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Amplicon Structure in Multidrug-Resistant Murine Cells: A Nonrearranged Region of Genomic DNA Corresponding to Large Circular DNA

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Multidrug resistance (MDR) in tumor cell lines is frequently correlated with amplification of one or more *mdr* genes. Usually the amplified domain also includes several neighboring genes. Using pulsed-field gel electrophoresis, we have established a restriction map covering approximately 2,200 kb in the drug-sensitive mouse tumor cell line TC13K. The mapped region is located on mouse chromosome 5 and includes the three *mdr* genes, the gene for the calcium-binding sorcin protein, and a gene with unknown function designated class 5. Long-range maps of the amplified DNA sequences in five of six MDR sublines that had been independently derived from TC13K generally displayed the same pattern as did the parental cell line. All six MDR sublines exhibited numerous double minutes, and one of them displayed a homogeneously staining region in a subpopulation. Large circular molecules, most likely identical to one chromatid of the double minutes, were detected in four of the sublines by linearization with γ irradiation. The size of the circles was about 2,500 kb, which correlated to a single unit of the amplified domain. We therefore propose that in four independent instances of MDR development, a single unit of about 2,500 kb has been amplified in the form of circular DNA molecules. The restriction enzyme map of the amplified unit is unchanged compared with that of the parental cell line, whereas the joining sites of the circular DNA molecules are not identical but are in the same region.

In chemotherapy, resistance of tumor cells to a single cytotoxic compound is often accompanied by cross-resistance to a broad range of structurally and functionally unrelated drugs (12, 25). This multidrug resistance (MDR) phenotype may bring about serious problems during chemotherapy (13). MDR can be experimentally induced in vitro and has been extensively studied in cell lines from humans, mice, and hamsters. MDR is caused by overexpression of a small group of membrane-bound P-glycoproteins (9). These P-glycoproteins consist of two homologous halves, each composed of three putative transmembrane loops and a nucleotide-binding fold (3, 11, 14). The P-glycoproteins act as energy-dependent unidirectional drug efflux pumps, preventing the accumulation of drugs in exposed cells (11).

The P-glycoproteins are encoded by members of a gene family. Several different nomenclatures have been used for the *mdr* genes, but in this report we have adopted the designations that have been proposed by Hsu et al. (18) and which were recently recommended by Kane and Gottesman (20). In the mouse, the *mdr* gene family consists of two closely related genes, *mdr1a* (6, 18) and *mdr1b* (14), and a third gene, *mdr2* (15, 18). Each of these genes has a direct counterpart in hamster cells (8, 26), whereas human cells display only two *mdr* genes (3, 36).

In many instances, P-glycoprotein overproduction is due to gene amplification, particularly in cultured cells in which very high levels of resistance can be attained. Amplification of the *mdr* genes, as well as of many other genes, is often associated with certain cytological structures, i.e., double minutes (DM) and homogeneously staining regions (HSR) (5, 17, 34, 35, 38). There is an intimate relationship between DM and HSR, and in some cell lines DM have been shown to act as precursors of HSR (2, 31). Observations of DM in different experimental systems have resulted in several models for the genesis of DM. The episome model suggested by Wahl and collaborators (2, 42) postulates as the initial event the excision of small circular DM precursors which enlarge into DM by recombination. In contrast, the model recently put forward by Sen and collaborators (29) suggests an instant formation of intact DM from prematurely condensed chromosomes.

To reach a better understanding of the *mdr* gene amplification process, we have constructed a physical map of the mouse *mdr* gene region and its surroundings, comprising in total about 2,200 kb. The region is located on mouse chromosome 5 and displays an organization which is similar to that of the hamster and, to some extent, human genomes. The map was compared with the amplified regions in six different MDR cell lines. In five of them, most of the amplified region was essentially unchanged compared with the arrangement of the corresponding sequences in the TC13K parental cells. Finally, we have shown that in four of these MDR cell lines, one unit comprising the entire region seems to be amplified as a circular DNA element, presumably corresponding to the DM detected in these cell lines.

MATERIALS AND METHODS

Cell lines and culture conditions. The murine SEWATC 13K cell line originates from a polyomavirus-induced osteosarcoma, which was later transformed into a transplantable ascites tumor (30). SEWATC13K cells display an HSR which carries about 40 copies of the *c-myc* gene. The SEWATC13K cell line was carried as a stationary suspension culture in McCoy 5A medium with 16% fetal calf serum. SEWATC13K cells were seeded in 15 separate bottles, which subsequently were exposed to either actinomycin D, colcemid, or vincristine (5 bottles of each). By increasing the drug concentration stepwise, all 15 sublines were induced to

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develop MDR (33). From these MDR sublines, six were chosen for further characterization. The MDR sublines were cultured as described above and supplemented with the respective selective agents: the A1 cell line with actinomycin D, the C1, C2, and C3 cell lines with colcemid, and the V1 and V3 cell lines with vincristine. The final maintenance drug concentrations were 0.1, 0.1, and 1.0 μg/ml, respectively. All of the MDR sublines displayed DM, and one of them, A1, also exhibited cells with an HSR (33).

Hybridization probes. The cDNA clone cp28, derived from an MDR Chinese hamster ovary cell line, CH^RC5 (41), is known to hybridize to all three *mdr* genes. In contrast, the genomic clone Ie7, derived from a DM-enriched fraction of an MDR SEWA cell line, is specific for the mouse *mdr1a* gene (32, 33) and was used to distinguish this gene from the two other members of the *mdr* gene family. The cDNA clones cp6 and cp30, also derived from CH^RC5 (41), detect two neighboring genes: the sorcin gene, which encodes a calcium-binding protein, and a gene designated class 5, with unknown function. A c-*myc* cDNA clone was used for control hybridizations.

All clones were digested with the appropriate restriction enzymes; the inserts were separated on agarose gels and isolated with the Bio 101 Geneclean kit. The inserts were labelled with [α-^{32}P]dCTP according to Feinberg and Vogelstein (10) and used as probes in the DNA blot analysis.

DNA blot analysis. High-molecular-weight DNA was prepared from cells immobilized in agarose plugs. The final cell concentration was 2×10^7 cells per ml except for the parental cell line TC13K, which was used at 6×10^7 to 8×10^7 cells per ml. Cellular proteins were degraded by proteinase K treatment as described by Van der Bliek et al. (41). Three separate DNA samples from each cell line were digested with the restriction enzymes *Mlu*I, *Not*I, and *Nru*I, respectively. Three additional DNA samples were double digested with the three possible combinations of the same enzymes. The protocol for the restriction enzyme digestions was as described by Van der Bliek et al. (41), with buffers and temperatures as recommended by the suppliers. In one experiment, partial *Nru*I digestions were performed by incubation for shorter times at room temperature as specified in the legend to Fig. 3. The DNA fragments were separated by pulsed-field gel electrophoresis (PFGE) in an LKB 2015 Pulsaphor system with a hexagonal electrode setting (Pharmacia LKB Biotechnology, Uppsala, Sweden). Agarose gels (1%) were prepared in a solution of 50 mM Tris, 50 mM boric acid, and 1 mM EDTA and run at 14°C and 350 V with an initial pulse time of 120 s for 24 h, followed by pulses of 180 s for 12 h. Gels were treated twice for 15 min each time in 0.25 M HCl and blotted to nylon filters (GeneScreen; Dupont) in 0.4 M NaOH–0.6 M NaCl. Conditions for hybridization were as described previously (33), and the final stringency washes were in 10 mM sodium phosphate (pH 7.2)–1% sodium dodecyl sulfate at 65°C. Prior to rehybridization, probes were removed as described previously (33).

Random linearization of circular DNA. To determine the appropriate radiation dose for linearization of putative circular DNA, gel plugs from the MDR sublines were immersed in 10 mM EDTA–10 mM Tris (pH 7.5) and placed in front of a ^{137}Cs source, and doses of γ irradiation from 5 to 125 Gy were administered. The resulting mixtures of linearized DNA circles and randomly fragmented DNA were separated by PFGE on 0.5% agarose gels, prepared in the same buffer. The gels were run at 14°C and 50 V for 90 h. Pulse time was 30 min. Hybridization and washing conditions were as described above. It was found that 10 Gy was an appropriate dose to generate a high-molecular-weight fragment detectable with the amplicon-specific probes (for an example, see Fig. 5, which shows a cp28 hybridization to DNA samples from subline C3, subjected to increasing γ-irradiation doses).

RESULTS

Restriction enzyme mapping of the amplicon. The six MDR sublines A1, C1, C2, C3, V1, and V3 and the parental cell line TC13K were treated with the restriction enzymes *Mlu*I, *Nru*I, and *Not*I alone or together in double digestions (*Not*I-*Nru*I, *Not*I-*Mlu*I, and *Mlu*I-*Nru*I). Hybridization with four gene probes, cp6, cp30, Ie7, and cp28, displayed bands which could be interpreted into restriction enzyme maps for each cell line with the exception of V3. In this particular line, a complex set of bands was found, which implied extensive rearrangements in the amplified domain (Fig. 1).

The majority of the bands for the remaining five sublines could be traced back to corresponding bands in the TC13K parental line. With minor exceptions in hybridizations to the *Mlu*I digestions and the *Not*I-*Mlu*I digestions (for *Not*I-*Mlu*I, the Ie7-cp28 hybridizations only) (Fig. 1 and 2), their overall maps were the same. Subline A1, specifically, also differed in the Ie7-cp28 hybridizations to the *Nru*I digestion and by lacking a 350-kb *Not*I fragment detectable with cp28. The impact on the amplicon maps of the exceptions is discussed below.

Since there was extensive agreement in banding pattern among sublines A1, C1, C2, C3, and V1 and the parental cell line TC13K, it is possible to describe the mapping procedure by using a single cell line as an example. For this purpose, the mapping of C1 is presented.

To distinguish between the different *mdr* genes, hybridizations were made with a cDNA probe (cp28) homologous to all three *mdr* genes as well as a genomic probe (Ie7) specific for *mdr1a*. These two probes yielded identical hybridization patterns, with the exception of a 350-kb *Not*I band. This band was detected only by cp28 and therefore was associated with the *mdr1b* and *mdr2* genes (Fig. 2).

In addition to the 350-kb *Not*I band, cp28 (and Ie7) revealed a *Not*I band in the compression zone (fragments larger than 1,600 kb). However, in the *Not*I-*Nru*I double digest, Ie7-cp28 detected a 650-kb band, which therefore represents the distance between two of these restriction enzyme sites across the *mdr1a* gene (Fig. 2). Ie7/cp28 hybridizations to a *Nru*I digest alone displayed a 1,000-kb band which represents the distance between the two adjacent *Nru*I sites surrounding the *mdr1a* gene, one of which must be identical to the *Nru*I site at one end of the 650-kb *Not*I-*Nru*I fragment (Fig. 2). Similar to the findings for the *Not*I-*Nru*I digests, Ie7-cp28 identifies a smaller band in the *Not*I-*Mlu*I digest (400 kb) than in the *Not*I or *Mlu*I digest alone (>1,600 and 1,200 kb, respectively). Therefore, an *Mlu*I site must be located 400 kb to the left of the *Not*I site next to the *mdr1a* gene. A second *Mlu*I site is located 1,200 kb from this *Mlu*I site across the *mdr1a* gene (Fig. 2, far right). In addition to the 1,200-kb fragment, Ie7-cp28 identifies a somewhat larger fragment and a band in the compression zone in the *Mlu*I digest (Fig. 2). This multitude of *Mlu*I fragments could be caused either by incomplete digestion or by heterogeneity in the amplicon population (see below). In the map, only the *Mlu*I site positioned by the 1,200-kb *Mlu*I fragment is shown. Finally, as predicted from the previously mapped *Mlu*I and *Nru*I sites, Ie7-cp28 detected a 750-kb fragment in the *Mlu*I-*Nru*I digest (Fig. 2).

The sorcin cDNA clone (cp6) was used to expand the

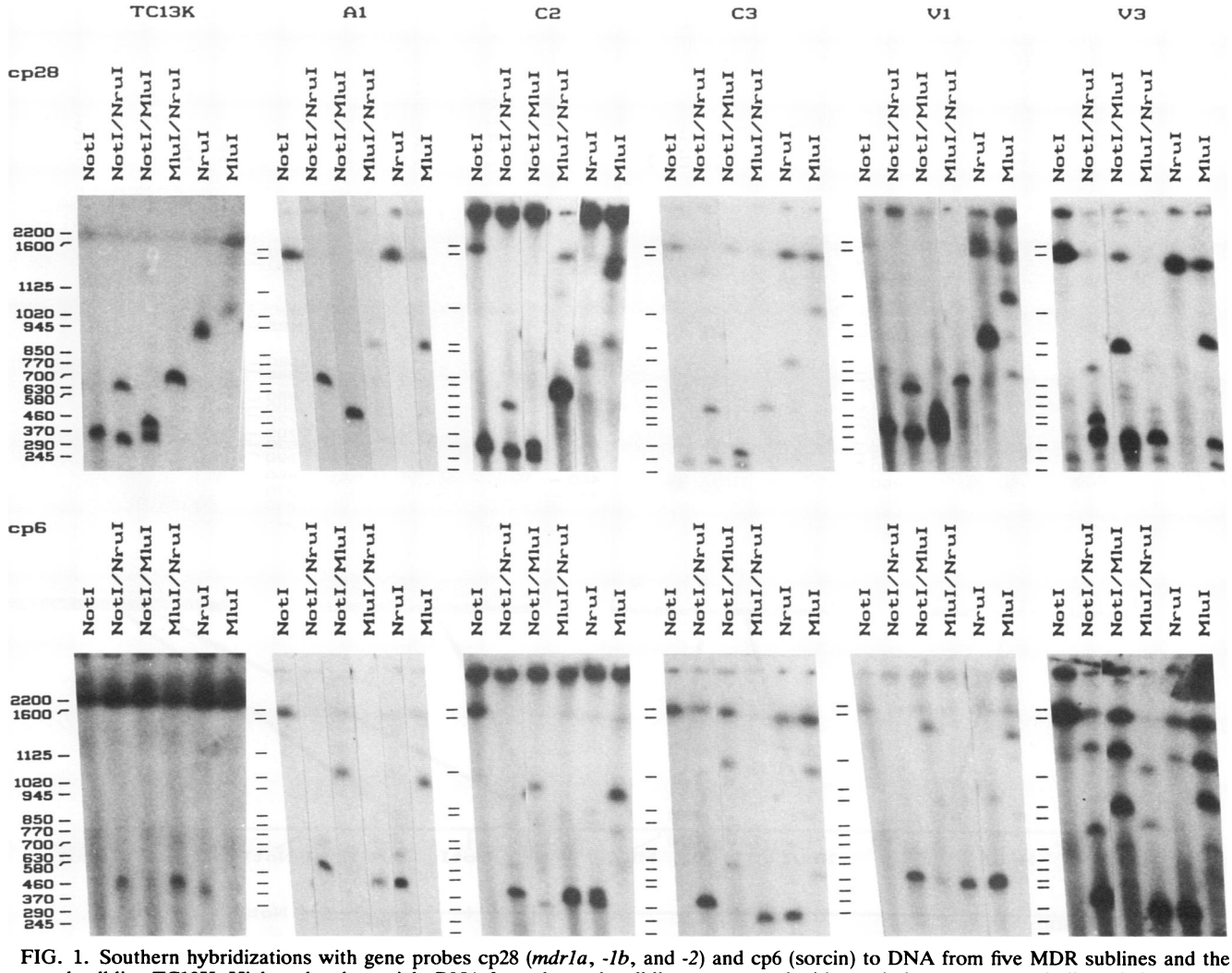

FIG. 1. Southern hybridizations with gene probes cp28 (*mdr1a*, *-1b*, and *-2*) and cp6 (sorcin) to DNA from five MDR sublines and the parental cell line TC13K. High-molecular-weight DNA from the each cell line was treated with restriction enzymes as indicated above each lane. The DNAs were separated by PFGE and hybridized with cp28 and cp6. Exposure time for each blot was about 2 days except for the TC13K blot, for which the exposure time was prolonged (up to 10 days). Size markers (indicated in kilobases) were chromosomes from *Saccharomyces cerevisiae*. Note that in the MDR sublines after *Mlu*I digestion, cp6 hybridization reveals bands (at different positions in the different sublines) which are not present in TC13K.

mapped region. This gene has repeatedly been found to be coamplified with the *mdr* genes (31, 40). Like the Ie7-cp28 probes, cp6 detected a *Not*I band in the compression zone (>1,600 kb), whereas the *Not*I-*Nru*I double digest and the *Not*I-*Mlu*I double digest generated bands of smaller sizes (475 and 900 kb, respectively; Fig. 2). In contrast to the Ie7-cp28 hybridizations, the sizes of these double-digest fragments did not differ from those obtained from digestion with *Mlu*I or *Nru*I alone (Fig. 2). Consequently, no *Not*I site is present in this region. Furthermore, in the *Mlu*I-*Nru*I double digest, cp6 revealed a band of the same size as in the *Nru*I digest. Thus, the two *Nru*I sites flanking this 475-kb fragment must be nested between the two *Mlu*I sites of a 900-kb fragment (Fig. 2). It is therefore not possible to determine the exact position of these *Nru*I sites relative to the *Mlu*I sites. This dilemma can be resolved if the region mapped with cp6 is located adjacent to region mapped with Ie7-cp28. In that case, the 475-kb *Nru*I fragment should be located next to the 650-kb *Not*I-*Nru*I fragment detected with Ie7-cp28. To investigate this matter, DNA was fully restricted with *Not*I and then partially digested with *Nru*I. A fragment of approximately 1,100 kb was detected, to which both the cp6 and the Ie7 probes hybridized (Fig. 3). This fragment matched the size of a predicted *Nru*I partial product, since the sizes of the cp6 *Nru*I 475-kb and the Ie7-cp28 *Not*I-*Nru*I 650-kb fragments add up to 1,125 kb (Fig. 2).

The connection between the maps generated by cp6 and Ie7-cp28 was further confirmed by a third probe (cp30). This probe represents a gene designated class 5, which has also been found to be coamplified in several MDR cell lines (31, 37, 41). cp30 revealed banding patterns which included bands identical in size with bands displayed both by cp6 and by cp28-Ie7 (for example, see Fig. 2, *Nru*I lanes). Thus, the two map regions could be brought together into a single map.

Structure of amplicons. The mapping procedure presented above reveals that the similarity in banding pattern among TC13K and MDR sublines A1, C1, C2, C3, and V1 involves restriction enzyme fragments which define the central part of

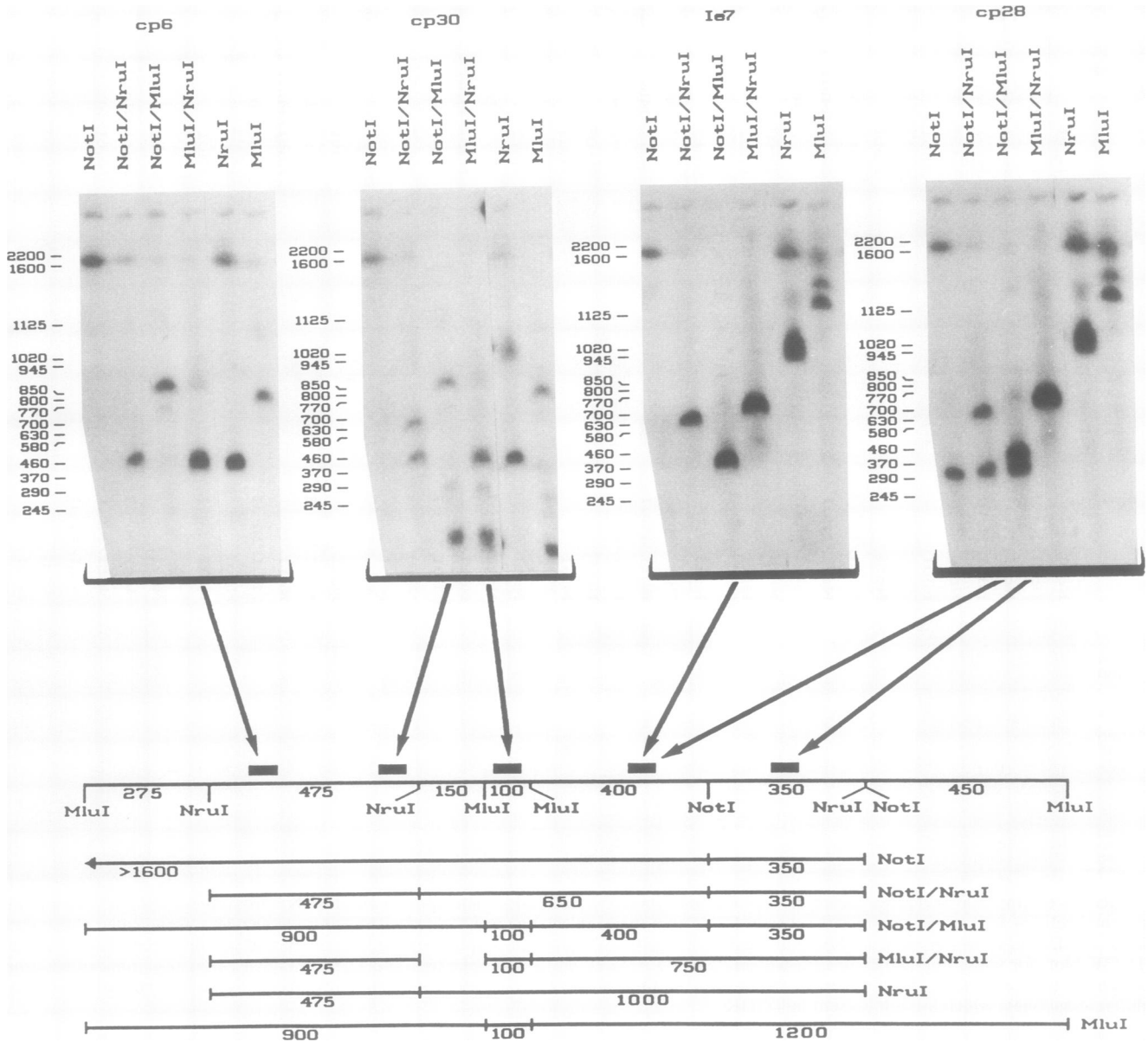

FIG. 2. Long-range mapping with PFGE of the MDR cell lines. The mapping of C1 is shown as an example of the procedure. Sublines A1, C2, C3, and V1, as well as the parental line TC13K, were subjected to the same procedure and gave the same results with the exceptions specified in the text. Two probes homologous to the *mdr* genes (cp28 to *mdr1a*, *mdr1b*, *mdr2*; Ie7 to *mdr1a*) and two probes for their neighboring genes (cp30 to class 5; cp6 to sorcin) were hybridized to six different digestions of high-molecular-weight DNA as indicated at the top. The derived map is presented below, with black boxes representing the approximate position of regions homologous to the probes. The lines below the map indicate the sizes (in kilobases) of the different restriction fragments detected with the four probes, as shown in their respective Southern blots. Only the smallest *Mlu*I fragment identified with Ie7 and cp28 is shown on the map. The larger *Mlu*I fragments presumably result from partial digestion at the rightmost *Mlu*I site and at one or more subsequent sites. Size markers (indicated in kilobases) were chromosomes from *Saccharomyces cerevisiae*.

the maps, whereas the differences in the fragments generated by the *Mlu*I digestion involve the distal parts of the maps. In Fig. 4, the derived restriction enzyme maps are aligned and the extent of these deviations can be compared.

At the left side of the map, defined by the cp6 probe, each of the five sublines A1, C1, C2, C3, and V1 displayed an *Mlu*I site that was not present in the parental cell line TC13K. The exact location of this *Mlu*I site varied among the sublines (Fig. 4). Weak additional *Mlu*I bands were observed occasionally with the cp6 probe in DNA blots from the five MDR sublines. These weak bands were possibly generated by cellular subpopulations with different amplicons.

At the right side of the map, defined by the cp28-Ie7 probes, four *Mlu*I sites are shown in the TC13K map (Fig. 4). This is an interpretation of the four bands repeatedly detected with the cp28-Ie7 probes in the *Mlu*I digestions of TC13K and in some of the sublines (Fig. 1). These four bands were probably caused by partial digestion of the *Mlu*I sites in this region. Since the cp28-Ie7 probes detected one

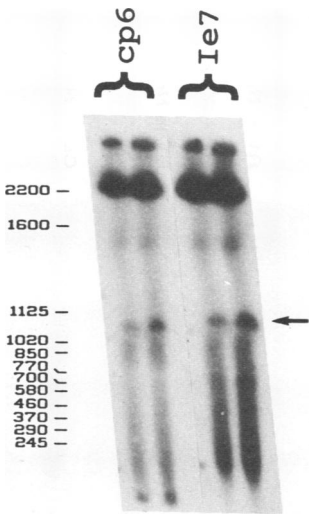

FIG. 3. Southern blots showing the association between the *mdr1a* gene (Ie7) and the sorcin gene (cp6). High-molecular-weight DNA from the MDR cell line C2 was fully digested with *Not*I and partially digested with *Nru*I, and the fragments were separated by PFGE. The two probes, cp6 and Ie7, were hybridized to two lanes differing only in digestion time with *Nru*I (left lanes, 15 min; right lanes, 30 min). Both probes identified a fragment of about 1,100 kb, indicated by an arrow. Size markers (indicated in kilobases) were chromosomes from *Saccharomyces cerevisiae*.

single double-cleavage product from the *Not*I-*Mlu*I double digestions (Fig. 1), the occurrence of partial digestion of the central *Mlu*I site (Fig. 4, position −400 kb) could be excluded. Thus, in order to generate the four *Mlu*I bands seen, the putative, infrequently digested *Mlu*I sites must be located toward the right hand side of the map. The relative intensity of the four *Mlu*I bands within each cell line differed among the sublines. One explanation of this observation could be the presence of different subpopulations within the sublines, with amplicons truncated at various positions.

Subline A1 differed from C1, C2, C3, and V1 by not showing amplification of the fragments detected with cp28-Ie7 in the *Mlu*I and *Nru*I cleavages, nor did it exhibit the 350-kb *Not*I fragment specifically detected by cp28 (Fig. 1). Thus, a recombinant site must be present relatively close to the remaining *Not*I site (Fig. 4). Hybridization to the compression zone in the *Nru*I digest with Ie7-cp28 as well as the unique 900-kb *Mlu*I fragment (also detected with Ie7-cp28) further confirms this notion (Fig. 1 and 4).

Linearization of circular amplicons. In all of the MDR sublines except A1, DM were the predominant cytological anomaly. Occurrence of DM is a well-known sign of gene amplification. Since DM have been suspected to be circular, a test for circularity in the mapped amplicons was carried out. Circular DNA can be linearized by γ irradiation, which introduces random double-stranded nicks into the DNA (39). DNA from the MDR sublines was treated with various doses of γ irradiation, subjected to PFGE, and probed with cp28. As examplified for subline C3, with which a fine adjustment of γ-irradiation dose was made (Fig. 5), a band of about 2,500 kb can be resolved at doses 5 and 10 Gy, reflecting a linearization of a circular molecule. This band becomes more difficult to observe at doses 30 and 50 Gy because of the background of degraded DNA. At doses of 70 and 90 Gy, the band is absent as a result of complete degradation from the γ irradiation. A very faint band, of 2,500 kb, can be detected in C3 even without γ irradiation (Fig. 5, lane 2). This band probably represents circular DNA, linearized by spontaneous degradation. At 30 to 90 Gy, some smaller bands can be seen. These may represent circles which have been nicked

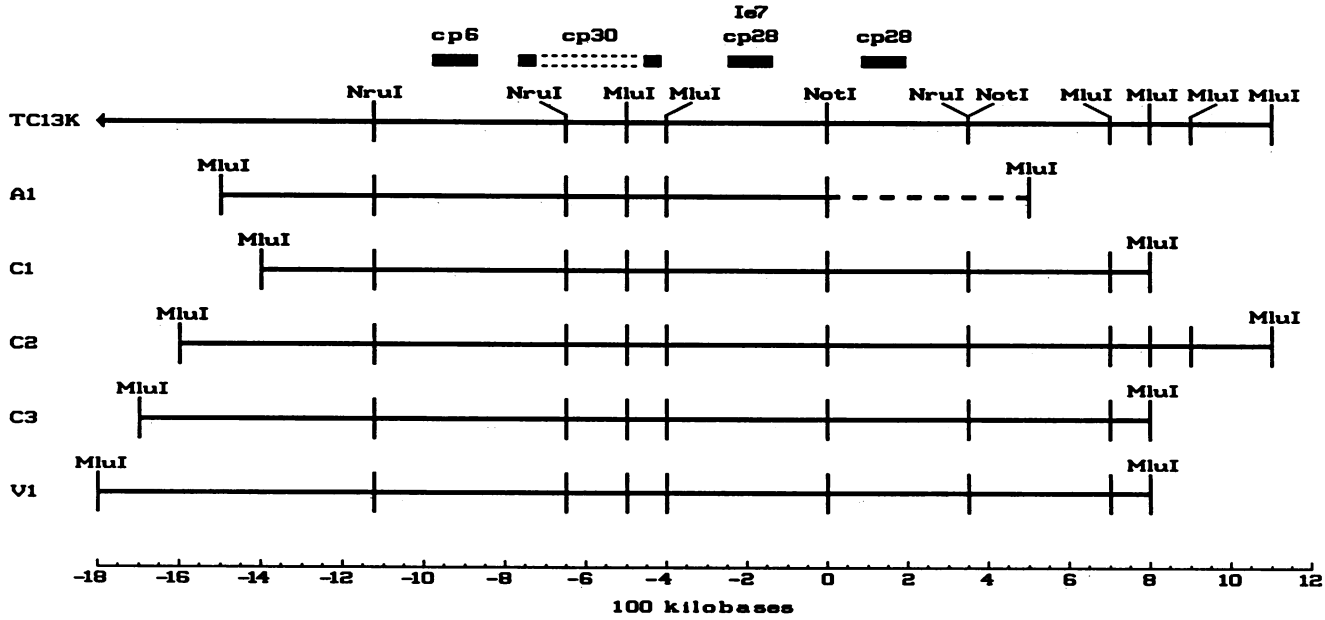

FIG. 4. Long-range restriction enzyme maps of five MDR sublines and the parental cell line TC13K. Black boxes represent the approximate positions of regions homologous to the probes. The box for cp30 is split, reflecting the hybridization pattern. When not indicated, the restriction enzyme sites in the maps of each MDR subline are the same as for the map of TC13K. The dashed line in A1 indicates that this region does not contain a region homologous to the cp28 probe positioned above. The zero point of the scale at the bottom of the figure was arbitrarily positioned at the central *Not*I site.

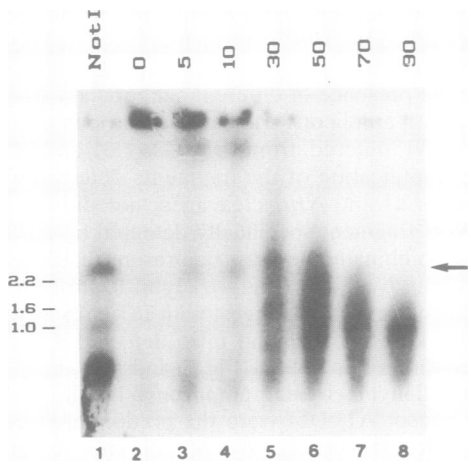

FIG. 5. Linearization of circular DNA molecules. The Southern blot of the MDR cell line C3 shows fine adjustment of the γ-irradiation dose. The DNA had been subjected to *Not*I digestion or increasing doses of γ irradiation (in grays), as indicated above the lanes. Fragments were separated by PFGE and hybridized with the *mdr* gene probe cp28. *Not*I digestion or γ-irradiation doses of 5 to 50 Gy generated an *mdr* gene-specific fragment of approximately 2,500 kb (marked by an arrow). Size markers (indicated in kilobases) were chromosomes from *Saccharomyces cerevisiae*.

more than once. Two hits would yield a population of molecules with the average size of approximately 1,250 kb, three hits would yield a population of about 800 kb, etc. Heterogeniety in the amplicon population is another explanation of these smaller fragments (see Discussion).

In Fig. 6A to C, hybridizations with cp28, Ie7, or cp6 to 10 Gy of γ irradiation on DNA from sublines A1, C1, C2, and V1, as well as the parental cell line TC13K, are shown. Like C3 (Fig. 5), sublines C1, C2, and V1 showed a band of approximately 2,500 kb, while A1 did not (see below). When digested with *Not*I under the same hybridization conditions, all five lines (including A1) displayed a band in the same size range (Fig. 5, lane 1; Fig. 6A to C). In addition, the *mdr1b*/*mdr2*-specific 350-kb *Not*I fragment is seen in the hybridizations with cp28 but not in the Ie7 and cp6 hybridizations (Fig. 5 and 6A). Taken together, these findings imply that in sublines C1, C2, C3, and V1, the amplicons are situated on circular DNA molecules of approximately 2,500 kb. The circles can be linearized either by an appropriate dose of γ irradiation or by digestion with *Not*I, which cuts at two sites 350 kb apart. The PFGE separation method was not sensitive enough to demonstrate the expected size difference between molecules linearized by the two different methods.

In contrast to the other sublines tested, no band was detected with the cp28, Ie7, and cp6 probes in γ-irradiated DNA samples from A1. Since A1 differed from the other sublines by exhibiting HSR in many cells, it is plausible that the HSR contained a substantial part of the amplified DNA sequences. Exposure of A1 DNA to γ irradiation would thus generate enhanced background hybridization caused by degraded HSR, which could cover linearized circles from other subpopulations. It is also conceivable that the amount of amplified DNA sequences residing in DM was too low in this subline to permit detection. However, like the other sublines, A1 displayed a band of about 2,500 kb when probed with cp28, Ie7, and cp6 after digestion with *Not*I (Fig. 6).

Control hybridizations were performed with a c-*myc*

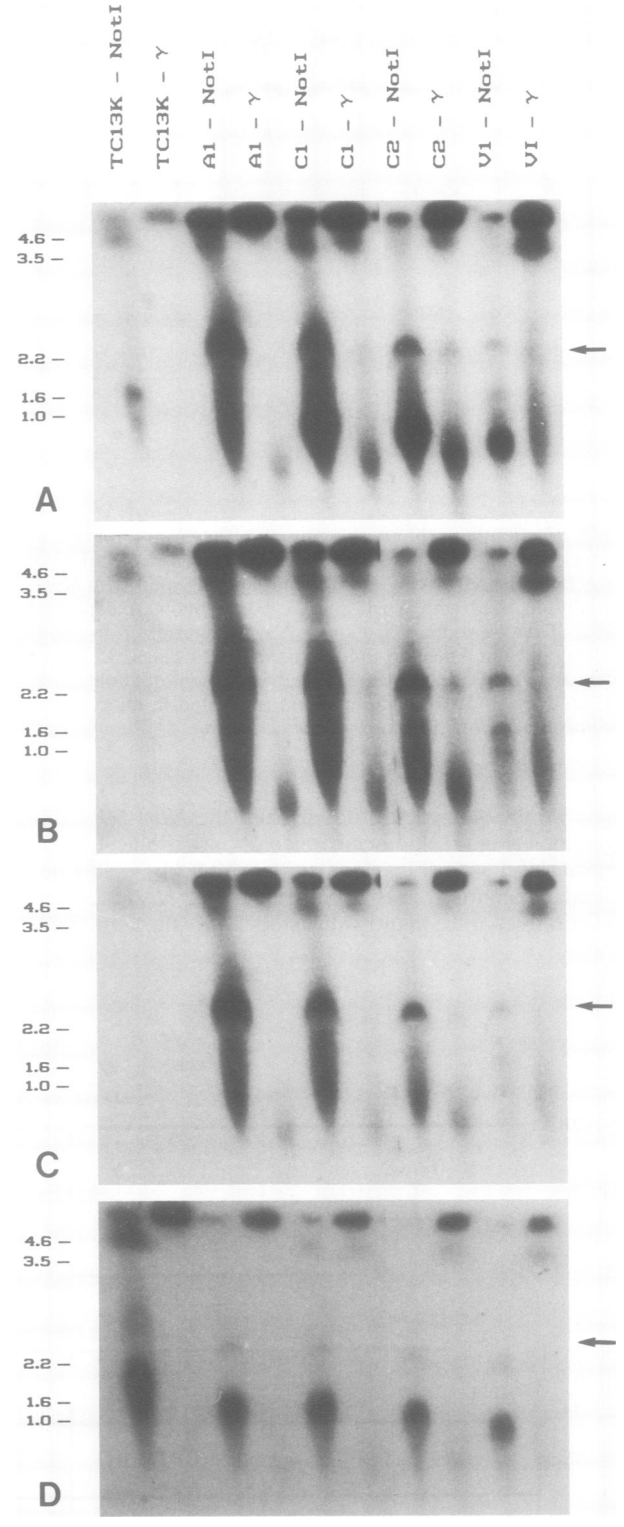

FIG. 6. Linearization of circular DNA molecules. Southern blots of four MDR sublines and the parental cell line TC13K were restricted with *Not*I or treated with γ irradiation (10 Gy). The resulting fragments were separated by PFGE and hybridized to cp28 (A), Ie7 (B), cp6 (C), and c-*myc* (D; control hybridization). Arrows at 2,500 kb indicate the approximate size of linearized circles (see text). Size markers (indicated in kilodaltons) were chromosomes from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

probe. The c-*myc* gene is known to be amplified to about 40 copies at one HSR in the parental line TC13K (22). As predicted, no c-*myc* fragment of any specific size was detected in DNA samples from the γ-irradiated sublines, whereas a 1,500-kb band and a weaker band, distinctly smaller than the 2,500-kb band, were found in the *Not*I-digested DNA samples (Fig. 6D). The more intense hybridization signal from TC13K corresponds to the threefold amount of DNA loaded in these lanes.

DISCUSSION

A restriction enzyme map of approximately 2,200 kb was constructed in the drug-sensitive SEWA mouse tumor cell line TC13K for the *mdr* gene family and its surroundings. Of six investigated MDR sublines, derived from TC13K, the five lines A1, C1, C2, C3, and V1 displayed amplification of this region without internal recombination. One subline, V3, showed extensive recombination in association with gene amplification.

Gene order. The linear order of the genes mapped could be determined as follows: sorcin (class 4)-class 5-*mdr1a-mdr1b-mdr2*. The relative order of the two latter *mdr* genes could not be resolved with the probes used, and the gene order shown was adopted from Raymond et al. (27). Since the *mdr* genes have already been mapped to chromosome 5 in the mouse (23), the sorcin and class 5 genes can be assigned to the same chromosome on the basis of the results presented here.

The gene order found was the same as in the hamster genome (7). In humans, a similar gene order has also been proposed, except for the fact that there is no gene that corresponds to the rodent *mdr1b* gene (37). This additional rodent *mdr* gene has been suggested to be due to duplication of a common ancestral gene of rodent *mdr1a* and *mdr1b* (4, 15, 26).

One circular amplicon per single DM unit. The occurrence of DM is the predominant cytogenetic sign of gene amplification in the majority of MDR cell lines examined. In general, DM have been shown to be the most common sites for gene amplification in mammals (42). DM are extrachromosomal chromatin structures without centromeres, and at cell division they are unequally distributed to the daughter cells (5, 16, 21).

Several authors have suggested that DM contain circular DNA (16, 28, 39). Utilizing the linearization effect of γ irradiation, we found that four (C1, C2, C3, and V1) of the five tested MDR sublines contained circular DNA molecules. These were all about 2,500 kb in size, and detection was accomplished with three of the probes from the amplicon region. When DNA from the same four cell lines was instead treated with the infrequently cutting *Not*I restriction enzyme, hybridizing fragments in the same size range were found with the same amplicon-specific probes. Thus, the amplified sequences in the four MDR sublines reside on circular DNA molecules, which contain two relatively adjacent *Not*I sites.

By comparing the results from the γ-irradiation linearization experiments of the four MDR sublines with the mapped amplicons of the same cell lines, further conclusions could be drawn. The rightmost *Mlu*I site mapped in these cell lines is unaltered in its locations compared with the parental cell line, TC13K. On the other hand, their leftmost *Mlu*I site is not found in TC13K, and its position varied among the four MDR sublines. If these *Mlu*I sites, located distally at both ends of the amplicon maps, in fact are one and the same, the distance between them defines the length of the amplicon (Fig. 4). For all MDR sublines, amplicon sizes calculated in this manner fall into the same size range as for their corresponding γ-irradiated, linearized circles. Since the leftmost *Nru*I site is unchanged, the joining site of the amplicon endings must be located somewhere between the leftmost *Nru*I and *Mlu*I sites in C1, C2, C3, and V1 (Fig. 4).

The limited region of recombination into circles in four MDR sublines indicates that sequences in this region might determine the site of circularization. Still, the *mdr1a* gene, which is actually selected for (33), is more than 1,000 kb from these recombination sequences. It may be that many different circular DNA molecules are generated from these sequences, but only those including the *mdr1a* gene prevail. This would explain both the very large size of the initial amplicon and the recurrent coamplification of genes located between the *mdr1a* gene and the presumed recombination sequences.

Our data thus indicate that in at least four independent instances, one single section of about 2,500 kb of nonrearranged DNA has been transferred into an extrachromosomal circular entity. This circular molecule is presumably identical to the single unit of a DM. These results are concordant with results of Van der Bliek and collaborators for a methotrexate-resistant mouse cell line (39). Using similar methods, these authors demonstrated the presence of a homogeneous population of circular DNA in the DM-carrying cells. Recently, Jongsma and collaborators (19) concluded that those circles were identical to single units of DM.

The occurrence of heterogeneity in amplicon sizes within the different MDR sublines cannot be excluded, not even in C1, which carries a specific marker chromosome in all cells (33). The γ irradiation revealed several smaller bands which did not match the size of the mapped amplicon and could be due to amplicon heterogeneity (Fig. 5). However, if several smaller amplicons coexist with the amplicon of approximately 2,000 to 2,500 kb that we have mapped, they must either display a nonrearranged subpart of this larger amplicon or be of such low frequencies that they are not recognized in the restriction enzyme mapping. Otherwise, a complex banding pattern, similar to that of V3, would appear. Furthermore, the band of approximately 2,500 kb recognized in the γ irradiations can be associated only with the 2,000- to 2,500-kb amplicon maps and not with smaller subparts belonging to amplicons of smaller sizes.

In the SEWA system, we have previously noted a decrease in the size of DM with time (24). Such a gradual decrease in the size of DM has also been suggested by Borst and collaborators (1). A gradual decrease in amplicon size could be due to recombination of large circular amplicons into smaller circles, which may differ among themselves, but only those that contain the *mdr1a* gene will prevail. Consistent with this notion, the four MDR SEWA cell lines (C1, C2, C3, and V1) exhibiting nonrearranged circles may be in a less advanced stage of MDR progression than is V3, which exhibits recombination and amplification of the *mdr1a* gene to a higher level than do neighboring, coamplified, genes (33).

Head-to-tail arrangements in HSR. In the fifth MDR subline (A1) subjected to γ irradiation, no 2,500-kb band was seen following hybridization with the amplicon-specific probes. However, this cell line differed from the other MDR sublines by exhibiting a subpopulation of HSR-containing cells that presumably contained a large proportion of the amplified sequences. When digested with *Not*I, a fragment of about the same size as for the four other MDR sublines

was detected in A1 with the amplicon-specific probes. Since only a single *Not*I site is present in the map of the amplified region of A1 (Fig. 4), this fragment probably reflected a head-to-tail arrangement of tandemly amplified regions in the HSR of A1.

Models for the genesis of DM. Several models have been put forward to explain the genesis of DM. The onion skin model, which requires rereplication during a single cell cycle (35), is a possible candidate. However, the large size of unrearranged amplicons in our MDR sublines would require the "misfiring" of several consecutive origins of replication, which seems rather unlikely. Another possibility is the formation of pre-DM bodies by the excision of the DNA sequence containing the gene selected for. These so-called episomes are proposed to consist of an entire replicon loop containing a single replication origin (42). Amplification of the human *mdr1* gene in an MDR human cell line was recently found to be in good agreement with the episome model (28). Since the episomes analyzed so far range in size from 120 to 750 kb, it is thought that these relatively small pre-DM bodies increase in size by a recombination process. This should generate DM with extensively recombined sequences such as those present in our V3 subline. However, the presence of large regions without recombination, such as the amplicons in our five other sublines, is not consistent with this model. Still, an initial step of excision cannot be excluded.

Another model suggested for the genesis of DM is the breakage of prematurely condensed chromosomes into DM. This model was recently proposed for the amplification of the *mdr* gene family in an MDR Chinese hamster ovary cell line (29). Although only circumstantial evidence exists for this model, it permits the generation of large unrearranged circles as an initial amplification product.

Species dependence in the pathway of DM generation? In a recent review by Wahl, the majority (five of seven) of the cell lines found to fit the episome model were of human origin (42). In the work presented here, gene amplification must have been generated by another mechanism in five of the six murine MDR sublines. Our data thus favor the notion that several different mechanisms may generate DM. Furthermore, our data indicate that in a specific cell line, a certain mechanism may be favored, a preference that might be species dependent.

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