RESEARCH

A YAC-based Contig of 1.5 Mb Spanning the Human Multidrug Resistance Gene Region and Delineating the Amplification Unit in Three Human Multidrug-resistant Cell Lines

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A contig of 21 nonchimeric yeast artificial chromosomes (YACs) has been assembled across 1.5 Mb of the multidrug resistance (MDR) gene region located at 7q21, and formatted with four previously reported probes, six newly isolated probes, and three sequence-tagged sites (STSs) from internal and end fragments of YACs. A physical map of rare cutter restriction enzyme sites across the region was also constructed by pulsed-field gel electrophoretic (PFGE) analysis of four overlapping YAC clones. The amplification unit of this region in different cell lines was then determined by Southern blot analysis on the basis of the physical map and probes. Amplified DNA was located in extrachromosomal elements in human MDR cell lines studied here, and the size of the amplification unit was determined to be discrete in one MDR amplification but variable in others.

The acquisition of multidrug resistance (MDR) in vitro is commonly associated with the increased expression of the cellular surface protein, P-glycoprotein, which functions as an energy-dependent drug efflux pump, resulting in a decrease of intracellular drug concentration (Pastan and Gottesman 1987; Bradley et al. 1988; van der Bliek and Borst 1989). Mammalian P-glycoproteins are encoded by families of linked genes, of which there are two known members in humans (PGY1 and PGY3 which are also called MDR1 and MDR3, respectively) (Roninson et al. 1984; Ueda et al. 1987; Chin et al. 1989), and three members in mice (mdrla, mdrlb, and mdr2) (Gros et al. 1986a, b; Devault and Gros 1990) and hamster (pnp1, pgp2, and pgp3) (Endicott et al. 1991).

PGY1 and PGY3 have been mapped to human chromosome 7 (Fojo et al. 1989; Chin et al. 1989), and their three rodent counterparts have been localized on mouse chromosome 5 (Hsu et al. 1989). The entire human MDR locus covers ~230 kb, and the human PGY1 and PGY3 genes are closely linked, separated by only 34 kb of intergenic DNA region and transcribed in the same direction (Chin et al. 1989; Lincke et al. 1991; Matsuda et al. 1993). Although no MDR-related genes other than PGY1 and PGY3 have been detected by Southern blotting (Riordan et al. 1985; Gros et al. 1986a, b; Roninson et al. 1986), other genes related to drug resistance might be in the region.

From a mechanistic point of view, two aspects have been shown to be involved in the acquisition of MDR. First, many experimental results indicate that trans-activation of the MDR genes is associated with exogenous stimulation, including anticancer agents, carcinogens, and...
with drug resistance, and analysis of the structure region. YACs were obtained from three different contig spanning 1.5 Mb including the MDR gene clones were isolated and assembled to construct a libraries (Brownstein et al. 1989; Scherer et al. 1987) have proven to be a powerful tool to clone large genomic fragments that cover extensive regions of DNA. As the first step in the isolation of genes, assessment of their correlation with drug resistance, and analysis of the structure of the MDR gene region in MDR cell lines, YAC clones were isolated and assembled to construct a contig spanning 1.5 Mb including the MDR gene region. YACs were obtained from three different libraries (Brownstein et al. 1989; Scherer et al. 1992; Green et al. 1995). Nonchimeric clones were selected, and the contig was then constructed and mapped further as part of the study: the extent of the amplification unit of the MDR gene region in two independent drug-resistant cell lines was then determined.

RESULTS

Construction of the YAC Contig

Three YAC libraries were used as sources to collect YAC clones carrying the human MDR genes (PGY1 and PGY3). A primer pair was designed from the promoter region of the PGY1 gene (Table 1) and was used to isolate YAC clones from Washington University total human library and chromosome 7-specific libraries. Additional clones were isolated from the libraries by PCR-based screening (Green and Olson 1990) using STSs produced from the end clones of yWSS167 and yWSS172. Clones were also isolated from another chromosome 7-specific YAC library (Scherer et al. 1992) using a hybridization-based approach (Table 2). The contig was assembled using criteria based on STS content (Fig. 1). Overlaps were detected by dot blot hybridization of probes, including end clones isolated from some YAC clones (Table 1), or by PCR for STSs (Table 1), and were confirmed by Southern blot analysis (data not shown). Clones that had been shown to be chimeric or gave ambiguous results were not analyzed further except for yWSS167 and yWSS2393, because the insert ends of these clones were used to extend the contig. Distances within the contig (Fig. 1) were estimated from the size of the YACs (Table 2), as determined by pulsed-field gel electrophoresis (PFGE).

The order of the DNA markers has been determined unambiguously by contig mapping as D7S2622–D7S574–D7S2625–D7S159–D7S2621–D7S1646–PGY3–PGY1–D7S2626–D7S2624–D7S2628–D7S1642–D7S2623–D7S2627. The direction of these markers along the chromosome could also be determined by multicolor fluorescence in situ hybridization (FISH) analysis on prophase chromosomes (Fig. 2A). The arrangement of green (HSC7E718, which localizes on 7q21.1, and RFC2, which localizes on 7q11.23) and red (yWSS3740, which also localizes on 7q21.1) signals indicated that the order is cen–HSC7E718–yWSS3740–ter. The order of some of the markers around the contig has been determined as follows: 7cen–D7S165–D7S177–D7S574–7q-ter by somatic cell hybrid analysis.
Table 1. List of previously cloned and newly isolated DNA probes and STSs

<table>
<thead>
<tr>
<th>DNA probe or STS</th>
<th>D-segment number</th>
<th>Source of DNA segment</th>
<th>Size (kb)</th>
<th>Method of isolation</th>
<th>PCR primer</th>
<th>Source</th>
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<td>E51ScdA-6</td>
<td>D7S574</td>
<td>HSC7ES15</td>
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<td>Scherer et al. (1993)</td>
</tr>
<tr>
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<td>flow-sorted chromosome 7</td>
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<td>random selection</td>
<td>—</td>
<td>Rommens et al. (1988)</td>
</tr>
<tr>
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<td>E66cd2</td>
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<td>HSC7ES66</td>
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<td>pMDR1-3&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>pMDR 105</td>
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<td>cDNA</td>
<td>—</td>
<td>Gottesman et al. (1987)</td>
</tr>
<tr>
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<td>PGY1</td>
<td>pMDR-H3</td>
<td>1.0</td>
<td>Cloning from genomic DNA</td>
<td>—</td>
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</tr>
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<td>HSC7E718</td>
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<td>—</td>
<td>this work</td>
</tr>
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<td>this work</td>
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<td>PGY1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Kohno et al. (1990)</td>
</tr>
<tr>
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<td>PGY3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Van der Bliek et al. (1988)</td>
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<td>D7S1646</td>
<td>yWSS899</td>
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<td>this work</td>
</tr>
<tr>
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<td>D7S1642</td>
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<td>this work</td>
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<sup>a</sup>Probe specific for 3' end of PGY1.
<sup>b</sup>Probe specific for 5' end of PGY1.
<table>
<thead>
<tr>
<th>YACs</th>
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<th>comments</th>
<th>YACs</th>
<th>length (kb)</th>
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<td></td>
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<tr>
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</tr>
</tbody>
</table>

*Definition of comments are as follows: (deletion?) Hybridization signal is not detected by the probes, which is expected to localize inside of the insert, although outside probes hybridize to the clone. Results were confirmed by Southern blotting. Shortening of size has not been determined yet. (Chimera) Determined by hybridization or PCR assay of probes or STSs isolated from end of each clones to hybrid cell panel. (Rearrangement) Unexpected size is detected by Southern blot analysis. (Ambiguous) Hybridization results are not explained simply. They may have some deletion, rearrangement, and/or chimerism.


Restriction Mapping of the YAC Contig

To confirm overlaps and consistency of the clones and to refine the contig map more accurately, restriction mapping was carried out on four clones, yWSS3740, HSC7E734, HSC7E656, HSC7E615, covering the entire region (Fig. 3), by indirect end labeling methods as described by Burke et al. (1987). Six putative CpG islands (Bird 1986) were observed (Fig. 3). The second one from the left was located very near the 5' end of the PGY1 gene, and likely is linked to the promoter of this gene (Ueda et al. 1987; Kohno et al. 1990; Uchiumi et al. 1993). Because CpG islands are usually linked to genes, the other five CpG islands may mark new genes that have not been identified yet.

Amplification Unit of MDR Gene Region in MDR Cell Lines

The availability of the YAC map facilitates the study of amplification units of the MDR gene region. Three MDR cell lines were analyzed by Southern blot hybridization using the probes described above (Fig. 4) and the amplification unit in each cell lines were estimated by the relative amplification ratio of the drug resistant cell lines to the parental cell lines according to the calculation described in Methods. The KB-C1 cell line was originally isolated as a colchicine-resistant cell, and the MDR1 region was amplified in an extrachromosomal DNA state (Akiyama et al. 1985; Fojo et al. 1985; Schoenlein et al. 1992). Kst-V100 cells were isolated by stepwise selection with increasing doses of vincristine (up to 100 ng/ml),
YAC CONTIG SPANNING HUMAN MDR1 GENE REGION

Figure 1 Contig map of YAC clones from the MDR region. Probes and STSs are shown at the top (see Table 1). (O) The presence of markers or STSs on the corresponding clones. Ambiguous clones, including chimeric, deletion, and rearrangement shown in the Table 2, were not included here.

Figure 5 summarizes the estimation of the amplification unit. The amplification unit of the MDR gene region in the KB-C1 cell was determined to be delimited in a 700- to 1300-kb range, consistent with Southern blot results on PFGE analyses of cellular DNA digested with NotI (890 kb; data not shown), as well as with results reported by Schoenlein et al. (1992). The amplification ratio of KB-C1 to parental KB is uniform (16.4 times) across the amplified region.

In contrast with KB-C1 cells, the amplification unit in Kst-V100 and Kst-V500 cells is longer, >1.5 Mb, with a gradient in the degree of amplification. The amplification ratio of Kst-V100 to parental Kst-6 is 1.5, 2.4, 2.8, 2.7, 2.3, and 2.0 times, and the ratio of Kst-V500 is 1.6, 3.8, 10.0, 7.4, 4.3, and 3.3 times at loci pE718R, pA35, PGY1, pWSS1336R, pWSS1336L, and pWSS3740L, respectively. Although amplification was detected in Kst-V100 at E66cd2 and pA164 loci, which are the outer-most markers used in this study, it was not detected in Kst-V500. These results suggest that the genomic region around the MDR genes is amplified as a huge zone at early stages (Kst-V100) of drug selection, and the unit becomes shorter and shorter during acquisition of higher resistance (Kst-V500). As a result, the ratio of amplification is highest at the middle of the amplification unit and lower at the ends.

DISCUSSION

The structure of human MDR genes PGY1 and PGY3 has been examined (Chin et al. 1989; Chen...
A long-range physical map of this region is now available. In this study, a contig and restriction map spanning the region are based on YACs. Moreover, ambiguous clones, including chimeras, were eliminated from the starting materials to build the contig, and the YAC clones and the map presented here are likely to be reliable sources for further detailed analysis of the region.

Because a physical map spanning 400 kb around PGY1 and PGY3 has been reported previously (Lincke et al. 1991), we have compared our map with it to assess the integrity and consistency of the YAC system. We detected all four SfiI clusters (five sites) and three BssHII clusters (four sites) reported by Lincke et al. (1991), as well as an additional BssHII site in the middle of the MDR1 gene. This new site probably is not a result of the experimental artifact because we detected this site in three independent YAC clones (Fig. 3). There may be some polymorphism among HepG 2 cell lines used earlier and the somatic cell hybrid cell line 4AF1/106/KO15 used for the YAC library constructed by Scherer et al. (1993). Another explanation for the difference in the restriction maps is that the new BssHII site may have been methylated in the study of Lincke et al. (1991) (genomic DNA was mapped). However, yeast DNA (which has no known methylation system) was used in our experiments, and the site is now susceptible to restriction enzyme digestion by BssHII.

At least six putative CpG islands were identified from the restriction map. Three MDR-related genes are located in tandem in the mouse and hamster genome (Gros et al. 1986a; Devault and Gros 1990; Endicott et al. 1991). The PGY1 gene encoding membrane P-glycoprotein is often
correlated with the acquisition of MDR phenotype in cancer cells, possibly because of the enhanced efflux pump of anticancer agents (Riordan et al. 1985; van der Bliek et al. 1988). P-glycoprotein also has chloride channel activity like the cystic fibrosis transmembrane conductance regulator (CFTR) (Gill et al. 1992; Higgins 1992). Isolation of genes linked to CpG islands thus may be useful in detecting any new MDR-related or ion channel gene.

We present here a molecular basis for two different mechanisms generating DMs. The amplification unit in the KB-C1 cell line was determined as a discrete 890-kb region encompassing the PGY1 and PGY3 genes. This is consistent with the results reported by Schoenlein et al. (1992). The amplification unit in the Kst-V500 cell line spans >1.5 Mb, and the middle of the unit, near the PGY1 gene, was amplified more than the end of the unit. DMs are often observed in KB-C4 cells, which were isolated by stepwise selection to further increasing doses of colchicine from KB-C1 cells (Fojo et al. 1985; Schoenlein et al. 1992). Although DMs have not been observed in the KB-C1 cells, the data reported by Schoenlein et al. (1992) and extended in this study, which show a

Figure 3 Restriction map of the contig. Restriction sites of three rare-cutter enzymes are shown (middle) with the relative positions of PGY1 and PGY3 (top) and the four YAC clones used in this analysis (bottom). Six putative CpG islands, which are defined as the cluster of two or more CpG-containing rare-cutter restriction sites are indicated by open boxes.

Figure 4 Southern blot analysis of the DNA isolated from drug-resistant cell lines. Genomic DNAs were isolated from KB-C1 and Kst-V500 and their parental cell lines, KB and Kst-6, respectively, and subjected to Southern blot analysis as described in Methods. Probes used are indicated by arrows.
discrete amplification unit of 890 kb, support a model of gene amplification in which small circular or submicroscopic extrachromosomal DNA precedes or multimerizes to form cytologically detectable DMs. Recently, a complete physical map of an amplicon was determined in two cases, the adenosine deaminase gene region (Nonet et al. 1993) and the \textit{N-myc} gene region (Schneider et al. 1992). Both results support the notion that the amplicon in DM-type cells is 500 kb and 1.2 Mb, respectively. A discrete circular configuration would be consistent with our results for KB-C1 cells.

Sen et al. (1989) proposed that DMs are generated from prematurely condensed replicating micronuclei in drug-treated CHO cells. This intriguing proposal would require that micronuclei are generated specifically from the MDR gene region (or perhaps from an adjacent region). The results presented here provide one molecular basis for the model proposed by Sen et al. (1989). An amplification unit can span a large zone of >1.5 Mb at the early step (Kst-V100) during stepwise selection of drug-resistant cell lines, but it is possible that the unit becomes shorter and the amplification ratio increases at later steps (Kst-V500). This pattern of amplification fits the model proposed by Sen et al. (1989) and is typically observed in HSR-type cells (Stark et al. 1989).

The YAC-based contig should help to analyze additional drug-resistant cell lines. These cell lines could further the analysis of the basis of amplification and could aid in the determination of a possible minimum sequence extent for amplicon.

**METHODS**

**YACs Containing Chromosome 7**

YAC clones were isolated from three different libraries; a total human library constructed at the Washington University (Brownstein et al. 1989), a chromosome 7-specific library containing human DNA derived from GM10791 somatic cell hybrid (Green et al. 1995), and a chromosome 7-specific library containing human DNA derived from the somatic cell hybrid cell line 4AF1/106/KO15 (Scherer et al. 1992).

**DNA Probes**

Detailed descriptions of four previously obtained probes are given in Table 1. The seven new probes were isolated by the bubble–PCR method (Riley et al. 1990).

**Synthetic Oligonucleotides**

Several primer pairs were prepared for PCR. YAC end clones were isolated by the bubble–PCR method, and the DNA sequence was determined to develop STSs (Olson et al. 1989). The DNA sequences of the oligonucleotides are described in Table 1.

**Cell Lines and Cell Cultures**

The parent cell line KB-3-1 was subcloned from a human KB epidermoid carcinoma cell line. The colchicine-resistant sublines KB-C1 and KB-C4 were kindly provided by S.I. Akiyama (Akiyama et al. 1985). Kst-6 was isolated from human KB cell lines after stable transfection of pMDRCAT1. The vincristine-resistant sublines, Kst-V100 and Kst-V500, were isolated by increasing the concentration of vincristine in the selection media to 100 or 500 ng/ml (Nacalai Tesque, Inc., Kyoto, Japan), respectively (Kohno et al. 1994). Cells were grown as described previously (Kohno et al. 1988, 1994).

**PCR Analysis**

Genomic DNA (100 ng) was mixed with 1 μM primer pairs and 1 unit of \textit{Tag} DNA polymerase (Amersham Co., Buck-
was normalized by radioactivity of the band hybridized by between lanes, which were caused by loading. The amplification ratio was then calculated from the normalized ratio by actinomycin D.

Southern Blot Analysis and Estimation of the Amplification Ratio

Genomic DNA digested with HindIII was separated on an 0.8% agarose gel and transferred to a nylon filter. DNA was cross-linked to the filter by UV irradiation. A 32p-labeled DNA probe was prepared by the random priming method (Feinberg and Vogelstein 1983). Gene amplification was determined by a bioimaging analyzer (BAS 2000, Fuji Film Co., Tokyo, Japan). Radioactivity of the bands hybridized by each probe was measured directly by the BAS 2000 and was normalized by radioactivity of the band hybridized by β-actin probe on the same filter to correct for differences between lanes, which were caused by loading. The amplification ratio was then calculated from the normalized radioactivity of the drug-resistant cell lines divided by that of the parental cell lines.

FISH Analysis

Probe labeling and in situ hybridization conditions were completed as described elsewhere (Lichter et al. 1988). About 200 ng of labeled probes, 2 μg of total yeast DNA without YAC, 2 μg of human COT-1 DNA (GIBCO BRL, Gaithersburg, MD), and 4 μg of salmon sperm DNA were ethanol-precipitated together and redissolved in 10 μl of hybridization mixture [50% (vol/vol) formamide, 2 x SSC, 10% dextran sulfate]. After denaturation at 75°C for 5 min, the probes were preannealed at 37°C for 20 min to block signals derived from repetitive sequences and hybridized onto metaphase chromosomes. Following incubation overnight and subsequent posthybridization washes and blocking with Block Ace (Dainippon Pharmaceutical Co., Ltd.) in 4 x SSC, probes were detected by means of fluorescein isothiocyanate (FITC)-conjugated avidin (Boehringer Mannheim). Chromosomes were counterstained with 0.2 mg/ml of 4',6-diamidino-2-phenylindole (DAPI, Sigma). Prophase chromosomes for the YAC orientation were prepared by the method of Inazawa et al. (1994) using ICRF193 to stop cell cycle to prophase. To determine the orientation of the MDR gene region, two YAC clones, HSCE718 and yWSS3740, were labeled with biotin-16-dUTP and digoxigenin-11-dUTP, respectively. The digoxigenin-labeled probe was detected with rhodamine-antidigoxigenin (Boehringer Mannheim). The Biotin-labeled RFC2 gene (a subunit gene of human replication factor C) was used as a marker for its location on 7q11.23 (Okumura et al. 1995). Fluorescence signals were imaged using a Zeiss Axioskop epifluorescence microscope equipped with a cooled charge coupled device (CCD) camera (Photometrics, PXL 1400). Image acquisition was performed on a Macintosh Quadra 840 AV computer with the software program IPLab (Signal Analytics Co.). The images were then pseudocolored and merged using Adobe Photoshop 2.5J (Adobe Systems, Inc.). DAPI, rhodamine, and FITC images were shown in blue, red, and green, respectively. The merged images of FITC and DAPI were printed directly using a Fuji Pictrography 3000 from a Macintosh computer.

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