Aberrant Transcription from an Unrelated Promoter Can Result in MDR-1 Expression following Drug Selection In vitro and in Relapsed Lymphoma Samples

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Abstract

The development of drug resistance in the treatment of cancer remains a major problem. The hallmark of multidrug resistance is cross-resistance to multiple structurally unrelated compounds. The MDR-1 gene encoding P-glycoprotein mediates one of the most extensively studied mechanisms of drug resistance. Previous studies led to the proposal that two promoters control expression of the MDR-1 gene, and these were designated the upstream and downstream promoters. In the present article, we provide evidence that transcripts originating from the putative upstream promoter of MDR-1 are in fact aberrant transcripts whose expression is regulated by nearby genomic sequences that include a human endogenous retroviral long terminal repeat (LTR). Expression of this LTR occurs in all cells. We show that following drug selection, especially in cases where gene amplification has occurred, MDR-1 transcripts can begin near this retroviral LTR with transcription proceeding in the direction opposite of the usual LTR transcription. Because expression of these aberrant MDR-1 transcripts (AMT) is found primarily in drug-resistant cell lines, we conclude that the development of drug resistance or the attendant drug exposure might have a role in the activation of this phenomenon or the selection of cells expressing AMTs. Demonstration of similar aberrant transcripts in tumor samples obtained from patients with relapsed lymphoma suggests that this phenomenon may also occur clinically. (Cancer Res 2005; 65(24): 11694-703)

Introduction

The development of drug resistance in the treatment of cancer continues to challenge the oncologist in the clinic. The hallmark of multidrug resistance is cross-resistance to multiple structurally unrelated compounds, such as the anthracyclines, taxanes, Vinca alkaloids, and epipodophillosoxins. The MDR-1 gene mediates one of the most extensively studied mechanisms of drug resistance. The protein product of the MDR-1 gene, P-glycoprotein (Pgp), is a 170-kDa ATP-dependent efflux pump located in the plasma membrane. Expression of this pump decreases intracellular drug accumulation rendering tumor cells drug resistant.

The cloning of MDR-1/Pgp in 1985 was followed shortly thereafter by cloning of the promoter region (1, 2). A region encompassing 324 bp, located 570 bp proximal to the start of translation, was found to contain several consensus sites, including a Y-box, GC-rich sequences, heat shock elements, and transcription factor binding sites, such as Sp1, YB-1, and c-EBP (3–6). In addition, 5’ extensions identified several putative and two major start sites, a pattern reminiscent of many housekeeping genes (7). In one drug-selected cell line, however, transcripts arising more 5’ were identified, and this led to the proposal that the MDR-1 gene contained a second promoter (8). This observation led to the designation of upstream and downstream promoters and stimulated a limited search for the putative upstream promoter (9). Although these studies failed to identify the putative promoter, they concluded it must be located a minimum of 30 kb upstream of the downstream promoter (10). Since those initial studies, evidence was presented for transcripts originating from more upstream promoters (8, 11). In many of these cell lines, studies have shown that the upstream promoter is in fact the promoter of another randomly located gene, which following chromosomal rearrangement “captures” the MDR-1 gene (12, 13). However, in several samples, evidence that a common upstream promoter was active was also documented.4

In the present article, we provide evidence that transcripts arising from the putative upstream promoter of MDR-1 are in fact aberrant MDR-1 transcripts (AMT) whose transcription is regulated by nearby genomic sequences that includes a retroviral element characterized previously in Genbank as contiguous expressed sequence tags. We show that, following drug selection, expression of these AMTs can be detected in both drug-resistant cell lines and clinical samples obtained from patients with refractory lymphomas. The very infrequent occurrence of this in unslected cell lines and untreated patient tumors, relative to its occurrence following drug exposure and the development of drug resistance, suggests that drug exposure or the acquisition of the drug-resistant phenotype might have a role in the activation of this phenomenon.

Materials and Methods

Cell culture. All cell lines were cultured in either RPMI 1640 or IMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and 100 units/mL penicillin/streptomycin. Drug-selected sublines were derived from parental cell lines by exposure to either Adriamycin (A, AD, ADR), vinblastine (V, VB), vincristine (VCR), actinomycin D (ACT-D), paclitaxel (TX), or colchicine (C). MCF-7 TH was selected by continuous exposure to 1 μg/mL Adriamycin. The cell lines are summarized in Table 1, with appropriate references (12–16).

Northern blotting. RNA was isolated from the cell lines described above using RNA Stat (Iso-Tex Diagnostics, Friendswood, TX) and analyzed using the 1.5-kb 5A probe. Probe synthesis, gel electrophoresis, and hybridization conditions were carried out as reported previously (17).

Note: The order of the first two authors is to be considered arbitrary.

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4 Unpublished observations.
RNase protection assays. Solution hybridizations were done as described previously (17) using one of three constructs: (a) a 929-bp PstI genomic construct that contains the MDR-1 promoter (17), (b) a construct from a genomic region located 112,277 bp 5′ of the normal start site of MDR-1 transcription containing 245 bp of the 251 bp found in AMTs (AMT probe shown in Fig. 3), and (c) a 458-bp construct containing a hybrid cDNA sequence (AMT/MDR-1) composed of 209 bp of the 251 bp found in AMTs fused to MDR-1 sequences from 112,277 bp 5′ of the normal start site of transcription containing 245 bp of the 251 bp found in AMTs (AMT probe shown in Fig. 3).

The constructs for the AMT and the hybrid AMT/MDR-1 RNase probes described above and shown in Fig. 3 were generated by reverse transcription-PCR (RT-PCR) using KB C-1 RNA as template. The 245-bp AMT riboprobe was generated using the following primers: AMT 5′-149076CCCAAACTTATCCTTGGGTTTTCACTG149050 and AMT 3′-148832TATCTGGTTGCTTCGAGTGAGTAC148858. The 458-bp hybrid AMT/MDR-1 riboprobe was generated using the following primers: AMT 5′-B149037AAGCCTGCCTGCCTTAGTCATGT149014 and MDR-1 R36496AAGGAAACGAACAGCGGCCTCTG36518 (numbered as per Genbank accession no. AC002457). Each PCR product was then TA cloned into the dual-promoter vector, pCRII (Invitrogen, Carlsbad, CA), and sequenced to obtain proper orientation for riboprobe synthesis. Probe synthesis and hybridization conditions were done as described previously (18, 19).

5′ Rapid amplification of cDNA ends isolation of aberrant MDR-1 transcripts. The 5′ rapid amplification of cDNA ends (RACE) kit (Invitrogen) was used to isolate the AMTs using the MDR-1 gene-specific primers GSP1 and GSP2 as reported previously (12).

Reverse transcription-PCR. First-strand cDNA synthesis using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) was done on 1 μg total RNA isolated from cell lines. The resulting cDNA was used as template in the PCR. Cycling conditions for the RT-PCR reactions followed by gel electrophoresis were carried out as described previously (12).

All primer numbers listed in this section were numbered as per Genbank accession no. AC002457 (36551 is the start of MDR-1 transcription). Primers used for PCR detection of the AMT sequence were those used previously to synthesize the 245-bp riboprobe (see riboprobe synthesis above).

Primers used to detect by PCR a 433-bp AMT/MDR-1 hybrid product (Fig. 2 D) were AMT+67 5′-149013AGCTCCTCCTCTGGTACTGGGA148992 and MDR-1 R36496AAGGAAACGAACAGCGGCCTCTG36518. Primers used for detection of the retroviral sequences (RVS) shown in Fig. 4 were RVS-F149961CCACGGTGCCAACAATGCAC149980 and RVS-R150550TCAGGGAGGTTTCACATC150533. Primers used for detection of MDR-1 expression have been described previously (20). Primer used for detection of additional aberrant transcripts (AAT) shown in Fig. 3 were AMT+132 5′-149076 CCCAAACTTATCCTTGGGTTTTCACTG149050, 180 AAT 3′-38700AATCTGCACCTTCTTGTCCTCCGC38723, 180 AAT 5′-38723GCGGAGGACAAGAAGGTGCAGATT38700, and MDR-1 R36496AAGGAAACGAACAGCGGCCTCTG36518.

Table 1. Expression and amplification of AMT in drug-resistant cell lines

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<th>RNA</th>
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* mRNA from these cell lines shown to have common AMT sequence at their 5′ ends.
† Shown previously to have hybrid MDR-1 mRNAs due to gene rearrangements.
Southern blotting. Genomic DNA was isolated from cell lines using Wizard Genomic Purification kit (Promega, Madison, WI) and digested with HindIII (Invitrogen). Genomic DNA (10 μg) was loaded per lane on a 1% agarose gel. After membrane transfer, blots were hybridized with either the *MDR-1* 5A probe or a 245-bp AMT probe synthesized by PCR using AMT 5' and AMT 3' primers described above. Hybridization and wash conditions were done as described previously (12, 19).

Intervening sequence green fluorescent protein cloning and transfection. To examine the direction of transcriptional activity of the 744-bp intervening sequence (IVS) in the *MDR-1* overexpressing cell lines, PCR was used to isolate an 815-bp fragment containing the 745 bp 5' UTR of another gene are identified in Table 1. However, 5' sequences proximal to residue 194 in several other cell lines (KB C-1, KB V-1, KB A-1, KB C-1, and KB V-1). All parental cell lines express *MDR-1* expression of the 4.5-kb *MDR-1* band is seen in all drug-resistant sublines. RNA (10 μg) was loaded per lane. The probe used encompasses residues in the middle third of the *MDR-1* RNA. B, expression of *MDR-1* as detected by RNAse protection analysis. Genomic structure of the *MDR-1* gene and its 5' UTR is shown schematically. Vertical arrow at –4/4/1, normal *MDR-1* transcription start sites. Location of the 929-bp Par genomic probe used in the RNAse protection analysis is shown. The RNA fragments protected by this probe are indicated (324, 134, and 130 bp). Cell lines that begin transcription at the normal *MDR-1* start sites (+1 and –4) protect fragments of 150 and 134 bp, respectively (as well as some slightly larger fragments). Five cell lines (KB C-1, ZR-75B TX320, DLD-1 VB300, MCF-7 TX400, and EW-36 VCR60) show protection of a 324-bp fragment (and slightly smaller fragments), indicating a more proximal start site.

**Results**

Ten parental and 22 drug-resistant cell lines derived from them were used in these experiments. Cytotoxicity profiling showed that these resistant cell lines were cross-resistant to the drugs comprising the multidrug-resistant phenotype mediated by *MDR-1* (14–16). All drug-resistant cell lines were shown to have increased expression of *MDR-1*. Figure 1A is a Northern blot analysis showing higher levels of *MDR-1* expression in the drug-resistant cell lines than in the parental cells from which they were derived.

When expression was analyzed by RNAse protection using a probe containing 194 bp of sequence 5' to the normal start of transcription (+1; Fig. 1B), the results shown in Fig. 1C were obtained. RNA from the cell lines expressing *MDR-1* was able to protect fragments of 130 and 134 bp long, consistent with the existence of transcripts originating at the start sites of *MDR-1* transcription (2, 9). However, some cell lines also protected longer fragments, including a 324-bp fragment. As shown in Fig. 1B, the 324 bp include 130 bp transcribed from exon 1 and an additional 194 bp from the putative *MDR-1* promoter region (–1 to –194). These promoter sequences are usually not detected with the RNase protection assay having been found in only 1 (HCT-15) of 17 unselected cell lines studied (includes the 10 parental cell lines in this study; data not shown). 5' RACE was used to identify additional sequences proximal to residue –194 of the *MDR-1* promoter. We reported previously that in some cell lines longer transcripts representing unique hybrid mRNAs contained sequences from the 5’ untranslated region (UTR) of another gene (10, 12). (MCF-7 TX400 and EW-36 VCR60) shown in Fig. 1 are two such examples). These cell lines with hybrid mRNAs containing sequences from the 5’ UTR of another gene are identified in Table 1. However, 5' RACE identified similar 5' sequences proximal to residue –194 in several samples, indicating that transcripts in these cell lines and patient samples shared a similar start site. This included five of the drug-resistant cell lines (KB C-1, KB V-1, KB A-1, DLD-1 VB300, and
ZR-75B TX160 identified in Table 1) as well as several RNA samples from patients with relapsed drug-resistant lymphoma.

The sequence shared by these five drug-resistant cell lines included 94 bp identified previously in multidrug-resistant KB cells (2) plus an additional 157 bp (251 total bases, Genbank accession no. AY452673). In the drug-resistant lymphomas, the additional 157 bp or a portion of these 157 bp was found when the 5' RACE products were sequenced (the start of shorter transcripts in three lymphoma samples are identified in Fig. 2.1 by boxed letters). In Genbank, the 157-bp sequence was contiguous with the 94 bp identified previously in KB C-1 cells, 112,277 bp 5' of the normal MDR-1 transcription start site. Figure 2.1 presents the sequence of the 251-bp AMT and a schematic of the genomic organization (Note: The 251 bp proximal to MDR-1 generate an AMT.) The term AMT refers to the 251-bp sequences whether found in genomic DNA or in mRNA either alone or as part of a hybrid transcript with MDR-1 designated AMT/MDR-1. When expression of the AMT sequences was examined in the cell lines under study, the results shown in Fig. 2B were obtained (PCR primers are underlined in the Fig. 2A sequence and identified by arrows in the Fig. 2A schematic). AMT expression was undetectable in parental cells, with the exception of ZR-75B and EW-36 cells. However, expression could be detected at levels higher than that of parental cells in 9 of 18 (50%) resistant cell lines (SW620 VB300, DLD-1 AD1000, DLD-1 VB300, KB C-1, KB V-1, KB A-1, MCF-7 TX400, MCF-7 TH, and ZR-75B TX160; not shown but negative: SW620 AD20, SW620 AD1000, SW620 VB2, and DLD-1 AD100). Similar studies in 15 normal tissues failed to identify AMT expression. These samples included 5 tissues that normally express...
MDR-1 at significant levels (colon, kidney, liver, adrenal gland, and intestine) and 10 tissues in which expression is not normally detected or is detected at very low levels (brain, spinal cord, lung, heart, spleen, bladder, s.c. tissue, breast, bladder, and skeletal muscle; normal tissue PCR not shown). These results suggest expression of AMT sequences alone or as an AMT/MDR-1 hybrid does not normally occur. Because the AMT sequences amplified in this assay are contiguous in genomic DNA, it should be noted that in all cases no PCR product was obtained from RNA samples when reverse transcription was omitted. However, a RT-PCR product was obtained with DNase-treated RNA samples.

In lymphoma samples identified by 5′ RACE as having AMT sequences contiguous with MDR-1, expression of AMT/MDR-1 RNA occurs without gene amplification (data not shown). We sought to determine if, in cell lines, expression of the AMT/MDR-1 correlated with amplification of this region. When DNA from the drug-resistant cell lines was examined (Fig. 2C), amplification of the region encompassing the AMT sequences was identified in the nine resistant cell lines expressing AMT sequences at a level higher than parental cells. High levels of amplification were seen in six cell lines (KB A-1, KB C-1, KB V-1, DLD-1 AD1000, MCF-7 TH, EW-36 VCB60, and SW620 VB300; latter not shown). Thus, the AMT region is coamplified with MDR-1 in these cell lines. Together, these results suggest that AMT transcription occurs preferentially after drug selection and is associated with amplification of the AMT sequences in drug-resistant cell lines. We also sought to determine if, in these resistant cell lines, AMT sequences were fused proximal to MDR-1. For these experiments, RT-PCR was done with a 3′ primer complementary to MDR-1 exon 1 and (MDR-1 R) and a 5′ primer complementary to residues in the 5′ region of the AMT sequence (AMT 5′). As shown in Fig. 2D, when the 11 drug-resistant cell lines expressing the AMT sequence were examined (SW620 VB300 and MCF-7 TH not shown), a 433-bp product composed of the AMT sequence fused to MDR-1 (AMT/MDR-1) was found in only 5 of the 11 cell lines (KB C-1, KB A-1, KB V-1, DLD-1 VB300, and ZR-75B TX160). Thus, although the AMT sequence is expressed in all cell lines in which it is amplified, in only a subset are these residues part of MDR-1 transcripts. We would note parenthetically that transcripts starting at the AMT do indeed read through into the coding sequence as shown by a PCR product using as 5′ primer sequences in the AMT and as 3′ primer sequences in exon 3 (data not shown).

Additional investigations were done to further characterize the acquired transcripts in the drug-resistant cell lines and to reconcile some discrepancies noted with the probes used in the RNase protection analyses. We detected in the lymphoma samples hybrid AMT/MDR-1 transcripts originating at different sites within the 251-bp AMT sequence. We also noted less than full-length protection of the probes in the RNase protection analyses, suggesting the existence of either shorter transcripts or alternate sites of splicing. These studies uncovered that hybrid transcripts in the drug-resistant cell lines were diverse as summarized in part in the Fig. 3 A schematic. As shown in Fig. 3B, close inspection of the products obtained when RT-PCR was done with a 5′ primer complementary to the AMT and a 3′ primer complementary to MDR-1 exon 1 (also used in Fig. 2D) revealed additional bands (identified in Fig. 3B gel by thin arrows). When RT-PCR products were cloned and individual colonies were isolated and sequenced, a variety of transcripts were identified. A Genbank search revealed additional sequences ~1.8 kb (180 bp) and 4.3 kb (130 bp) 5′ of the MDR-1 promoter (Fig. 3B). These additional hybrid transcripts (depicted in the Fig. 3B schematic and starting in the AMT) were present in the drug-resistant cell lines as shown by the additional PCR reactions in Fig. 3B. PCR products were obtained with a 5′ primer complementary to the AMT and a 3′ primer complementary to the 180-bp fragment as well as with a 5′ primer complementary to the 180-bp fragment and a 3′ primer complementary to MDR-1 exon 1. Similar results confirmed the existence of the other hybrid transcripts in the Fig. 3B (data not shown for all products). Finally, RNase protection experiments conducted with a probe either complementary to the 251-bp sequence (AMT; left gel) or complementary to a hybrid sequence composed of the 251 bp at the 5′ end and exon 1 sequences at the 3′ end (AMT/MDR-1; middle gel) gave the results shown in Fig. 3C. The differing size fragments protected in the experiments indicated that although a start site at the most 5′ end of the AMT sequence was the most common start in KB V-1 cells (left gel) multiple start sites exist along the AMT sequence and protect shorter fragments in the KB V-1 and KB C-1 cells. In addition, the different size fragments protected with the AMT/MDR-1 probe (middle gel) were consistent with the existence of multiple start sites as well as splicing into exon 1 principally at residue −194 but also at sites closer to the normal start of MDR-1 transcription (+1). Alternate splicing into exon 1 at sites other than −194 is likely responsible for the protection of fragments less than 324 bp but longer than 134 bp in the RNase protection shown in Fig. 1 and in the right gel of Fig. 3C, both of which used the same 929-bp Ps tI genomic construct that contains the first 134 bp of MDR-1 and the 194 bp of sequence 5′ to the normal start of transcription (+1). With transcripts in the drug-resistant cell lines originating at an aberrant promoter (see below), it was not surprising to find different transcript lengths and variable splicing.

Genbank accession no. AC002457 revealed that a human endogenous retroviral LTR (HERV LTR) is contiguous with the AMT sequence in genomic DNA. When expression of this RVS was examined by RT-PCR, a product was obtained only when the primer closest to the AMT sequence was used as the 5′ primer, indicating that this RVS is transcribed retrograde to the AMT sequence. These observations are depicted schematically in Fig. 4A. In addition, HERV LTR expression was shown by PCR in 15 cell lines and normal tissues, suggesting that this LTR is widely expressed as shown in Fig. 4B. Furthermore, a 605-bp probe containing the HERV LTR detected a 1.5-kb message on Northern analysis, indicating that additional sequences are cotranscribed (data not shown).

We thus had a situation where 251 bp (AMT) not normally transcribed were transcribed after drug selection, and these 251 bp were separated from a RVS (HERV LTR) by 745 bp of genomic sequence (designated the IVS; see below). Several putative promoter elements, including a CCAAT box and consensus sequences for the binding of transcription factors (c-Myb, GATA-1, TFID, C/EBP, and SP1), were identified in the 745-bp IVS. We considered the possibility that together with a portion of the HERV LTR the intervening 745-bp IVS could function as a promoter for the AMT sequence in a retrograde direction to the transcription of HERV LTR. To examine this, the promoter activity of genomic fragments 5′ to the AMT sequence was determined as summarized in Fig. 4C. Several fragments containing RVS (fragments E, F, I, and J) had high levels of promoter activity, as did an 815-bp fragment containing the 745-bp IVS and 71 bp of contiguous RVS (fragment IVS). Additional studies with the IVS cloned into a luciferase reporter vector, pGL3-Base (pGL3-B), both in an orientation that would readily detectable, indicating that this 815-bp IVS could function as a...
promoter. Ten cell lines were examined, including (a) cells that do not normally express MDR-1 or express MDR-1 at very low levels (MCF-7, T47D, ZR-75B, and KB 3-1), (b) cells with endogenous expression of MDR-1 (HCT-15 and DLD-1), (c) a multidrug-resistant cell line overexpressing MDR-1 in which neither expression of the AMT sequence nor AMT transcripts could be identified (DLD-1 VB30), and (d) three multidrug-resistant cell lines with high levels of expression of the AMT sequence and high levels of expression of AMTs containing the AMT sequence proximal to MDR-1 (KB A-1, KB C-1, and KB V-1). The results in the figure are depicted as the relative ratio of luciferase expression. This ratio was obtained by dividing the RLU of the pGL3-BRVS construct with the 815 bp in the direction of the RVS (as a promoter this would transcribe retrograde to the AMT sequence and MDR-1) by the RLU obtained with the pGL3-BMDR-1 construct with the 815 bp in the direction of the AMT sequences and MDR-1. An expression ratio of >1 indicates greater luciferase activity for the pGL3-B RVS construct that initiates transcription in the direction of the RVS retrograde to AMT sequences and MDR-1. As shown in Fig. 4D, the expression ratio was >1.0 in 7 of the 10 cell lines, indicating preferential transcription in the direction of the RVS retrograde to AMT sequences and MDR-1. In contrast, in KB A-1, KB C-1, and KB V-1 cells, in which high levels of expression of AMT sequences were detected along with high levels of AMT/MDR-1 transcripts, the expression ratio was <1.0, indicating preferential
expression of the construct with luciferase driven by the 815 bp oriented in the direction of the AMT sequences and MDR-1. These results indicate that, following drug selection, aberrant transcription of MDR-1 can originate at the AMT sequences, as a putative promoter initiates transcription.

The possibility that a promoter >112 kb 5’ to the normal MDR-1 start site could regulate expression of the MDR-1 gene caused us to think that perhaps a deletion in this region could have brought the putative 815-bp IVS promoter and the AMT closer to MDR-1. There are 42 Alu sequences in these 112 kb, providing possible sites for gene rearrangements and deletions to occur (ref. 22; Genbank accession no. AC002457). To examine this possibility, we used three probes without Alu sequences designated P-1, P-2, and P-4 that are located 15, 47.5, and 91 kb 5’ to MDR-1 exon 1, respectively, and the MDR-1 probe used in Fig. 4 to examine DNA. Probes P-1, P-2, and P-4 detected DNA amplification in KB C-1, KB V-1, and KB A-1 comparable with that detected with the MDR-1 probe. This indicates a large portion of the 112 kb was included in each ampiclon, reducing the likelihood a substantial deletion occurred during drug selection (data not shown).

Finally, because the 5’ RACE indicated that AMT/MDR-1 hybrid transcripts occur in clinical samples, we used the primers in Fig. 2D to examine RNAs isolated from drug-resistant lymphomas. The patients from whom these samples were obtained had received multiple cycles of chemotherapy with the Pgp substrates, doxorubicin (Adriamycin), and vincristine, and a majority had received etoposide (VP16). As shown in Fig. 5B, expression of hybrid AMT/MDR-1 transcripts was found in a subset of the drug-resistant lymphoma samples (B and G-K). Similar studies using lymphoma samples obtained before any therapy (Fig. 5C) and also circulating lymphocytes from nine individuals without cancer (data not shown) did not find these hybrid sequences.

Discussion

Although there is extensive evidence that increased expression of MDR-1 occurs in both hematologic and solid tumor malignancies,
the mechanism(s) whereby this occurs is less well understood (23–26). Numerous studies have identified likely transcription factors that mediate the expression of MDR-1 under normal circumstances, but in the majority of cases the role of these factors in acquired overexpression of MDR-1 is less clear (27–31). Instead, it seems more likely that these transcription factors, which mediate the expression of MDR-1 under normal circumstances, have a limited or no role in acquired overexpression. Indeed, in the drug-resistant cells, constructs containing the putative “normal” MDR-1 promoter are usually expressed at levels comparable with that in parental cells, although MDR-1 levels are several hundred-fold to several thousand-fold higher (27, 31). Although drug-resistant reversal strategies have employed antagonists of drug transport, a greater understanding of how overexpression occurs may eventually lead to strategies that prevent or delay the emergence of drug resistance. In the present study, we provide evidence that, in some drug-selected cell lines, aberrant transcription from an aberrant start site with promoter activity can generate MDR-1 transcripts. The evidence indicates this aberrant transcription occurs from a putative promoter located 112 kb upstream from the normal start site of MDR-1, adjacent to an inserted RVS. Transcription from this aberrant site occurs primarily in drug-selected cell lines, and in a fraction of these, a hybrid MDR-1 transcript is generated (summarized in Table 1). The latter has an intact start of translation and should generate a normal protein.

In previous studies, we showed that acquired overexpression of MDR-1 can occur as a result of gene rearrangements leading to capture of the MDR-1 promoter by an unrelated active promoter (12). In numerous cell lines derived from parental cells with low to absent expression of MDR-1, unique gene rearrangements generated unique hybrid transcripts, all of which in turn were translated into functional proteins (Pgp). Although the current results show a similar capture of MDR-1 by an aberrant promoter, several differences indicate activation of MDR-1 occurs by a different mechanism. Unlike our previous observations, gene rearrangements did not occur possibly because of the close proximity of the "capturing promoter" to the normal start of MDR-1 transcription. Furthermore, the hybrid transcripts were not unique but rather were similar, differing only slightly in their 5' extension. Unlike our previous observations, the "capturing promoter" is not that of a normally transcribed gene but seems aberrantly activated with drug selection. The data suggest a transcriptional regulatory role for nearby sequences encoding several putative promoter elements (the IVS sequence) as well as an adjacent RVS (HERV LTR; see below). These sequences are contiguous to the 251 bp we have designated the AMT sequence that becomes fused 5' of the MDR-1 RNA.

The data indicate that a portion of the RVS is needed for promoter activity; however, the retroviral LTR does not contribute sequences to the aberrant transcripts, although it may contribute sequences where RNA polymerases and transcription factors bind and activate transcription. In humans, ~8% of the genome is derived from sequences with similarity to infectious retroviruses. HERVs are the remnants of ancestral retroviral infections that have integrated into germ-line DNA. Retroviral proviruses are encoded by three genes designated gag, pol, and env, with flanking 5' and 3' LTR. These flanking repeats contain promoter, enhancer, hormone-responsive elements, and polyadenylation signals responsible for viral protein expression. HERVs are usually inactive as infectious retroviruses. Frequently, the entire viral core is lost by homologous recombination leaving behind a solitary LTR. A recombination event between the provirus 5' and 3' LTR yields the solitary LTRs commonly found in the human genome. Enhancer and promoter elements within solitary retroviral LTRs retain their transcriptional properties and regulate expression of neighboring genes. In addition, activity of HERV LTRs can also be modulated by methylation or polymorphisms that occur within the RVS (32, 33). We would note that our observations are reminiscent of previous studies in murine cell lines that reported activation of MDR-1 by randomly inserted MMLV LTR sequences (22, 34). These observations can also be considered in the context of attempts at retroviral gene therapy where MDR-1 expression was driven by a viral LTR. These studies showed changes in the biology of the

**Figure 5.** A, genomic organization and putative mRNA of MDR-1. Horizontal arrows, PCR primers (described in Materials and Methods). These primers were designed to detect hybrid AMT/MDR-1 transcripts and MDR-1 expression in the RNAs under study. B, RT-PCR results obtained when RNA from drug-resistant lymphoma samples were examined. MDR-1 expression was detected in 8 of 12 resistant lymphoma samples (B, C, and G-L). In 6 of 8 patient RNAs, hybrid AMT/MDR-1 products were also obtained, showing expression of these hybrid transcripts (B and G-K). C, RT-PCR results obtained from RNA isolated from 14 untreated lymphoma samples using MDR-1-specific primers and hybrid AMT/MDR-1 primers used in (B). In 13 of 14 untreated tumors, detectable levels of MDR-1 were observed, although no hybrid AMT/MDR-1 transcripts were detected. Controls used are unselected SW620 cells that express MDR-1 and drug-selected KB 8-5 and KB C-1 cells that express MDR-1 and hybrid AMT/MDR-1 transcripts.

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transduced hematopoietic cells manifested as altered engraftment potential and proliferation. Therefore, LTR-driven transcription could be relevant to areas of cell biology in addition to drug resistance (35).

In this present study, the AMT sequence fused to the 5' end of the MDR-1 RNA is located 112 kb from the normal start of transcription. Although this represents a greater distance than usually exists between promoter and coding regions, there are precedents in the literature, including cases where RVS affects expression. In one report, the chimeric transcript (Genbank accession no. AF041208) included an HERV-E LTR sequence at its 5' end contiguous with sequences of exon 2 of the Mid1 gene. This 4-kb LTR located 16 kb upstream functions as an alternative tissue-specific promoter for the Mid1 gene splicing into exon 2 containing the Mid1 ATG start site. Likewise, a 5' variant transcript of the human endothelin B receptor gene initiates in a HERV-E LTR located 57.6 kb upstream of the endothelin B gene and splices into the first coding exon (36–38). Finally, precedent can be found in the CYP19 gene encoding aromatase P450, the key enzyme in estrogen biosynthesis. CYP19 is expressed primarily in the gonads and brain of most mammals and is also expressed at high levels in placenta in primates. Placental transcription of CYP19 is driven by an alternative promoter located ~100 kb upstream of the coding region and this promoter is an endogenous LTR (39). We now add acquired MDR-1 transcription to this growing list of genes whose transcription can be affected by RVS. In the present example, aberrant transcription is acquired during drug selection and provides a survival advantage.

Soon after the MDR-1 gene was cloned, the putative promoter was identified (2, 8). Longer transcripts isolated from KB C-1 cells led to the conclusion that a second promoter, designated the upstream promoter, existed for MDR-1. In the present study, we have characterized the full length of these longer transcripts and localized them to a site 112 kb 5' of the normal start site for MDR-1 transcription, where an aberrant promoter recruited in drug-resistant cells directs transcription. As with other aberrant promoters, this promoter generates transcripts differing in start sites, length, and composition (38). Whether drug selection induces the expression of transcription factors that bind to and recruit this aberrant promoter or whether changes in chromatin structure expose an otherwise hidden promoter leading to its activation cannot be ascertained from the present data. Differential mRNA display experiments have shown activation of endogenous reverse transcriptases as well as increased SINE transcription following exposure to genotoxic agents, such as cisplatin, etoposide, γ-radiation, and UV radiation (40, 41). Perhaps in our cancer cell lines, drug exposure could alter the status of the genomic sequences (including the neighboring HERV LTR) 5' to the AMT and activate this AMT/MDR-1 transcript. The demonstration of hybrid MDR-1 transcripts in drug-resistant lymphomas suggests that a similar activation may occur in clinical samples. Although the lymphoma samples were not "paired" samples obtained from the same patient before and after therapy, these results suggest that expression of MDR-1 in these drug-resistant lymphomas was acquired. Further studies are needed to characterize the prevalence of these hybrid transcripts in drug-resistant tumors and to determine the extent of its correlation with drug resistance.

Finally, we would note that data in Genbank indicate that 27 kb 5' of MDR-1, an unrelated gene stretching more than 107 kb, transcribes retrograde to MDR-1. Designated RPIP9 for Rap2-interacting protein 9, its function is unknown but its high homology to RPIP8 suggests a role in the inhibition of the RAS signaling pathway (42). It is composed of nine exons, from which four different transcripts are generated. The 251-bp sequence that constitutes the AMT is found between exons 4 and 5 of RPIP9.

In conclusion, we have shown that the putative upstream transcripts of MDR-1 are likely regulated by nearby genomic sequences possibly including a retroviral element. We have shown expression of hybrid transcripts starting at this aberrant promoter primarily in drug-resistant cell lines and have found these also in tumor samples from patients with relapsed lymphomas. The clinical importance of this observation and how drug exposure might facilitate the activation of this phenomenon remains to be determined.

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