Amplification of DNA sequences in human multidrug-resistant KB carcinoma cells
(adriamycin/colchicine/vinblastine/gene amplification)

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ABSTRACT Four KB carcinoma cell lines selected independently for resistance to either colchicine, adriamycin, or vinblastine were studied. All cell lines showed high levels of resistance to the selecting drug and cross-resistance to the other drugs and to actinomycin D. Double-minute chromosomes could be identified on chromosomal spreads of these multidrug-resistant KB cell lines. Amplification of specific DNA sequences was demonstrated by using the technique of in-gel renaturation. All the cell lines share common amplified sequences. There are also amplified sequences that are specific for each cell line. A revertant cell line that has reacquired drug sensitivity has lost its amplified sequences. Specific probes obtained by cloning amplified sequences from the cell line selected in vinblastine recognize amplified sequences in all the resistant lines. The presence of common amplified sequences in these cell lines is strong evidence for the importance of these regions in multiple drug resistance.

The development of resistance to multiple drugs is a common clinical problem in the treatment of various cancers. To study this problem, workers in several laboratories have used increasing concentrations of a single agent to select cell lines resistant to several drugs (1–8). Although the precise mechanism of multiple drug resistance is unknown, decreased drug accumulation, increased efflux, and altered metabolism, or a combination of these have all been proposed. One means by which cells can become drug resistant is by specific gene amplification; this phenomenon has already been demonstrated in a number of systems with acquired resistance to a single drug (9–14). Double-minute chromosomes and homogeneously staining regions are thought to be cytogenetic manifestations of the phenomenon of gene amplification (12, 15–17). Several approaches have been used to identify amplified sequences in drug-resistant cell lines. The most sensitive of these appears to be that of DNA renaturation in agarose gels as described by Roninson (19). With this technique, amplification of specific sequences has been shown to correlate with multidrug resistance in Chinese hamster cells (20).

We have described elsewhere the isolation, genetic characterization, and biochemical analysis of four mutants of the human KB carcinoma cell line obtained by successive single-step selection with colchicine (21–23). The resistance of these cells has been increased further and, in addition, we have isolated two additional cell lines exhibiting pleiotropic drug resistance by selection with adriamycin or vinblastine. In the present study, we show that these independently selected cell lines have amplified >100 kilobases (kb) of DNA. Some of the amplified regions are common to all the cell lines, while others are cell-line specific.

MATERIALS AND METHODS

Materials. Deoxycytidine 5'-triphosphate, tetra(triethylammonium) salt, [α-32P] (specific activity, 3000 Ci/mmol; 1 Ci = 37 GBq) in 0.01 M N-Tris(hydroxyethyl)methylglycine was obtained from New England Nuclear. T4 DNA polymerase, restriction enzymes, and competent Escherichia coli HB 101 were purchased from Bethesda Research Laboratories. Formamide was a Fluka product. Nuclease S1 was obtained from Sigma. T4 DNA ligase was a Pharmacia product. P-L Biochemicals was the source of PUC-13 digested with HindIII and treated with bacterial alkaline phosphatase. All other reagents were of the highest purity available.

Cell Lines and Cell Culture. Details of the isolation and characterization of the various cell lines used are described elsewhere (21–23). Briefly, KB 3-1, the parent cell line, was derived from a single clone of human KB carcinoma cells after two subclonings. Ethyl methanesulfonate was used to enhance the mutation rate in the first two steps of isolation when colchicine was the selecting agent and in the first step only when adriamycin or vinblastine was the selecting agent. After the initial steps, the most cross-resistant lines, as mass populations, were subjected to stepwise increases in the concentrations of the selecting drugs until the time of harvesting. The revertant cell line used in these studies was a spontaneous revertant of the colchicine-resistant mutant that was isolated and subcloned twice in the absence of colchicine and has been carried in colchicine-free medium. Relative resistance was determined by measuring the amount of drug that reduced the cloning efficiency of the cells to 10% of the value in the absence of drug (LD10) and dividing the LD10 of the resistant cells by the LD10 of the parental cells. Details of this assay have been described (21, 22).

Cytogenetic Studies. After subculture, cells were grown for 48–72 hr, at which time metaphase arrest was initiated by the addition of colchicine (0.2 μg/ml; 3.3 μg/ml for the cell line selected in colchicine), and the incubation continued for 1 hr. Chromosome preparations were made according to the air-drying technique of Tjo and Whang (24). Both conventional Giemsa stain and trypsin Giemsa banding (25) were performed.

DNA Extraction and Restriction Enzyme Digestion. DNA extracted from the various cell lines was treated with ribonuclease, spooled, and digested with 10 units of enzyme per μg of DNA at 37°C for 8 hr under conditions recommended by the manufacturer. After extractions with phenol and chloroform, and ethanol precipitation, the DNA was resuspended in 10 mM Tris, pH 7.5/1 mM EDTA (TE buffer) and the concentration was determined spectrophotometrically at 260 nm. A 13-cm gel was run to confirm the completeness of the digestion and to compare quantities before the in-gel renaturation experiment.

Abbreviation: kb, kilobase(s).
In-Gel Renaturation. By using the restricted DNA, in-gel renaturation experiments were performed as described by Roninson et al. (19, 20). This technique has been used to identify amplified DNA sequences. After restriction enzyme digestion, a mixture of labeled and unlabeled DNA is subjected to electrophoresis followed by two cycles of in-gel denaturation and renaturation with S1 nuclease digestion. Amplified sequences are more likely to find a complimentary strand after denaturation and can resist subsequent digestion by S1 nuclease, which cleaves single-stranded fragments. After a final wash, the gel is dried and an autoradiogram is obtained showing a pattern of bands that represents the amplified sequences. For the labeling reaction, 2 units of T4 DNA polymerase was added to 2 μg of the restricted DNA, and the reaction was allowed to proceed for 5 min before the addition of free nucleotides. Under these conditions, the terminal 100 residues are removed by the T4 DNA polymerase before the resynthesis phase begins. Eighty to 250 μCi of [32P]dCTP (specific activity, 3000 Ci/mmol) was added in the initial phase of resynthesis, which was allowed to proceed for 50–60 min. Then, a 100-fold excess of unlabeled dCTP was added, and the reaction was incubated for an additional 20 min. The specific activity of the labeled product varied depending on the amount of labeled nucleotide added, with values of $30 \times 10^{10}$ cpm per μg of DNA obtained when 100 μCi was used. Lower specific activities were associated with increased backgrounds and were not used.

DNA was separated in a 1% agarose gel (40 × 20 × 0.4 cm) in 22.5 mM Tris acetate/10 mM sodium acetate/1 mM EDTA, pH 8.2, ethidium bromide at 1 μg/ml. Each lane contained 10 μg of "driver" DNA and 15 × 10^6 cpm of "tracer" in 500 ng of DNA restricted and labeled as described above. The wells were 9 × 1.5 × 3 mm, and the DNA was loaded in a vol of 14–16 μl. Electrophoresis was carried out for 22–24 hr at 80 V with recirculation of the buffer until the 2.3 kb HindIII fragment of λ phage was at the midpoint of the gel. The gel slab was cut to 35 cm in length and subjected to two cycles of in-gel denaturation/renaturation and nuclease S1 digestion. All steps were carried out in a warm room (37°C) with the gel in a box 37 cm long, on a rocker (50 times per min); nevertheless, the solutions had a temperature of 34°C when directly measured. The pH of the washes was 6.0 × 10^-3. Equilibrations were performed by changing the buffer five times at 15-min intervals. The DNA fragments in the gel were initially denatured in 0.5 M NaOH/0.6 M NaCl, with 0.004% thymol blue as indicator. Two 600-ml washes (30 min each) were performed. Neutralization was then carried out by equilibrating the gel with hybridization buffer. The composition of the hybridization buffer was 0.9 M sodium chloride/0.5 mM EDTA/50% formamide/0.05 M sodium phosphate, pH 7.0. The buffer was changed five times at 15-min intervals during which time the gel color changed from blue (alkaline) to yellow (neutral). If the gel was not poured on a level surface, the thicker areas did not equilibrate in this time. Next, the gel was incubated for 2 hr in 600 ml of hybridization buffer to allow renaturation to occur. Then the gel was equilibrated for S1 nuclease digestion with 0.2 M sodium chloride/50 mM sodium acetate, pH 4.5.5/1 mM zinc sulfate. Again, the buffer was changed five times at 15-min intervals. Nuclease S1 digestion was performed by adding 45,000 units of enzyme in 600 ml of buffer, for a final solution/gel concentration of 50 units/ml, and incubating for 2 hr. Longer incubations or the addition of fresh S1 nuclease at 1 hr did not change the results. The DNA was then again denatured as described above. After equilibration with hybridization buffer, the second hybridization was carried out overnight. The following morning, another cycle of S1 nuclease digestion was carried out, followed by five changes with 3 × NaCl/Cit (1 × NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate)/0.1% NaDodSO4 over a 3-hr period. The gel was then placed on 3-mm Whatman filter paper and was dried on a slab gel drier before autoradiography.

Cloning of Amplified Sequences. For cloning the amplified sequences, isolation, digestion, and electrophoresis of DNA were carried out as described above. A 3-cm strip of gel identified by the 4.4-kb HindIII fragment of phage λ was cut from lanes, each of which had been loaded with 10 μg of DNA. The region chosen is indicated by the vertical lines on either side of the VB lane of Fig. 3. It was picked because it contained a large number of amplified sequences that had appeared in the drug-resistant line. The strip was subjected to two cycles of in-gel denaturation and renaturation without nuclease S1 treatment. It was then rehydrated with ethidium bromide, cut into three 1-cm pieces, and the DNA was electroeluted. Carrier tRNA (20 μg) was added and the DNA was extracted six times with phenol and once with phenol/chloroform. After ethanol precipitation, the pellet was resuspended in TE buffer and ligated to commercially available dephosphorylated HindIII-digested pUC 13 plasmid DNA. Competent E. coli HB 101 were then transfected with DNA following the protocol provided by the manufacturer, and ampicillin-resistant colonies were isolated. Plasmid DNA was isolated from resistant bacteria by miniscale extraction and digested with several restriction enzymes to identify plasmids with identical inserts. Two hundred sixty colonies were screened; inserts were found in 83 of them. Eighteen were unique. The others belonged to one of eight groups that contained 2 or more members; the largest group was composed of 19 members. Two plasmids were used as probes for Southern blot analysis after nick-translation: one belonged to a group with 7 members; the other appeared only twice.

RESULTS

Fig. 1 and Table 1 provide a summary of the isolation steps for the various cell lines and a comparison of their resistance to several agents. All three isolates demonstrate resistance to colchicine, vinblastine, adriamycin, and actinomycin D, although the ratios of resistance vary depending on the selecting agent. Detailed properties of these cell lines are described elsewhere (21).

Fig. 2 shows the chromosome spread from the cell line selected in colchicine while it was being carried in 2.5 μg of colchicine per ml (KB-C2.5). Double-minute chromosomes are easily identified. Double-minute chromosomes could also be seen in spreads from the cell lines selected with adriamycin and vinblastine, but they were not seen in spreads from the revertant (data not shown).

Fig. 3 shows the result of the in-gel renaturation experiment. The pattern obtained when DNA was restricted with HindIII is shown. The parental cell line, KB-3-1, has a much simpler pattern of amplified sequences than the multidrug-resistant cell lines. Identical patterns were seen for the cell lines selected in colchicine with and without mezerin (mezerein line not shown). In addition, many similarities can be seen when the cell line selected with colchicine is compared with the cell lines selected with adriamycin and vinblastine. The amplified sequences are shown schematically on the right side of the figure. The schematic shows the pattern of amplified sequences seen in the parental cell line (KB-3-1) on the left. The labeled Com demonstrates the additional amplified sequences that are common to the three multidrug-resistant cell lines depicted; in some cases, these bands are best seen in Fig. 4. Lanes C4, Ad, and VB show the amplified sequences unique to each of these three cell lines. The schematic representation is a compilation of results from many experiments and different exposures and depicts only bands that were seen frequently. Most of the bands shown can be easily seen in Figs. 3 and 4, but not all are clearly
visualized. This is particularly true for the larger fragments, which were seen in many of our renaturation experiments although they were the most difficult to visualize. For example, the largest band in the lane labeled Com is seen in Fig. 3 in the Vb lane and the Ad lane, but not in the C4 lanes; however, these bands are seen in Fig. 4 in the two lanes where C4 is the tracer (Tr) and Vb is the driver (Dr). Another example is the three top bands in lane C4, where only the lower one is seen in this exposure, although two additional bands were usually present. Likewise, for the Ad lane the upper two bands are not seen clearly in this exposure.

Fig. 4 shows the autoradiograph of a mixed renaturation experiment. The various combinations of unlabeled carrier DNA and radiolabeled tracer DNA are described in the legend. In this experiment, tracer (Tr) amounts of labeled DNA from one cell line were mixed with unlabeled driver (Dr) DNA from a different cell line. This type of analysis helps to emphasize the sequences that are amplified in common among the drug-resistant lines. It is evident that the three cell lines have amplified some sequences in common, but some differences are evident as well. In comparing any two cell lines, the intensity of the bands varies depending on the source of the DNA, especially the tracer DNA. In addition, some bands that are specific to the cell line selected in vinblastine can be seen when vinblastine DNA is the source of the tracer DNA. Examples of this are seen in bands between the 6.6-kb and 9.5-kb HindIII fragments of phage λ and represent sequences unique to the cell line selected in vinblastine whose renaturation has been "driven" by the tracer DNA.

To examine further the amplified bands demonstrated by the in-gel renaturation experiments, repeated sequences from the cell line selected in vinblastine were cloned as described. A 3-cm strip of gel identified by the 4.5-kb HindIII fragment of λ phage (shown in Fig. 3 by vertical lines on either side of lane Vb) was used to isolate the repeated sequences. Plasmid DNA was isolated from resistant bacteria by miniscale extraction and digested with several restriction enzymes to identify plasmids with identical inserts. Clones that gave similar restriction patterns were assumed to be derived from the same region of amplified DNA. Of the 260 colonies screened, inserts were found in 83 of them. The plasmid used as a probe in Fig. 5A belonged to a group with seven members; the plasmid used as a probe in Fig. 5B had only one pair. Fragments recognizing amplified sequences included some for which no pair could be found (unpublished observations). The results in Fig. 5A show that the sequence cloned recognizes amplified DNA sequences in the three independently derived drug-resistant lines, but not in the revertant. The results in Fig. 5B show that the second probe recognizes sequences amplified only in the vinblastine-selected cell line from which it was obtained. In both A and B, single-copy sequences are not seen in these exposures, although they could be visualized in longer exposures; however, longer exposures also had a higher background, probably as a result of the presence of repetitive sequences in the amplified segments. The plasmid used as a probe in Fig. 5B detects several bands. This is likely due to restriction fragment polymorphism secondary to rearrangement during the selection for multidrug resistance, because in the parental cell line, where there is no amplification of this DNA sequence, only a single band is seen just above the 4.4 kb fragment of λ phage (data not shown).

**DISCUSSION**

This paper describes gene amplification in four independently isolated human cell lines selected for pleiotropic drug resistance. Gene amplification has been previously demonstrated

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**Table 1. Relative resistance of KB sublines to various agents**

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<th>Adriamycin</th>
<th>Vinblastine</th>
<th>Actinomycin D</th>
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**Fig. 2.** Chromosome spread from the cell line selected in colchicine, showing double-minute chromosomes.
sequences to double-minute chromosomes and to homogeneously staining regions with radiolabeled probes (26). We found the multiple drug-resistance phenotype in KB cells to be unstable with loss of resistance when the cells were maintained in drug-free medium. For example, the cell line selected with colchicine, which was selected in several steps and from which the revertant line shown was obtained, gradually lost its high level of resistance when maintained in a drug-free medium for a few months. In the absence of selective pressure, reversion to a lower more stable level of resistance occurred. Our revertant cell line, KB-Cl-R1 was cloned from such a revertant population.

Rapid loss of resistance in the absence of selective pressure has been shown previously for various cell lines in which double-minute chromosomes are present, and it is the probable explanation for our results (12, 15–18). Stabilization at a low level of resistance may indicate that more than one mechanism is responsible for the multiple drug-resistance phenotype. Another explanation is that a lower copy number of the amplified gene(s) remains stably integrated in the chromosome, as has been suggested by others. A third possibility is that the early steps represent a regulatory change with a gene “turn on,” while gene amplification
cloning amplified the near lines. Not fragment occurred in gene that be generated. This mixed renaturation (11, 13), may of differences presence regions drug-resistance underestimation although of limitations the segment not the size of the amplified unit(s). Some copies the amplified. The interpretation of the in-gel renaturation technique is that each cell line selected with vinblastine, from which it was obtained.

In summary, we have demonstrated the presence of amplified sequences in four independently isolated cell lines exhibiting resistance to multiple drugs. Some amplified sequences are shared by all the cell lines, indicating the importance of these regions in acquisition of the multiple drug-resistance phenotype. Our current efforts are to use the in-gel renaturation technique to obtain specific human probes, which may help us to identify the gene(s) and gene product(s) responsible for the multiple drug-resistance phenotype and provide information to aid cancer chemotherapy in the future.

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