[CANCER RESEARCH 47, 2875–2878, June 1, 1987]

Chromosomal Localization of Three Genes Coamplified in the Multidrug-resistant CH^{R}C5 Chinese Hamster Ovary Cell Line[1]

A. P. M. Jongsma,[2] B. A. Spengler, A. M. Van der Bliek, P. Borst, and J. L. Biedler

Department of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands [A. P. M. J., A. M. V. d. B., P. B.], and Memorial Sloan Kettering Cancer Center, Walker Laboratory, Rye, New York 10580 [B. A. S., J. L. B.]

ABSTRACT

At least five gene classes are amplified in the multidrug-resistant CHO cell line CH^{R}C5. Protein products have been identified for two classes; class 2 codes for the large membrane P-glycoprotein, whereas class 4 encodes the small cytoplasmic calcium-binding protein sorcin (V19). By DNA analysis we have shown previously that these five genes are linked in two groups: class 1 + 2 + 3; and class 4 + 5. By use of *in situ* hybridization with complementary DNAs derived from the resistant cell line we demonstrate here that genes from both linkage groups are amplified and situated together in each of two different chromosomal regions of the resistant Chinese hamster cell line. The positions of the amplicons correspond to cytogenetically identified homogeneously staining regions in an altered 7q$^+$ chromosome and in a rearranged Z-7 [t(3;4)] chromosome. The native genes were mapped both in the CH^{R}C5 line and in a normal diploid Chinese hamster cell strain, CHNF 86. We confirm the position of the class 2 gene on 1q26 and we show that class 4 and 5 genes are located in the same region of 1q.

We conclude that the gene classes 2, 4, and 5 are closely juxtaposed in the normal Chinese hamster genome and comprise one amplicon in resistant cells. Our results are compatible with the hypothesis that multidrug resistance is due to overexpression of P-glycoprotein genes and that the other genes amplified in the CH^{R}C5 line are coamplified because they happen to lie close to the P-glycoprotein genes.

INTRODUCTION

Multidrug resistance can potentially limit the effectiveness of cancer chemotherapy, because treatment with one drug can cause resistance to a wide variety of other drugs (1–4). This type of resistance results in reduced intracellular drug accumulation (3, 5–7), which suggests the presence of membrane alterations. Indeed, a M_r 170,000 glycoprotein, variously termed the P-glycoprotein, gp150–180, or a similar designation is overproduced in many if not all multidrug-resistant cell lines (8–11). We have previously isolated complementary DNAs derived from five gene classes that are amplified and overexpressed in the multidrug-resistant Chinese hamster ovary cell line CH^{R}C5 (12). Of these five genes, class 2, representing most likely a gene family, is homologous to the P-glycoprotein gene, which encodes the M_r 170,000 glycoprotein(s) (13). In line with a central role for these proteins in multidrug resistance, the class 2 genes are the only ones consistently amplified in a series of independently derived, multidrug-resistant Chinese hamster cell lines (14). A second gene, the class 4 gene, has recently been shown to encode the cytosolic protein sorcin (V19) overexpressed in many but not all multidrug-resistant cell lines (15, 16). The products of the other genes are unknown.

Variation in cross-resistance levels among cell lines selected with different drugs suggests that multiple components modulate the resistant phenotype (see Refs. 9, 10, 17, and 18). Differential amplification of the five gene classes could provide one explanation for this variability and would be substantiated if there is selective amplification of two or more independent loci. On the other hand linkage of all five gene classes in one locus would be compatible with selection for one of these genes and coamplification of a varying number of flanking genes (12).

We have previously shown by pulse field gradient gel analysis that gene classes 1, 2, and 3 are linked on a 440-kilobase *Sac*II fragment and that gene classes 4 and 5 are both present on a 190- and a 300-kilobase *Sfi*I fragment (12). In this paper we present cytogenetic evidence that all five gene classes are linked in one large (>1000-kilobase) amplicon in the CH^{R}C5 cell line and that the amplified genes reside in two chromosomal aberrations, HSRs,[3] previously identified in this cell line.[4]

MATERIALS AND METHODS

Cell Lines and Culture. The multidrug-resistant CH^{R}C5 cell line was derived from the Chinese hamster ovary line Aux-B1, by stepwise selection for increasing colchicine resistance (19). CH^{R}C5 and CHNF 86, a chromosomally normal fibroblast Chinese hamster cell strain, were cultured in a 1:1 mixture of Eagles' minimum essential medium and Ham's F-12 medium supplemented with 5% fetal bovine serum. Chromosome preparations were made according to standard procedures. Earlier cytogenetic studies of CH^{R}C5 cells had revealed two HSRs in a 7q$^+$ marker chromosome and in a rearranged Z-7 [t(3;4)] chromosome in addition to the various chromosomal aberrations which are observed in the sensitive parental Aux-B1 (Chinese hamster ovary) cells (20, 21).[4] Because of these translocations, all chromosomes of CH^{R}C5 can be identified without G-banding, except for chromosomes 9 and 10. These chromosomes are combined together with the small marker chromosomes in the statistical analysis.

Labeling of cDNA Clones. The cDNAs used as probe were cloned in the *Pst*I site of pUC9 by deoxycytosine-deoxyguanosine tailing. Plasmid DNA with cDNA inserts cp28 (0.9 kilobase), cp36 (2.1 kilobases), and cp30 (2.5 kilobases) were used as probes for gene classes 2, 4, and 5, respectively (see Ref. 12). The DNA was labeled by nick translation with four ^{3}H-labeled nucleotides (New England Nuclear) (22) and was separated from unincorporated nucleotides by repeated ethanol precipitation in the presence of an excess of salmon sperm DNA. Specific activities of the probes were 1.9×10^7, 2.3×10^7, and 2.2×10^7 cpm/μg DNA for cp28 (class 2), cp36 (class 4), and cp30 (class 5), respectively.

***In Situ* Hybridization.** *In situ* hybridization was performed as described by Chandler and Yunis (23) with some minor modifications. Briefly the method was as follows. Chromosome slides were treated with 100 μg pancreatic RNase A (Sigma) per ml 2 × SSC at 37°C for 2 h. Chromosomal DNA was denatured by immersing the slides in 70% formamide/2 × SNP at 70°C, quickly chilled in −20°C ethanol, dehydrated, and air-dried. The slides were prehybridized with hybridization mix containing 100 μg denatured salmon sperm DNA per ml for 2 min at room temperature. The probes were resuspended at a concentration of 100 ng/ml hybridization mix composed of 50% formamide, 2 × SNP, 10% dextran sulfate (Sigma) (D-6001), 1 × Denhardt's

Received 11/11/86; accepted 3/4/87.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by Grant NKI 84-20 of the Queen Wilhelmina Fund to P. B. and by grants from The National Cancer Institute to the Sloan Kettering Institute (CA-08748) and to J. L. B. (CA-28595).

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: HSR, homogeneously staining region; SSC, 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0; SNP, SSC-0.04 M phosphate, pH 6.0; cDNA, complementary DNA.

[4] J. L. Biedler, T-d. Chang, K. W. Scotto, P. W. Melera, and B. A. Spengler. Chromosomal organization of amplified genes in multidrug-resistant Chinese hamster cells, submitted for publication.

solution (24), and 100 μg denatured salmon sperm DNA per ml; denatured at 70°C for 15 min; and used immediately for hybridization. Slides were incubated in moist chambers for 17 h at 37°C. After hybridization the slides were washed in three rinses of 5 min each in 2 × SNP, four changes of 15 min each in 50% formamide/2 × SNP at 42°C, and two 30-min washings in 2 × SSC at room temperature.

Autoradiography. Chromosome preparations were dipped in liquid nuclear track emulsion (Kodak NTB-2) and exposed at 4°C for 8 to 21 days. After development, slides were stained with Giemsa (Fisher) diluted 1:150 in phosphate-citrate buffer, pH 6.8. For each experiment 100–200 metaphase spreads were photographed and analyzed.

Analysis of Results. The position of the grains on the chromosome arms was recorded relative to the centromere and the telomere, as was the location of chromosome band interfaces. The assignment of the grains to the major bands was realized by transferring the data to G-banded chromosomes. Nomenclature of chromosomes and bands was adapted from Ray and Mohandas (25). The visual data were statistically supported by using the χ^2 test to map the native genes in CH^{R}C5 and confirmed in the wild-type hamster cell line using the two-sample χ^2 test.

RESULTS

Localization of the Amplified Genes. Fig. 1 illustrates representative metaphase cells of CH^{R}C5 hybridized with probes for gene classes 2, 4, and 5, respectively. The background is less than 2–3 grains/spread outside of the chromosomes, 100–200 mitoses of each hybridization experiment were photographed and analyzed for chromosomal localization of the grains. Pseudodiploid CH^{R}C5 cells are characterized by two resistance-related abnormalities, a relatively long, darkly staining HSR with an adjacent strongly banded abnormal region on chromosome 7 distal to band q27 and a short HSR on the long arm of the Z-7 chromosome replacing most of the long arm of chromosome 4 (Fig. 2). The distal part of the chromosome 7q$^+$ and the long arm of the rearranged Z-7 [t(3;4)] clearly show specific labeling with all probes (Fig. 2).

Table 1 summarizes the data from hybridizations of class 2, 4, and 5 cDNAs with CH^{R}C5 chromosomes. All chromosomes 7q$^+$ and about 80% of Z-7 chromosomes were labeled, having together 80, 72, and 89%, respectively, of the total number of silver grains. Thus, both chromosomes are carriers of amplified genes of classes 2, 4, and 5. The difference in the total grain numbers suggests a 2–3-fold greater degree of amplification in chromosome 7q$^+$ than in the Z-7 chromosome, a finding in accordance with the approximate 3-fold difference in the length of the HSRs.

Fig. 2 shows the distribution of the silver grains over the 7q$^+$ and Z-7 marker chromosomes. In 7q$^+$ the majority of the grains are found over the cytogenetically recognized HSR. Most of the grains over the Z-7 are found on the distal half of the long arm, where the second HSR is identified.[4] The distributions of the different gene classes over the amplified regions are quite similar, suggesting a concerted amplification of the whole gene cluster.

Determination of the Wild-type Position of the Genes. By excluding the HSR-bearing chromosomes from analysis, we were able to localize the nonamplified genes of classes 2, 4, and 5 in CH^{R}C5 (Table 2). The corresponding genes could be assigned to chromosome 1 ($P < 0.0005$, $P < 0.001$, and $P < 0.0005$, respectively). Fig. 3 illustrates the grain distribution over chromosome 1 in the three hybridization experiments with CH^{R}C5 cells. The gene sites were determined to lie within bands 1q26–1q31. The localization of the class 2 (*i.e.*, the membrane glycoprotein) gene to 1q confirms the results obtained with the pCHP1 probe by Riordan *et al.* (13) and the

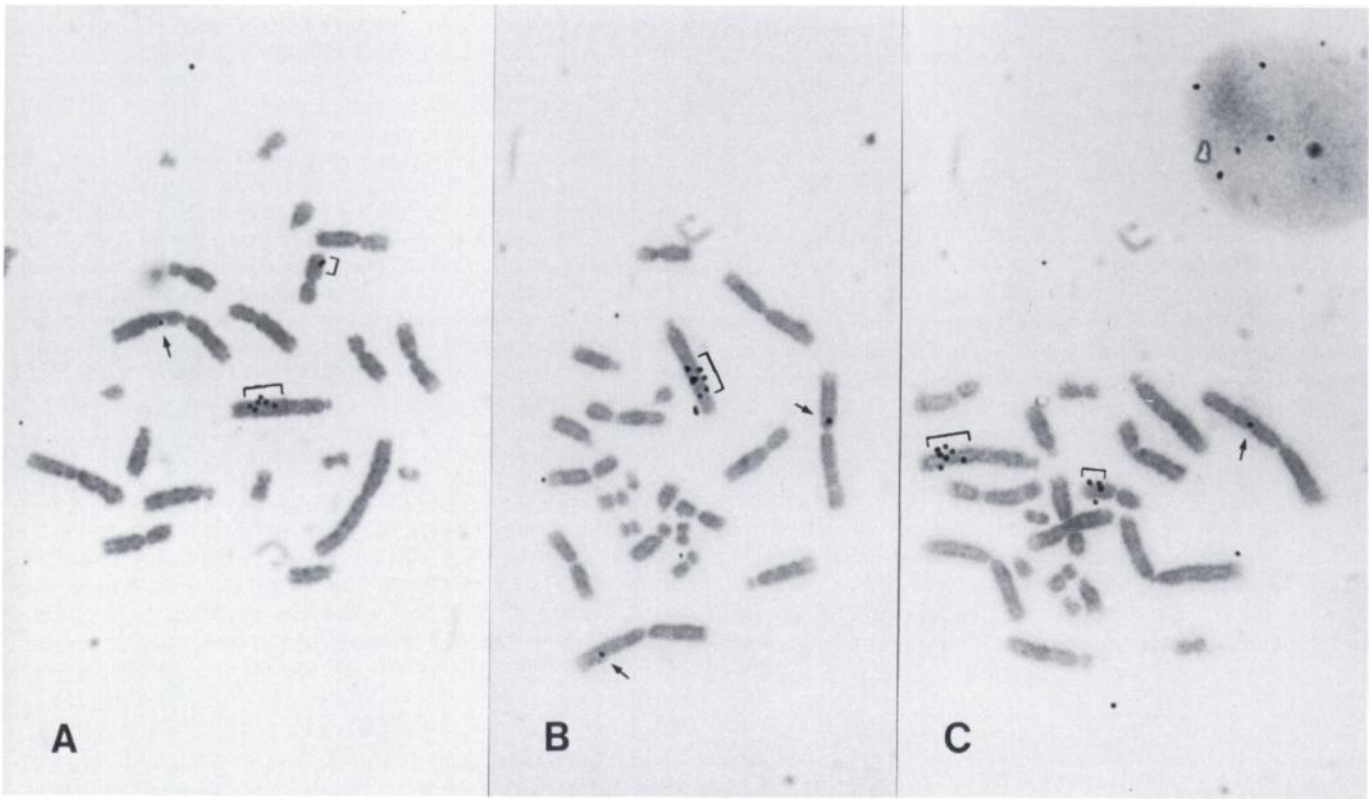

Fig. 1. Three representative mitoses of CH^{R}C5 hybridized with ^{3}H-labeled cDNA probes for class 2 (*A*), class 4 (*B*), and class 5 (*C*). Significant labeling is found over the two HSRs (*brackets*). *Arrows*, single grains over 1q (*A*); over 1q and 1p (*B*), and over 1q (*C*). The hybridized preparations were exposed for 3 weeks (*A*) and 2 weeks (*B* and *C*).

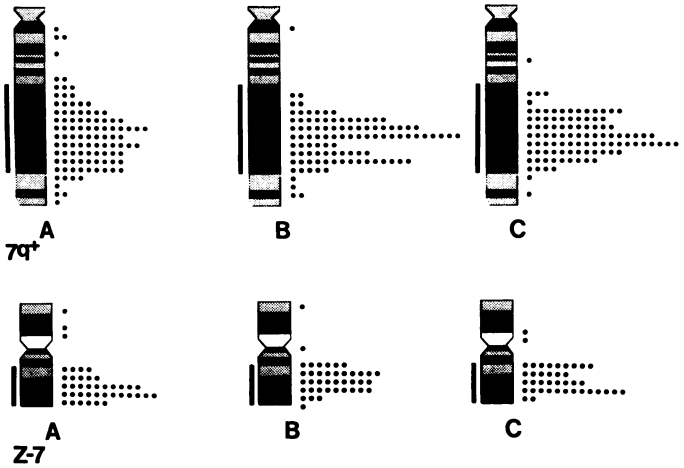

Fig. 2. Localization of class 2 (*A*), 4 (*B*), and 5 (*C*) genes over the HSR-bearing chromosomes 7q+ and Z-7 after *in situ* hybridization. The positions of 100 grains over chromosome 7q+, observed in about 25 cells, were measured for each of the genes and plotted in the diagrams showing that the amplified genes are scattered over the major part of the HSR. The positions of 40 grains for each of the genes plotted over chromosome Z-7 fits with the location of the HSR in this chromosome. HSRs are indicated (*bars*).

Table 1 *Localization of the amplified genes in CH^{R}C5 by in situ hybridization*

The percentage of grains over the HSRs and the percentage of HSR-bearing chromosomes labeled demonstrate the presence of class 2, 4, and 5 genes in both the HSRs.

Gene	No. of cells	No. of grains	Grains (%)		Chromosomes labeled (%)	
			7q+ (HSR)	Z-7 (HSR)	7q+ (HSR)	Z-7 (HSR)
Class 2	84	548	54	26	100	79
Class 4	153	1578	55	17	100	78
Class 5	145	1838	67	22	100	81

Table 2 *Assignment of class 2, 4, and 5 genes to chromosome 1q*

The native genes were assigned to chromosome 1 in CH^{R}C5 cells by using the χ^2 test after exclusion of the HSR-bearing chromosomes. The more precise location on chromosome 1q was determined in wild-type CHNF 86 cells by use of the two-sample χ^2 test.

Cells	Class	No. of cells	No. of grains		*P*
			Total	Over 1	
CH^{R}C5	2	84	117	47	<0.0005
	4	153	444	144	<0.001
	5	145	223	111	<0.0005
				Over 1q	
CHNF 86	4	139	428	94	≪0.0005
	5	155	330	164	≪0.0005

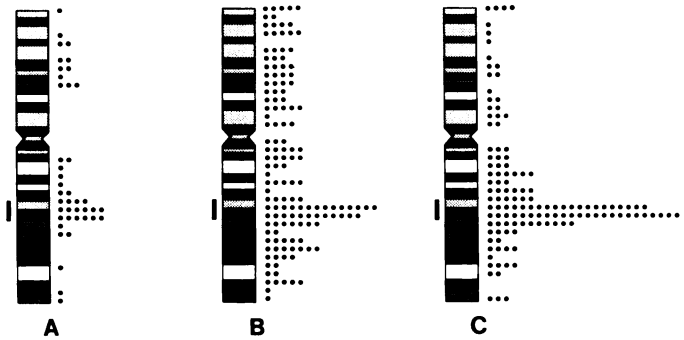

Fig. 3. Sublocalization of the class 2, 4, and 5 genes over the normal chromosome 1 in the resistant CH^{R}C5. For classes 2 (*A*), 4 (*B*), and 5 (*C*) the positions of, respectively, 41, 141, and 127 grains were measured and plotted on the diagrams. The single gene loci on chromosome 1 are most probably on the bands q26 or q27–31 (*bars*). The distribution of grains over 1p is representative for nonspecific labeling in these experiments.

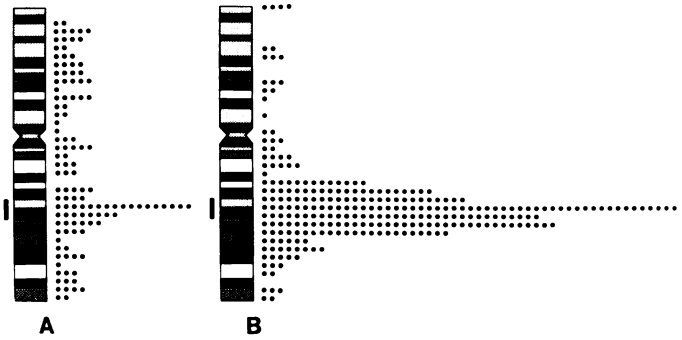

Fig. 4. Sublocalization of the class 4 and 5 genes over chromosome 1 in the wild-type CHNF 86 cells. For class 4 (*A*) and 5 (*B*) genes, respectively, 121 and 265 grains were plotted on the diagrams of chromosome 1. The single gene loci are determined to lie in the region 1q26–31 (*bars*).

p5L-18 probe by our group.[4] To exclude mislocalization due to rearrangements in CH^{R}C5, the class 4 and 5 probes were also hybridized with normal Chinese hamster chromosomes of CHNF 86 cells. By using the two-sample χ^2 test, the assumed position of the class 4 and 5 genes on chromosome 1q could be confirmed [$P \ll 0.0005$ each (Table 2)]. The distribution of the grains over the wild-type chromosome 1 indicates the same gene positions, *i.e.*, 1q26-q31, as observed in CH^{R}C5 cells (Fig. 4). The pattern of grains in the short arm of the chromosome is representative of the background labeling over the chromosomes. A relatively high background over the chromosomes is found with the class 4 probe in the CH^{R}C5 as well as in the CHNF 86 cells. Cross-hybridization of class 4 cDNAs with nonamplified repeats has also been observed with digests of genomic DNA.[5] Even though the number of CH^{R}C5 cells hybridized with class 2 gene probe is rather low for sublocalization purposes, the distribution clearly indicates that all three nonamplified genes, classes 2, 4, and 5, are located in the middle half of 1q, most probably within the bands 1q26 or 1q27–31.

DISCUSSION

We have used *in situ* hybridization techniques to demonstrate linkage of three gene classes, classes 2, 4, and 5, amplified in the multidrug-resistant cell line CH^{R}C5 (12). By pulsed field gradient gel analysis we had already shown that five amplified genes in CH^{R}C5 are linked in two groups, one including gene classes 1, 2, and 3 and the other including classes 4 and 5. The combined results of the two sets of experiments unambiguously show that all five genes are linked in the same amplicon.

Previous experiments with two different class 2 gene probes, pCHP1 and p5L-18, have shown that these amplified genes in CH^{R}C5 are present in HSRs on chromosome 7q+ (13) and on the Z-7 [t(3;4)] chromosome.[4] Our data show that genes 4 and 5 are also present in these abnormal chromosome regions. All three genes show the same relative distribution and sublocalization over the two HSRs. This indicates that these genes coamplified in each round of selection for increased colchicine resistance and migrated together to the new locations. This conclusion is further supported by our observation that all three native genes are located in 1q23–1q31 region as was previously shown for class 2 with the probes pCHP1 (13) and p5L-18.[4] The grain distribution over the HSR of chromosome 7q+ suggests that the proximal part of the cytogenetically determined HSR does not carry amplified class 2, 4, and 5 genes. Therefore,

[5] A. M. Van der Bliek and P. Borst, unpublished data.

this chromosome region might be derived from darkly stained bands of another chromosome or might be a result of reintegration of coamplified DNA sequences not comprising class 1–5 genes.

Our demonstration that all known amplified genes in the CH^{R}C5 line are linked within the amplicon and the suggestive evidence that these genes are already linked in the normal Chinese hamster genome strongly support the idea that multidrug resistance is caused by overexpression of only one of these gene classes and that the amplification of neighboring genes is the fortuitous consequence of the size of the amplicon. Amplification of different subsets of the genes 1–5, as observed in other multidrug-resistant hamster lines (14), mouse lines,[6] and one human cell line[7] support this interpretation. Indeed only one class of genes, the class 2 gene family, is consistently overexpressed in all multidrug-resistant cell lines (12–14, 26–28). This indicates that the drug primarily selects for overexpression of the class 2 genes and that the amplification of the other gene classes is due to their linkage to the class 2 genes. Whether overexpression of the other genes modulates the exact pattern of cross-resistance remains to be seen.

ACKNOWLEDGMENTS

We thank Dr. V. Ling for providing the CH^{R}C5 cell line; A. Riethorst and T. van der Velde-Koerts for technical assistance; J. M. Lomecky for help with the illustrations; N. van Nuland-van Wijngaarde and M. Sonne-Gooren for typing the manuscript; and Drs. M. B. Meijers, F. Baas, and M. H. L. de Bruijn for many helpful discussions.

REFERENCES

1. Bell, D. R., Gerlach, J. H., Kartner, N., Buick, R. N., and Ling, V. Detection of P-glycoprotein in ovarian cancer. A molecular marker associated with multidrug-resistance. J. Clin. Oncol., *3:* 311–315, 1985.
2. Biedler, J. L., Chang, T., Peterson, R. H. F., Melera, P. W., Meyers, M. B., and Spengler, B. A. Gene amplification and phenotypic instability in drug-resistant and revertant cells. *In:* B. A. Chabner (ed.), Rational Basis for Chemotherapy-UCLA symposium Vol. 4, pp. 71–92. New York: Alan R. Liss Inc., 1983.
3. Riordan, J. R., and Ling, V. Genetic and biochemical characterization of multidrug-resistance. Pharmacol. Ther., *28:* 51–75, 1985.
4. Gudkov, A., and Kopnin, B. Gene amplification in multidrug-resistant cells: molecular and karytypic events. Bioessays, *3:* 68–73, 1985.
5. Biedler, J. L., and Riehm, H. Cellular resistance to actinomycin D in Chinese hamster cells *in vitro*: cross-resistance, radioautographic and cytogenetic studies. Cancer Res., *30:* 1174–1184, 1970.
6. Danø, K. Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. Biochim. Biophys. Acta, *323:* 466–483, 1973.
7. Inaba, M., Fujikura, R., and Sakurai, Y. Active efflux common to vincristine and daunorubicin in vincristine-resistant P388 leukemia. Biochem. Pharmacol., *30:* 1863–1865, 1981.
8. Juliano, R. L., and Ling, V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim. Biophys. Acta, *455:* 152–162, 1976.
9. Kartner, N., Riordan, J. R., and Ling, V. Cell surface P-glycoprotein associated with multidrug-resistance in mammalian cell lines. Science (Wash. DC), *221:* 1285–1288, 1983.
10. Biedler, J. L., and Peterson, R. H. F. Altered plasma membrane glycoconjugates of Chinese hamster cells with acquired resistance to actinomycin D, daunorubicin and vincristine. *In:* A. C. Sartorelli, J. S. Lazo, and J. R. Bertino (eds.), Molecular Actions and Targets for Cancer Chemotherapeutic Agents, Vol. 2, pp. 453–482. New York: Academic Press, 1981.
11. Shen, D.-W., Cardarelli, C., Hwang, J., Cornwell, M., Richert, N., Ishii, S., Pastan, I., and Gottesman, M. M. Multiple drug-resistant human KB carcinoma cells independently selected for high level resistance to colchicine, Adriamycin or vinblastine show changes in expression of specific proteins. J. Biol. Chem., *261:* 7762–7770, 1986.
12. Van der Bliek, A. M., van der Velde-Koerts, T., Ling, V., and Borst, P. Overexpression and amplification of five genes in a multidrug-resistant Chinese hamster ovary cell line. Mol. Cell. Biol., *6:* 1671–1678, 1986.
13. Riordan, J. R., Deuchars, K., Kartner, N., Allon, N., Trent, J., and Ling, V. Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. Nature (Lond.), *316:* 817–819, 1985.
14. De Bruijn, M. H. L., Van der Bliek, A. M., Biedler, J. L., and Borst, P. Amplification and disproportionate expression of five genes in three multidrug-resistant Chinese hamster lung cell lines. Mol. Cell. Biol., *6:* 4717–4722, 1986.
15. Meyers, M. B., and Biedler, J. L. Increased synthesis at a low molecular weight protein in vincristine-resistant cells. Biochem. Biophys. Res. Commun., *99:* 228–235, 1981.
16. Van der Bliek, A. M., Meyers, M. B., Biedler, J. L., Hes, E., and Borst, P. A 22-kDa protein (sorcin/v19), encoded by an amplified gene in multidrug-resistant cells, is homologous to the calcium-binding light chain of calpain. EMBO J., *5:* 3201–3208, 1986.
17. Meyers, M. B., Spengler, B. A., Chang, T. D., Melera, P. W., and Biedler, J. L. Gene amplification-associated cytogenetic aberrations and protein changes in vincristine-resistant Chinese hamster, mouse and human cells. J. Cell Biol., *100:* 588–597, 1985.
18. Martinsson, T., Dahllöf, B., Wettergren, Y., Leffler, H., and Levan, G. Pleiotropic drug resistance and gene amplification in a SEWA mouse tumor cell line. Exp. Cell Res., *158:* 382–394, 1985.
19. Ling, V., and Thompson, L. H. Reduced permeability in CHO cells as a mechanism of resistance to colchicine. J. Cell. Physiol., *83:* 103–116, 1974.
20. Siciliano, M. J., Stallings, R. L., and Adair, G. M. The genetic map of the Chinese hamster and the genetic consequences of chromosomal rearrangement in CHO cells. *In:* M. M. Gottesman (ed.), Molecular Cell Genetics, pp. 95–135. New York: John Wiley and Sons, Inc., 1985.
21. Deaven, L. L., and Peterson, D. F. The chromosomes of CHO, an aneuploid Chinese hamster cell line: G-band, C-band, and autoradiographic analysis. Chromosoma (Berl.), *41:* 129–144, 1973.
22. Rigby, P. W. J., Dieckmann, M., Rhodes, C., and Berg, P. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick-translation with DNA polymerase I. J. Mol. Biol., *113:* 237–251, 1977.
23. Chandler, M. E., and Yunis, J. J. A high resolution *in situ* hybridization technique for the direct visualization of labeled G-banded early metaphase and prophase chromosomes. Cytogenet. Cell Genet., *22:* 352–356, 1978.
24. Denhardt, D. T. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun., *23:* 641–646, 1966.
25. Ray, M., and Mohandas, T. Proposed banding nomenclature for the Chinese hamster chromosomes (*Crisetulus griseus*). Cytogenet. Cell Genet., *16:* 83–91, 1976.
26. Roninson, I. B., Abelson, H. T., Housman, D. E., Howell, N., and Varshavsky, A. Amplification of specific DNA sequences correlates with multi-drug resistance in Chinese hamster cells. Nature (Lond.), *309:* 626–628, 1984.
27. Scotto, K. W., Biedler, J. L., and Melera, P. W. The differential amplification and expression of genes associated with multidrug resistance in mammalian cells. Science (Wash. DC), *232:* 751–755, 1986.
28. Shen, D-W., Fojo, A., Chin, A. E., Roninson, I. B., Richert, N., Pastan, I., and Gottesman, M. M. Human multidrug-resistant cell lines: increased mdr 1 expression can precede gene amplification. Science, *232:* 643–645, 1986.

[6] G. Levan *et al.*, personal communication.
[7] Unpublished results.