note that a previous report of the mouse MDR-1 homolog, mdr1a, described a gene rearrangement between mdr1b and mdr1a in mouse multidrug-resistant J7.T1 cells selected with taxol. The rearrangement resulted in joining most of the mdr1b gene 5’ to the intact mdr1a promoter, and the breakpoint occurred within a LINE-1 repetitive element in intron 4 of mdr1b (52). We also note that NHEJ has been reported to be the dominant repair mechanism in most translocations found in pediatric leukemias where microhomologies and no specific motifs have been identified at the breakpoints (53). Our results provide further support for the thesis that although the relative contribution of HR and NHEJ varies, mammalian cells often prefer NHEJ over HR (54, 55). Indeed, the preference for NHEJ by a cancer cell is not surprising, since HR is most efficient when it acts in coordination with the S and G2 checkpoint controls, and these are often defective in malignant cells (56), whereas the NHEJ pathway is preferentially used during the G1 to early S phase (55). Although we favor NHEJ as the mechanism involved in the repair of the breaks, we cannot exclude other mechanisms of nonhomologous end joining as has been described with unbalanced translocations and amplification in mice deficient for a NHEJ DNA repair protein and the tumor suppressor, p53 (57, 58).

We conclude that rearrangements that lead to capture of MDR-1 by an active, but unrelated promoter can lead to acquired drug resistance. The rearrangements arise principally as a result of nonhomologous recombination and occur following exposure to a variety of agents, including some that are not thought to induce significant DNA damage. By identifying the breakpoints, we have been able to more reliably examine parental cell DNA and conclude that the rearrangements are acquired and probably drug-induced. Our ultimate goal has been to understand the molecular events leading to acquired drug resistance with the hope of averting its development. Because the data presented herein using genomic DNA suggest that the chromosomal breaks and in turn the rearrangements may be caused by the drugs to which the cells are exposed, we would suggest that their occurrence may be lessened by administering drugs as a continuous infusion (43). Furthermore, although we report these rearrangements exclusively in cells expressing MDR-1, this paradigm can apply to other mechanisms of drug tolerance that involve overexpression of a resistance gene. The challenges that remain are to determine the frequency with which this occurs in clinical samples and attempt to develop strategies to prevent its occurrence.

Note Added in Proof—HSCARG has been renamed NMRAL-1 (NmrA-like family domain-containing 1 gene; Gene ID: 57407) since the original submission of the manuscript.
