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Differential Amplification and Disproportionate Expression of Five Genes in Three Multidrug-Resistant Chinese Hamster Lung Cell Lines

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At least five linked genes are amplified in the multidrug-resistant Chinese hamster ovary cell line CH^{R}C5, selected with colchicine (A. M. Van der Bliek, T. Van der Velde-Koerts, V. Ling, and P. Borst, Mol. Cell. Biol. 6:1671–1678, 1986). We report here that only a subset of these, encoding the 170-kilodalton P-glycoprotein, are consistently amplified in three different multidrug-resistant Chinese hamster lung cell lines, selected with vincristine, daunorubicin, or actinomycin D. Within each cell line, genomic sequences homologous to the P-glycoprotein cDNA probe were amplified to different levels. The pattern of differential amplification was consistent with the presence of at least two and possibly three P-glycoprotein genes. In the actinomycin D-selected cell line, these genes were disproportionately overexpressed relative to the associated levels of amplification. These results underline a central role for P-glycoprotein in multidrug resistance. In the daunorubicin-selected cell line, another, as yet uncharacterized, gene was amplified but disproportionately underexpressed. Its amplification was therefore fortuitous. We present a tentative map of the region in the hamster genome that is amplified in the multidrug-resistant cell lines which were analyzed.

Drug resistance constitutes a serious obstacle to effective cancer chemotherapy. When induced experimentally in mammalian cells by selection with a specific drug, it is frequently accompanied by a complex pattern of cross-resistance to various functionally and chemically unrelated drugs (4). This phenomenon of multidrug resistance may explain why some cancer patients who have undergone successful initial drug treatment relapse and fail to respond to subsequent combination chemotherapy (25). Since the highest resistance in vitro is usually found to the drug used in selection, the pattern of cross-resistance is not constant, suggesting that the underlying mechanism is a multicomponent system. Resistance is the result of a decrease in the intracellular drug concentration (8, 13), presumably due to alterations in the cellular membrane. A potential component of this system, the 170-kilodalton (kDa) P-glycoprotein, is overproduced in many multidrug-resistant cell lines; the level of overproduction correlates well with the degree of resistance (3, 24–26). Variations in the level of a number of other proteins have also been associated with multidrug resistance but were specific for either the selective drug or the cell type (18–21).

The overproduction of proteins associated with mammalian drug resistance is often the result of gene amplification (7, 12, 29, 33). To identify the genes determining the multidrug-resistant phenotype, we have constructed a cDNA library from the resistant Chinese hamster ovary (CHO) cell line CH^{R}C5 and isolated cDNA clones derived from overproduced transcripts (36). These could be grouped into five classes by the length of represented transcripts and correspond to genes that are amplified 10 or 30 times in CH^{R}C5 DNA. Class 2 cDNAs encode the overproduced P-glycoprotein. By pulsed-field gradient analysis we have shown that the genes in the CH^{R}C5 cell line are located on two large DNA fragments, which are probably linked to form an amplicon at least 1,100 kilobases (kb) long. The genes are also transcribed in the drug-sensitive AuxB1 parental line, and sequences of all five cDNA classes are conserved in mouse and human DNA (36; unpublished results). This suggests that the encoded products are normal components of some differentiated cells.

Important questions remain. Is amplification of all five gene classes essential for multidrug resistance per se? Is there any correlation between amplification of one or more genes and the specific pattern of cross-resistance displayed by a cell line? Are any of the genes nonessential and fortuitously amplified because they happen to be located close to essential genes? To answer these questions, we have extended our analysis of the five gene classes identified in the CH^{R}C5 line to a series of three multidrug-resistant Chinese hamster lung (CHL) cell lines (4, 19, 20, 22), each of which was selected stepwise with a different drug and has a specific pattern of cross-resistance (Table 1). Each of these lines bears the cytogenetic hallmarks of gene amplification (5), e.g., homogeneously staining regions in the VCRd-5L and DMXX lines (2, 19) and an abnormally banding region in the ADX line (2).

MATERIALS AND METHODS

Cell culture. The cell lines were cultured as described previously (4, 15, 19, 20, 22) by standard procedures. The media for the cell lines DC-3F/VCRd-5L, DC-3F/DMXX, and DC-3F/ADX contained vincristine sulfate (50 μg/ml; Oncovin, Eli Lilly & Co.), daunorubicin (10 μg/ml; Cerubidine, Rhône Poulenc), and actinomycin D (10 μg/ml; Sigma Chemical Co.), respectively. All media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml.

DNA preparation, blotting, and nick translation. The isolation of plasmid DNA from *Escherichia coli* and of genomic DNA from cultured cells and the digestion of these DNAs with restriction endonucleases have been described (6, 16).

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TABLE 1. Cross-resistance in multidrug-resistant CHL cell lines

Cell line	Relative resistance[a]			
	Vincristine	Daunorubicin	Actinomycin D	Colchicine
DC-3F	1	1	1	1
DC-3F/VCRd-5L	2,750[b]	178	267	1,000
DC-3F/DMXX	285	883[b]	263	700
DC-3F/ADX	1,400	54	2,450[b]	280

[a] Ratio of the 50% effective dose for a resistant line and for the control line DC-3F. Values are taken from reference 15 and references therein.
[b] Drug used in selecting that resistant cell line.

Digests of genomic DNA were considered complete if phage lambda DNA added to a sample of the incubation mixture was completely digested. The DNA fragments were fractionated in 1% agarose gels at 2.25 V/cm for 20 h and transferred to nitrocellulose (Schleicher & Schuell, 0.45-μm pore size) by standard procedures (16, 32). The blots were prehybridized for 2 h at 65°C with 50 μg of sonicated and denatured salmon sperm DNA per ml in 3× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.5)–0.1% sodium dodecyl sulfate (SDS)–5× Denhardt solution. Hybridizations were done for 20 h at 65°C with gel-purified and nick-translated (23) cDNA fragments. The final wash following hybridization was for 30 min at 65°C in 0.1× SSC–0.1% SDS. Autoradiography was done at −70°C on Kodak X-Omat S or AR-5 film with Dupont Cronex Lightning-Plus screens.

RNA isolation, fractionation, and blotting. For isolation of RNA the LiCl-urea method was used (1). DNA was removed by DNase I treatment, and polyadenylated [poly(A)$^+$] RNA was purified by oligo(dT)-cellulose column chromatography (16). RNA samples were denatured and glyoxylated by the method of Thomas (35) and fractionated at 1.5 V/cm for 20 h in gels containing 1.5% agarose and 10 mM phosphate buffer, pH 6.5. After transfer to nitrocellulose (35) the RNA blots were prehybridized for 2 h at 42°C with 100 μg of sonicated and denatured salmon sperm DNA in 40% formamide–0.9 M NaCl–5 mM EDTA–0.1% SDS–0.1% Ficoll 400–0.1% polyvinylpyrrolidone–10% dextran sulfate–50 mM sodium phosphate buffer (pH 6.5). Hybridizations were done for 20 h at 42°C. Probes and further treatment of RNA blots were as described for DNA blots.

To quantitate the degree of overproduction of a particular transcript in a resistant cell line relative to the parental line, 6-μl samples of a dilution series of poly(A)$^+$ RNA in water from the resistant line and of undiluted parental poly(A)$^+$ RNA were denatured by boiling for 1 min and immediately chilled. The samples were then combined with 9 μl of ice-cold 5 M NaCl and spotted onto a nitrocellulose filter under mild suction. The filter had been prewetted in 20× SSC. Hybridization and further treatment were as described for RNA blots. After autoradiography the RNA-containing areas were excised from the filter, and the amount of radioactivity was quantitated by liquid scintillation counting.

RESULTS

High-molecular-weight DNA and poly(A)$^+$ RNA were prepared from cultures of the three resistant CHL cell lines DC-3F/VCRd-5L, DC-3F/DMXX, and DC-3F/ADX, the drug-sensitive parent line DC-3F, and the resistant CHO line CH^{R}C5. Equal amounts of *Eco*RI-digested DNA from each cell line were fractionated on agarose gels and transferred to nitrocellulose. The blots were hybridized to a labeled cDNA corresponding to one of the five transcript classes detected in CH^{R}C5 cells (Fig. 1). Each probe detected a unique set of DNA fragments which were identical in each of the cell lines compared. However, corresponding fragments in the five cell lines were mostly amplified to different levels and in many instances were not amplified at all. Fragments that were never amplified (Fig. 1, class 1 and 4) were presumably derived from homologous genes or pseudogenes. Equal amounts of DNA were used in each lane as judged by rehybridization of the blots to labeled dihydrofolate reductase (DHFR) cDNA from plasmid pR400-12 (31) (data not shown). Levels of amplification were determined by comparing a dilution series of *Eco*RI-digested DNA from a resistant CHL cell line to undiluted digested DC-3F DNA following hybridization to a cDNA from one of the five classes. This is illustrated for class 2 in Fig. 2. The amplification levels are summarized in Table 2. No assumptions are

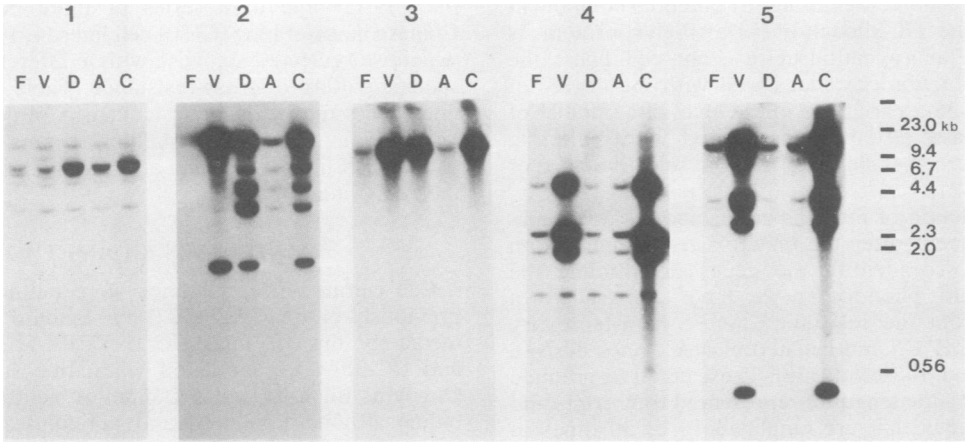

FIG. 1. Autoradiograms showing differential gene amplification in multidrug-resistant Chinese hamster cells. The resistant CHL lines DC-3F/VCRd-5L (lanes V), DC-3F/DMXX (lanes D), and DC-3F/ADX (lanes A) were compared with the drug-sensitive parental line DC-3F (lanes F) and the resistant CHO line CH^{R}C5 (lanes C). The lanes contain 10 μg of *Eco*RI-digested genomic DNA. The *Eco*RI fragments were visualized by hybridization to the cDNA probes cp19 (class 1), cp28 (class 2), cp16 (class 3), cp6 (class 4), and cp30 (class 5) derived from five classes of transcripts that are overproduced in the CH^{R}C5 line (36). The class is indicated above each panel. The relatively weak signal with the class 2 probe in lanes F and A is more clearly visible in Fig. 2. The size marker was *Hin*dIII-digested phage lambda DNA. See reference 36 for other details.

made about the ploidy of the cell lines or the mechanism of amplification. DC-3F cells have chromosome numbers in the diploid range (4), and if amplification only affects one allele, the actual levels of amplification could be twice those listed. However, these cells do contain structurally altered chromosomes (19), and therefore the correction factor may not be the same for all five gene classes. This is illustrated by the class 4 and 5 gene copy numbers in the DMXX line, which were only half those found in the parental cell line (Fig. 1, Table 2).

A similar analysis was carried out at the RNA level. Since the five cell lines contain the same number of DHFR gene copies, the concentrations of the poly(A)$^+$ RNA preparations were normalized by dot blot hybridization (14) to the labeled DHFR probe mentioned above (data not shown). Equal amounts of RNA were then fractionated on agarose gels and transferred to nitrocellulose filters. Each RNA blot was hybridized to one of the nick-translated cDNAs also used for the DNA blots (Fig. 3). With the exception of the class 4 probe the cDNAs recognized one major specific transcript each. Between cell lines, these transcripts could differ in quantity but not in size. The class 4 probe recognized two major transcripts, and the relative amounts of these were not constant between cell lines. The relationship between these transcripts is not known. The degree of overexpression of the five classes of transcripts in each resistant CHL line relative to the drug-sensitive parent line DC-3F (Table 2) was assessed by comparing a dilution series of poly(A)$^+$ RNA from a resistant cell line to undiluted DC-3F poly(A)$^+$ RNA by dot blot hybridization. The RNA levels in the three resistant CHL lines were roughly proportional to the corresponding levels of gene amplification, with two exceptions that will be discussed below.

DISCUSSION

It is immediately apparent from Fig. 1 and 3 that the multidrug-resistant CHL cell lines did not contain the same complement of amplified genes as the CHO line CH^{R}C5, although all five gene classes were present in the parent CHL line DC-3F and were amplified in one or more of the resistant lines. Only class 2 cDNA, encoding the 170-kDa P-glycoprotein, corresponded to sequences that were consistently amplified in all three resistant CHL cell lines. In these lines a 150- to 180-kDa glycoprotein is overproduced (20), which presumably is identical to the P-glycoprotein.

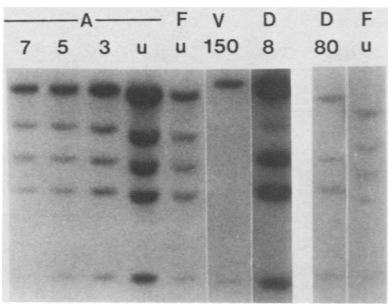

FIG. 2. Autoradiograms showing quantitation of the amplification levels detected with the class 2 probe in the resistant CHL lines. Lanes designated by u contain 10 μg of *Eco*RI-digested DNA from the cell line indicated. Other lanes contain this amount of DNA diluted by the factor indicated above individual lanes. The electrophoretic mobility of DNA fragments varied by lane with the amount of DNA applied. For other details see the legend to Fig. 1.

TABLE 2. Gene amplification and overexpression in multidrug-resistant CHL cell lines[a]

Gene class	DC-3F/VCRd-5L		DC-3F/DMXX		DC-3F/ADX	
	DNA	RNA	DNA	RNA	DNA	RNA
1	1	2	6	7.5	4	6.5
2[b]	1, 150	53	8, 80	85	4, 6	44
3	40	69	30	3	1	3.5
4	30	51	0.5	0.5	1	1.5
5	30	61	0.5	1	1	3

[a] Amplification (DNA) and overexpression (RNA) levels are expressed as the ratio of DNA or RNA concentrations for the control cell line DC-3F and for a resistant line needed to obtain equal hybridization signals with a cDNA probe corresponding to the gene class indicated.

[b] The amplification levels given for class 2 genes represent the two different levels seen for class 2 gene fragments (Fig. 1). They are proposed to correspond to three different class 2 genes as indicated in Fig. 4.

Multiplicity of P-glycoprotein genes. The VCRd-5L, ADX, and CH^{R}C5 lines differed widely in the actual levels of amplification detected with the class 2 probe, but were qualitatively similar in displaying two levels of amplification that affected identical DNA fragments. Of the five fragments in the *Eco*RI digest (Fig. 1 and 2), the three of intermediate size were not amplified in the VCRd-5L line, amplified 4-fold in the ADX line, and amplified 10-fold in the CH^{R}C5 line (Table 2) (36). The two remaining fragments were amplified 150-fold, 6-fold, and 30-fold, respectively, in the three cell lines. Different levels of amplification within a single domain can arise through gain or loss of amplified DNA during the multiple-step selection process imposed and can be due to DNA rearrangements. These frequently accompany amplification in drug-resistant cell lines generated by stepwise selection (33). The identical outcome in three independently selected cell lines, however, strongly suggests the presence of at least two genes.

In the DMXX line the three intermediately sized DNA fragments themselves were amplified to different levels (Fig. 2). This could be due to rearrangement within the second gene, which by coincidence did not generate a change in size distribution of the DNA fragments (Fig. 1). We favor the alternative interpretation, that our class 2 probe recognized at least three P-glycoprotein genes. This was corroborated by the finding of sequence heterogeneity within P-glycoprotein cDNAs, which indicates the presence of at least three partially homologous genes in CHO cells (10).

Disproportionate overexpression of P-glycoprotein genes. The degree of class 2 mRNA overproduction in DC-3F/ADX cells was 7.5- to 11-fold higher than the level of gene amplification, depending on how many different P-glycoprotein genes were expressed (Table 2). In the VCRd-5L and DMXX lines this difference was at most threefold and within the limits of experimental error for the titration methods used. The high level of P-glycoprotein mRNA in ADX cells therefore cannot be explained by constitutive expression of extra gene copies alone. One possible explanation is that the promoter region of a P-glycoprotein gene has been affected by a rearrangement or mutation, leading to an increased rate of transcription. Although other explanations cannot be ruled out and remain to be tested, the normally small chance of a promoter upmutation may have increased by the combined presence of multiple gene copies and a selective environment. Adenosine deaminase overproduction in deoxycoformycin-resistant CHO cells (27) and argininosuccinate synthetase overproduction in canavanine-resistant human cells (34) have been observed without corresponding

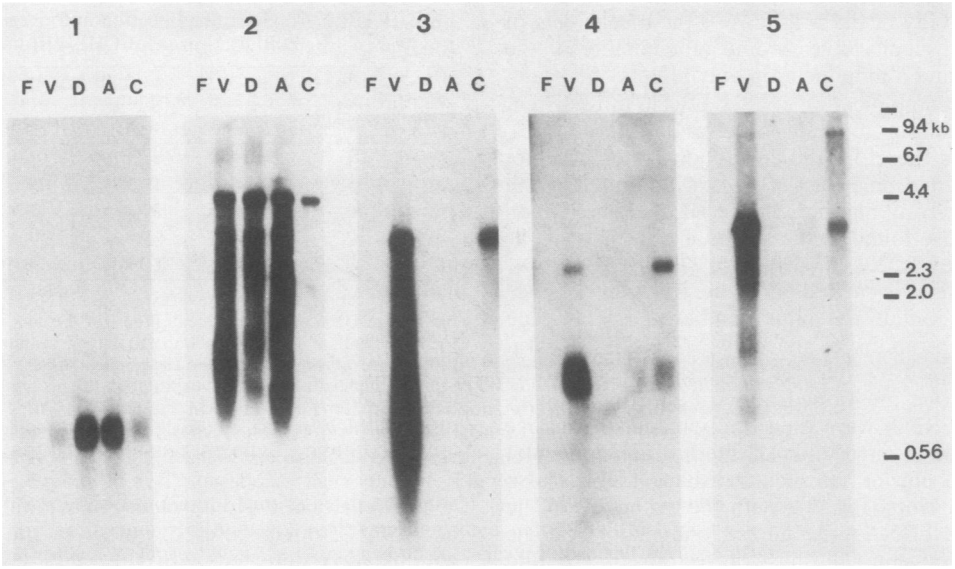

FIG. 3. Autoradiograms showing differential overproduction of five classes of mRNA in multidrug-resistant Chinese hamster cells. The lanes contain 1 μg of poly(A)$^+$ RNA. The size marker was *Hin*dIII-digested phage lambda DNA, which was labeled by filling in of fragment ends (28) and treated as poly(A)$^+$ RNA. Specific RNAs were visualized as described in the legend to Fig. 1.

gene amplification and may therefore be achieved in a way similar to P-glycoprotein overproduction in ADX cells. Disproportionate overproduction of mRNA in the ADX line and a more resistant subline has recently also been observed with another cDNA probe which appears to recognize class 2 genes (30).

Disproportionate underexpression of the class 3 gene. DMXX cells failed to express a large number of class 3 gene copies (Table 2). Since this gene was both amplified and overexpressed in VCRd-5L cells, the gene copies in DMXX cells must have been inactivated. Loss of function would occur if only part of the gene was amplified or if the amplified gene contained internal rearrangements. While the proposed rearrangement of P-glycoprotein genes in ADX cells may have occurred at any time during the amplification process and may have affected as little as one gene copy, the class 3 inactivation in DMXX cells must have taken place at a very early stage to have silenced most of the gene copies present in the amplified domain. The underexpression of the class 3 gene proves that this gene plays no role in the multidrug resistance displayed by DMXX cells and must have been fortuitously coamplified due to its proximity to essential genes. Precedents for such coamplification have been found with functional amplification of the adenylate deaminase gene and of the CAD gene complex (9, 37).

Molecular basis for cross-resistance. No clear correlation has emerged between the patterns of cross-resistance displayed by the three resistant CHL lines (Table 1) and the overexpression of one gene or another (Table 2). In fact, the DMXX and ADX cells corresponded closely in terms of overexpression, but varied widely in their level of cross-resistance to the four drugs tested. We therefore tentatively

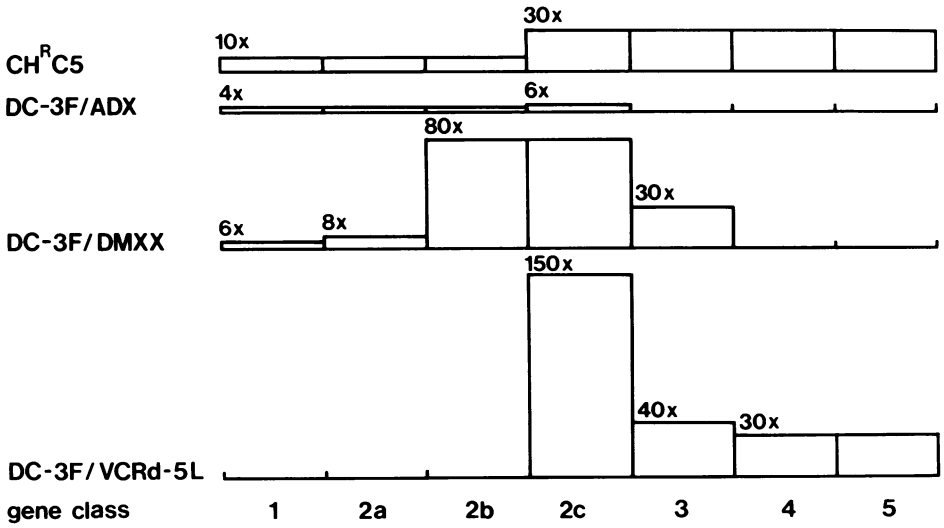

FIG. 4. Tentative map of genes amplified in multidrug resistance. The gene order (bottom line) is based on pulsed-field gradient analysis of the CH^{R}C5 genome (36) and on the amplification levels of the individual genes (represented by blocks) in the four resistant cell lines compared. The amplification levels are indicated above the blocks. The order of genes 4 and 5 may be reversed.

conclude that the four gene classes other than class 2 are fortuitously coamplified. It is possible that other genes in the amplicon, not included in the five classes cloned thus far, will correlate with cross-resistance patterns. An attractive alternative, also suggested by others (24), would be that the P-glycoprotein gene family itself is responsible for the pattern of cross-resistance by differential overexpression of its members. Regardless, the central role of the P-glycoprotein genes in multidrug resistance emerges clearly from our analysis and was accentuated by the disproportionate overexpression of these genes in ADX cells. This does not imply that coamplified genes are nonfunctional. They could be responsible for many of the variations in protein levels and other phenotypic characteristics observed in multidrug-resistant cells.

Organization of the amplified domain. Coamplification of genes implies that they are linked in the genome of the parental cell. This linkage may have been obscured in the final amplicon of the resistant cells discussed here by rearrangements accompanying repeated rounds of stringent selection. Nevertheless, pulsed-field gradient analysis of the CH^RC5 genome, using cDNAs corresponding to the five gene classes as probes, provided evidence for linkage of class 1, 2, and 3 genes on the one hand and of class 4 and 5 genes on the other (36). One of the arguments for linkage between these two sets of genes is that classes 2 to 5 were amplified to the same level. This would be unlikely if the two sets of genes belonged to different amplicons. The data now available allow a rough prediction of how the five gene classes are ordered in a normal hamster cell. One should include at least three P-glycoprotein genes (genes 2a to 2c), which are probably clustered. Individual genes within the cluster that were amplified to the same level were assigned to neighboring positions. Because genes 1, 2a, and 2b in CH^RC5 cells were amplified 10-fold and the remaining genes 30-fold and with the assumptions made above, the genes can be ordered as 1-(2a, 2b)-2c-3-(4, 5). The amplification patterns of the independently selected ADX, DMXX, and VCRd-5L cell lines are consistent with this map (Fig. 4). Since DMXX cells amplified genes 2a and 2b to different levels, their order relative to genes 1 and 2c becomes fixed while genes 4 and 5 remain interchangeable. The predicted gene order in the parental genome therefore would be 1-2a-2b-2c-3-(4, 5). The size of the region amplified in the CH^RC5 line is at least 1,100 kb (36).

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