the intact R body–coding sequence. The results (Figs. 1B and 2) confirm that the R body–coding region of pKAP169-1 is split by the 7.5-kb transposon-like sequence.

The 7.5-kb inserted sequence found in pKAP169-1 is similar to transposon-like sequences observed in the R body–coding plasmids pKAP47 and pKAP298 with respect to both size and distribution of restriction sites (*8*). To determine whether these sequences are related, Southern blots containing restriction endonuclease–digested pKAP169 and pKAP169-1 were hybridized to a radiolabeled DNA probe that included about two-thirds of a 7.5-kb element from pKAP47 (Fig. 1A). The results (Figs. 1B and 3) demonstrate that these sequences share a high degree of homology and strongly support the hypothesis that they are transposons. We previously reported that, in several strains of *C. taeniospiralis*, sequences having homology with the 7.5-kb transposon-like elements occur in other replicons within the endosymbiont genome, most likely in the chromosome (*8*). Thus pKAP169-1 probably resulted from transposition of a 7.5-kb transposon-like element residing in the *C. taeniospiralis* 169 chromosome into the R body–coding sequence of pKAP169.

We realize that the results presented here do not absolutely preclude the possibility that *C. taeniospiralis* 169-1 may be the result of two separate mutational events, one inactivating the R body–coding sequence and the other inactivating the toxin-coding sequence, the nature and location of which are unknown. However, the probability of both events occurring in the same cell is extremely low. In addition, our results are consistent with previous reports that killing activity is affected by the rate of R body production in *Caedibacter* (*3, 5*).

It is our conclusion that, although the genetic loci for R-body and toxin synthesis are probably separate, synthesis of intact R bodies is required for the toxin component to be active against sensitive paramecia. The evidence also suggests that (i) synthesis of intact R bodies is not required for resistance of the ciliate host to killing mediated by bright forms of *C. taeniospiralis* and (ii) the killer trait is an example of cytoplasmic inheritance with respect to both the eukaryotic host and the bacterial endosymbiont.

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Human Multidrug-Resistant Cell Lines: Increased *mdr*1 Expression Can Precede Gene Amplification

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The development of simultaneous resistance to multiple structurally unrelated drugs is a major impediment to cancer chemotherapy. Multidrug resistance in human KB carcinoma cells selected in colchicine, vinblastine, or Adriamycin is associated with amplification of specific DNA sequences (the multidrug resistance locus, *mdr*1). During colchicine selection resistance is initially accompanied by elevated expression of a 4.5-kilobase *mdr*1 messenger RNA (mRNA) without amplification of the corresponding genomic sequences. During selection for increased levels of resistance, expression of this mRNA is increased simultaneously with amplification of *mdr*1 DNA. Increased expression and amplification of *mdr*1 sequences were also found in multidrug-resistant sublines of human leukemia and ovarian carcinoma cells. These results suggest that increased expression of *mdr*1 mRNA is a common mechanism for multidrug resistance in human cells. Activation of the *mdr*1 gene by mutations or epigenetic changes may precede its amplification during the development of resistance.

SIMULTANEOUS RESISTANCE TO MULtiple cytotoxic drugs occurs frequently in tissue culture cells selected for resistance to a single agent and has been suggested as a mechanism of resistance to chemotherapy in human tumors. Multidrug-resistant cell lines have been isolated in tissue culture by multistep selection. Early steps occur with very low frequency in most cell lines unless the cells are treated with a mutagen; later steps occur more readily and cells can be rapidly adapted to relatively high levels of the selecting drug (*1*). Recently, we found that two DNA sequences, referred to as *mdr*1 and *mdr*2, are amplified in multidrug-resistant human KB carcinoma cell lines (*2*). These sequences were detected and cloned by hybridization with a homologous probe derived from the *mdr* gene, which is amplified in multidrug-resistant Chinese hamster cell lines (*3*). A human genomic clone, corresponding to one of the amplified *mdr* sequences and designated pMDR1, hybridizes to a messenger RNA (mRNA) of 4.5 kb that is expressed in the multidrug-resistant cell lines (*2*). We now examine expression of the *mdr*1 mRNA during the development of multidrug resistance in KB carcinoma cells, as well as in two other human multidrug-resistant cell lines of different origin. Since even low levels of cellular multidrug resistance may result in clinically refractory tumors, we were especially interested in analyzing *mdr*1 expression in the sublines with low (two- to sixfold) relative drug resistance.

The isolation and some properties of the human multidrug-resistant KB carcinoma cell lines have been described (*4, 5*). The KB cell lines used in this study, the manner of their selection, and their relative resistance to various drugs are shown in Table 1. The agents used in selecting different sublines in multiple steps were colchicine, Adriamycin, and vinblastine. In the first two steps of colchicine selection, clones could only be obtained if the cell populations were first mutagenized with ethyl methane sulfonate (EMS). Similarly, KB cell lines selected independently for resistance to Adriamycin or vinblastine (*4, 5*) were obtained only after mutagenesis with EMS in the first step. Subsequent selection, up to very high levels of resistance, was possible without mutagenesis, and occurred at high frequency (KB-A1, KB-V1). Also shown in Table 1 are the

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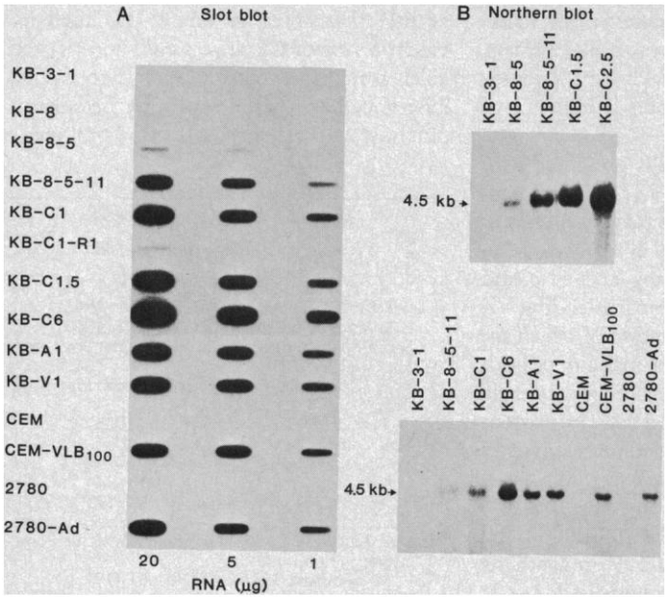

Fig. 1. Expression of *mdr*1 sequences in human multidrug-resistant cell lines. (A) Slot blot of total RNA from various human cell lines blotted on nitrocellulose and hybridized with the *mdr*1-specific probe (*2*). (B) Northern hybridization of poly(A)$^+$-selected RNA (upper part) and total RNA (lower part) from various human multidrug-resistant cell lines. RNA prepared as described (*2*) was applied to filters using a Schleicher & Schuell slot blot apparatus (A) or by blotting after electrophoresis in 1 percent agarose containing 13.4 percent formaldehyde. The probe used was the gel-purified insert from a pBR322 subclone containing a 0.8 kb Pvu II fragment of the human *mdr*1 locus (*2*). Nitrocellulose filters were baked and preincubated for 4 to 6 hours at 42°C in 50 percent formamide, 5× SSC (standard saline citrate), 10× Denhardt's solution, 0.1 percent sodium dodecyl sulfate (SDS) and salmon sperm DNA at 100 μg/ml. Filters were hybridized overnight in the above solution containing ^{32}P-labeled probe. Filters were washed three times for 10 minutes at room temperature in 2× SSC, 0.1 percent SDS, and three times for 20 minutes at 50°C in 0.1× SSC, 0.1 percent SDS.

relative degrees of drug resistance of the human leukemic lymphoblast and ovarian carcinoma cell lines used in our study.

To determine the extent to which *mdr*1 sequences were expressed in these cell lines and the size of the corresponding RNA's, we performed Northern hybridization with total RNA and polyadenylated [poly(A)$^+$] RNA from these cells (Fig. 1B). A 4.5-kb RNA, which migrates just below the 28*S* ribosomal RNA marker, is visible in all the lanes containing either total or poly(A)$^+$ RNA from the resistant lines but is not seen, even after prolonged exposure, in any of the sensitive cell lines. There is a good parallel between extent of multidrug resistance and levels of *mdr*1 mRNA (Table 1). In addition to the 4.5-kb RNA, a 10.5-kb RNA band was detected in other experiments. This band may correspond to a nuclear precursor of the 4.5-kb RNA.

Slot blot hybridization of total RNA was used to quantitate the expression of *mdr*1 in various sensitive and resistant cell lines (Fig. 1A). Little or no expression of the *mdr*1 sequences is seen in the parental, drug-sensitive cell lines, but increasing expression occurs as the cell lines become more resistant to the drugs. A revertant cell line, KB-C1-R1, subcloned in the absence of colchicine from the colchicine-resistant cell line KB-C1, still expresses *mdr*1 sequences at reduced levels, consistent with its low multidrug resistance. It is not possible to calculate the exact extent of increased expression in the resistant cell lines relative to the parental line since the hybridization signal from the parental RNA is too weak. We have calculated the extent of expression relative to the KB-8 cell line (Table 1). Expression appears to correlate well with increasing drug resistance for every step of selection in KB cells and reaches very high levels in our most resistant KB cell lines.

Two other human cell lines of different origin, selected for multidrug resistance, also express high levels of the 4.5-kb mRNA (Fig. 1 and Table 1). Little or no expression of this RNA was detected in the parental cell lines. The human leukemic lymphoblast cell line CEM (ATCC CCL119) and its resistant derivatives CEM-VLB$_{100}$, selected for resistance to vinblastine (*6*), and the ovarian cell line 2780 and its resistant derivative 2780-Ad, selected for resistance to Adriamycin (*7*), both showed high levels of expression of the 4.5-kb mRNA.

To compare the levels of expression with the extent of amplification of the *mdr*1 sequences, we isolated genomic DNA from all of the cell lines described above and subjected it to digestion with Hind III. We then looked for amplification of *mdr*1 by Southern blot analysis (Fig. 2). No amplification of *mdr*1 was found in the KB cell lines with low levels of resistance (KB-8, KB-8-5, and the revertant subline, KB-C1-R1), even though these cell lines expressed increased levels of *mdr*1 mRNA. The apparent variation in intensity in the 4.4-kb Hind III fragment detected by the *mdr*1 probe in parental cell lines (KB-3-1) is probably the result of variation in loading or transfer during the blotting procedure. To quantitate precisely the amount of *mdr*1 DNA present in the parental KB-3-1 cell line and at the first three steps in selection for multidrug resistance, KB-8, KB-8-5, and KB-8-5-11, we prepared slot blots of Eco RI–digested DNA from these cell lines (Fig. 3). The signal intensity remains approximately constant among KB-3-1, KB-8, and KB-8-5 DNA, whereas the signal is increased in KB-8-5-11 DNA, where the *mdr*1 gene is ampli-

Table 1. Derivation and cross-resistance of multidrug-resistant cell lines used.

Cell line	Selecting agent	Relative resistance to			Reference	*mdr*1 mRNA expression*
		Col	Adr	Vbl		
KB-3-1	Parental KB	1	1	1	(*4*)	ND†
KB-8	Colchicine (5 ng/ml)	2.1	1.1	1.2	(*4*)	1
KB-8-5	Colchicine (10 ng/ml)	3.8	3.2	6.3	(*4*)	3
KB-8-5-11	Colchicine (100 ng/ml)	40	23	51	(*4*)	80
KB-C1	Colchicine (1 μg/ml)	260	160	96	(*4*)	270
KB-C1-R1	Revertant of KB-C1	6	3	4	(*4*)	1
KB-C1.5	Colchicine (1.5 μg/ml)	320		140	(*4*)	340
KB-C6	Colchicine (6 μg/ml)	2100	320	370	This work	820
KB-A1	Adriamycin (1 μg/ml)	19	97	43	(*4*)	270
KB-V1	Vinblastine (1 μg/ml)	170	420	210	(*4*)	320
CEM	Parental leukemic	1	1	1	(*6*)	ND
CEM-VLB$_{100}$	Vinblastine	45	120	420	(*6*)	250
2780	Parental, ovarian	1	1	1	(*7*)	ND
2780-Ad	Adriamycin		170	15	(*7*)	260

*Slot blots of total RNA were hybridized to the ^{32}P-labeled *mdr*1 probe as shown in Fig. 1. Levels of *mdr*1 expression were determined by densitometry of the autoradiograms. Tracings of peaks were cut out, weighed, and compared to the KB-8 peak, which was arbitrarily assigned a value of 1. †ND, none detected.

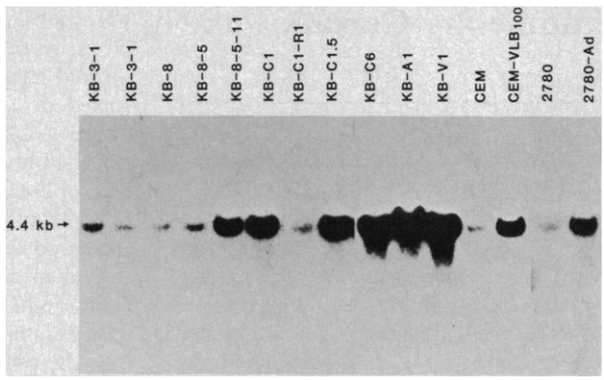

Fig. 2. Southern blot hybridization of DNA from human multidrug-resistant cell lines. DNA, prepared as described (*2*), was digested with Hind III and separated by electrophoresis in 0.8 percent agarose gels before Southern transfer to GeneScreen Plus (New England Nuclear). Each lane contained 5 μg of DNA, except for KB-V1, which contained 2.5 μg. The blots were hybridized with the *mdr*1 probe for 18 hours at 42°C in 50 percent formamide, 5× SSC, 1 percent SDS, and 100 μg/ml salmon sperm DNA. They were then washed with 2× SSC at room temperature for 10 minutes, 2× SSC, 1 percent SDS at 42°C for 60 minutes, and 0.1× SSC at room temperature for 60 minutes prior to autoradiography.

fied five- to tenfold. When the same slot blots are hybridized to a complementary DNA (cDNA) clone for the epidermal growth factor (EGF) receptor gene as a control for DNA loading (Fig. 3B), ratios of *mdr*1 to EGF-receptor gene signals average 1.8 for KB-3-1, 1.4 for KB-8, and 1.5 for KB-8-5 as determined by densitometry, indicating no amplification of the *mdr*1 gene in these cell lines (*8*). These results demonstrate that increased expression of *mdr*1 sequences in human cells can occur prior to gene amplification.

Amplification of the *mdr*1 gene was detected in highly resistant sublines of KB cells selected in colchicine, vinblastine, or Adriamycin, as well as in CEM-VLB_{100} and 2780-Ad cell lines (Fig. 2) [see also (*2*)]. In the latter two sublines, the degree of gene amplification was estimated by densitometry to be approximately 5- to 10-fold for 2780-Ad and 10- to 15-fold for CEM-VLB_{100}. In all cases, the increase in mRNA expression was greater than the extent of amplification. These results suggest that the evolution of these lines involved a step or steps in which expression was increased out of proportion to gene amplification. Similar dissociation of amplification and expression of the *dhfr* gene has been reported for tumor cells selected for resistance to methotrexate in vitro (*9*). The development of multidrug resistance in human KB cells differs in this respect from Chinese hamster V79 cells, where a low (five- to sevenfold) degree of relative drug resistance is accompanied by a similar (five- to tenfold) degree of amplification of *mdr* DNA (*3*).

Our inability to select multidrug-resistant cells without mutagen treatment suggests a model to account for its development. The initial steps of selection could require activation of the *mdr*1 gene either by a regulatory mutation or by epigenetic mechanisms, such as changes in DNA methylation. Amplification alone may not be sufficient to activate expression of a gene that is normally totally turned off or expressed at very low levels. Once activation of the *mdr*1 gene has been achieved, an obvious cellular mechanism for further increased expression would be to amplify the activated gene, as occurs in the cell lines reported here. Mutagenesis may also be needed to introduce a structural mutation in the *mdr*1 gene or into another cellular gene whose altered expression may also be required to develop the full multidrug resistance phenotype.

These studies demonstrate a parallel between expression of the *mdr*1 gene and the expression of resistance to multiple agents in five independently derived human cell lines of different origins selected for resistance to three different cytotoxic drugs. Expression of *mdr*1 may therefore represent a common mechanism of multidrug resistance in human cell lines. Increased expression of *mdr*1 in at least some cases occurs initially without gene amplification and may be a prerequisite for the development of multidrug resistance. This observation may be especially relevant for the analysis of the role of the *mdr*1 gene in the development of multidrug resistance by human tumors in the course of chemotherapy and may have diagnostic potential. Since the tumor cells are expected to have a relatively low degree of resistance, such an analysis would require quantitation of *mdr*1 RNA expression rather than of gene amplification in tumor samples.

Recently, a segment of cDNA encoding a cell surface glycoprotein, p170, known to be increased in membrane preparations from multidrug-resistant rodent and human cells, was shown to hybridize to amplified DNA sequences in several multidrug-resistant cell lines, including cell lines with amplified *mdr*1-related sequences (*11*). The identification of the protein product of the *mdr*1 gene will help in defining its relationship to the cell surface glycoprotein p170, in determining its function in multidrug-resistant cells, as well as in defining its role in the development of multidrug resistance in human cancer.

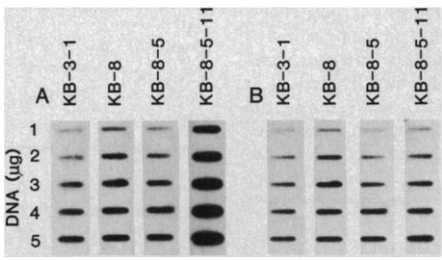

Fig. 3. Slot blot hybridization of DNA from KB cell lines isolated in the first three steps of selection for multidrug resistance. DNA digested with Eco RI (1 to 5 μg, as indicated) was applied to nitrocellulose filters using a slot blot apparatus. (A) The DNA was hybridized with the *mdr*1 probe. (B) The same filter was hybridized with a partial cDNA clone for the EGF-receptor (pE7) (*10*) as described (Fig. 1). The filters were washed four times for 15 minutes at room temperature in 5× SSC, 0.1 percent SDS, and then twice for 30 minutes at 50°C in 1× SSC, 0.1 percent SDS.

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8. Although not clearly seen in every lane on these photographs, the signal intensity when DNA is hybridized with either the *mdr*1 probe or the EGF receptor probe is proportional to the amount of DNA applied to the nitrocellulose, as determined by densitometry of the original autoradiograms. Because of this proportionality, these data can be used to calculate the relative degree of amplification of the *mdr*1 gene.
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