MOLECULAR AND CELLULAR BIOLOGY, Sept. 1983, p. 1634–1647
0270-7306/83/091634-14$02.00/0

Vol. 3, No. 9

Correlation of Unstable Multidrug Cross Resistance in Chinese Hamster Ovary Cells with a Homogeneously Staining Region on Chromosome 1†

STEPHEN H. GRUND,[2] SHIVA R. PATIL,[1] HARSHAD O. SHAH,[1] PETER G. PAUW,[2] AND JOAN K. STADLER[2]*

Division of Medical Genetics, Department of Pediatrics, University of Iowa Hospitals, Iowa City, Iowa 52242,[1] and Department of Genetics, Iowa State University, Ames, Iowa 50011[2]

Received 20 January 1983/Accepted 7 June 1983

An enrichment selection method using repeated pulses of low drug concentration (1 μg/ml) was used to isolate CHO (AK412) variants that are 20-fold more resistant to cytochalasin D (CD). CD-resistant (Cyd^R) variants possess a unique unstable phenotype, including a longer doubling time in nonselective medium, a higher frequency of multinucleate cells in the population (probably due to a defect in cytokinesis), an altered morphology, and increased resistance or sensitivity to a number of unrelated drugs. In each of two variant lines examined cytologically, this multiple phenotype is associated with a small homogeneously staining region on chromosome 1. The homogeneously staining region is present in the Cyd^R variants, but absent both in the CD-sensitive parent and in a CD-sensitive revertant subpopulation. Studies of CD-displaceable binding of [^{3}H]cytochalsin B show a fourfold reduction in CD binding or uptake when whole cells of the variant line were examined. Lactoperoxidase-catalyzed iodination and metabolic labeling with [H^3]fucose of cell surface proteins of the Cyd^R variants showed multiple differences in electrophoretic band migration when compared with parental proteins.

Detailed studies of methotrexate resistance in several variant cell lines of different species have shown that gene amplification is the basis of an acquired ability to grow in the presence of steadily increasing toxic concentrations of drug (1, 25). This drug resistance phenotype was found to be due to an increased number of genes per cell coding for production of dihydrofolate reductase, the enzyme targeted by methotrexate. (1).

Similar enrichment selections for growth resistance to other drugs have produced gene amplification at additional gene loci (32). There is, for example, clearly demonstrated amplification of the CAD (36) and metallothionein I genes (5). In other instances, induced overproduction of gene product has been the first indicator of putative gene amplification, e.g., overproduction of aspargine synthetase (2), 3-hydroxy-3-methylglutaryl coenzyme A (30), and ribonucleotide reductase (21, 26).

Enrichment selections have also yielded cell lines with pleiotropic impermeability to vincristine, maytansine, adriamycin, and other drugs (4, 7). These latter lines have altered karyology and include one of two unique nuclear structures, double minute chromosomes (DMs) or homogeneously staining regions (HSRs); both chromosomal structural variations have been shown to contain amplified gene sequences in other drug-resistant lines (DM, [14]; HSR, [28]). Whether DMs and HSRs always contain amplified genes is unknown, but their common association with gradually acquired drug resistance implies that this may be so (32).

Because DMs and HSRs may both always contain amplified gene sequences, it is tempting to believe that there is some kind of causal relationship that links these two phenomena (3, 19). Furthermore, because these structures do not usually coexist in the same cell, or even the same cell line, it is also tempting to seek a rationale for the independence of these occurrences.

Although the number of reports of putative gene amplification continues to increase (32), the sample size probably still is too small to allow completely valid generalizations regarding DM and HSR relationships. However, two points already seem to emerge. First, there may be some real differences along species lines. When both lung and ovary lines from Chinese hamster (*Cricetulus griseus*) have yielded drug-

† Journal paper no. J-10874 of the Iowa Agriculture and Home Economics Experiment Station, Ames. Project 2441.

resistant mutants after intensive enrichment selection, careful cytology almost always demonstrates the presence of a newly developed HSR (2, 7, 28). DMs are uncommon in CHO and seemingly are unassociated with the selected genetic variation (18). Conversely, mouse (*Mus musculus*) cell lines, including neuroblastoma, lymphoma, and fibroblastic lines, have either HSRs or DMs in association with similarly selected drug resistance phenotypes (33). Human cell lines selected for methotrexate resistance also have variable numbers of DMs (24).

Second, there may be, within a species, some significance in the association of one chromosome structure rather than another with the stability of the drug resistance phenotype. In mouse cell lines, for example, DMs are often associated with unstable phenotype (17), whereas HSRs (17) or extremely low numbers of DMs (4) are associated with stable drug resistance. In Chinese hamster cell lines, on the other hand, either stable or unstable drug resistance phenotypes occur in the presence of an HSR (28; J. L. Biedler and B. A. Spengler, J. Cell Biol. **70**:117a, 1976).

We report here on an unstable, complex, multiple-drug resistance phenotype associated with the presence of a subterminal HSR on the long arm of chromosome 1 in CHO cells (cell line AK412) (9). This is the first demonstration of an HSR at that chromosomal location in this species; previous studies have shown HSRs at the site of a terminal long arm deletion on chromosome 1 (2), chromosome Z-7 (2), chromosome 2 (28), and chromosome 9 (7). Taken together, these findings continue to illustrate the remarkable flexibility of the mammalian genome. Our work also supports the hypothesis that the usual primary cellular response of Chinese hamster cell lines to enrichment drug selection is the development of an HSR containing amplified gene sequences.

MATERIALS AND METHODS

Cell lines and culture conditions. AK412 and variant sublines were stock cultured in alpha minimal essential medium (GIBCO Laboratories) containing 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (nonselective medium, αNS). The occurrence of the cytochalasin D (CD) resistance phenotype was regularly monitored by growing the Cyd^R lines in alpha minimal essential medium containing 1 μg of CD per ml (selective medium, αS-1 CD). All cells were routinely grown at 37°C in 5% CO_2 and were subcultured after trypsinization and dilution.

Chemicals. CD was obtained from Aldrich Chemical Co. or Sigma Chemical Co., and cytochalasin B (CB) was obtained from Aldrich. Colcemid, actinomycin D, procaine, adriamycin, vincristine, lactoperoxidase, galactose oxidase, and glucose oxidase were purchased from Sigma. All radioactive compounds, [^{3}H]CB, Na^{125}I, [^{3}H]$NaBH_4$, and [^{3}H]fucose, were obtained from New England Nuclear Corp. Protein standards were purchased from Bio Rad Laboratories. Giemsa, used in chromosome banding, was supplied by Fisher Scientific Co.

Selection of CD-resistant variants. CD variants were obtained by an enrichment selection procedure involving 16 cycles of growth with drug, followed by outgrowth in drug-free medium. First, 10^6 cells per 75-cm^2 culture flask were grown in αS-1 CD for 4 to 6 days. The αS-1 CD was then removed, the cells were washed 3 times with αNS, and fresh αNS was added. These cells were grown to confluency (2 to 5 days) and then subcultured into two 75-cm^2 flasks at a concentration of 3×10^5 cells per flask. The population again was grown to confluency in αNS, and the cycle was repeated by dividing this regrown population into two 75-cm^2 flasks, each containing 10^6 cells in αS-1 CD. The gross morphology of cells was routinely examined with phase contrast on a Zeiss inverted microscope.

Isolation of resistant variants. After 16 rounds of selection, more than 95% of the cells in the treated population were resistant to 1 μg of CD per ml. This population was diluted and cloned in αNS in 96-well microtiter dishes (Costar). Wells containing one attached cell were marked; cells in the resulting clone were picked, grown to confluency in 25-cm^2 flasks in αNS, and tested for resistance to 1 μg of CD per ml by determining cloning efficiency in the presence of drug. These isolated variant sublines then were subcloned again in αNS or αS-1 CD and retested for CD resistance. Separately retrieved CD-resistant isolates may, because of the enrichment selection method, be sister subclonal lines. Three of these, Cyd^R-8, -10, and -20, were chosen for further analysis and comparison.

Isolation of sensitive revertants. Subpopulations of 100 cells from a freshly recloned Cyd^R variant line were cloned again into αNS in 25-cm^2 flasks. At the time of this subcloning, the largely CD-resistant population, derived from a single resistant cell, had been grown in nonselective medium for only 15 population doublings and had a cloning efficiency of nearly 100% in drug. After 7 to 10 days, when clones were easily visible, the nonselective medium was gently removed and replaced with αS-1 CD. The flasks were incubated 1 to 4 h at 37°C and then examined with phase-contrast microscopy. Positive morphological reactions to CD (CD sensitivity; see below for description) or the absence of these morphologies (CD resistance) were clear by 4 h, especially in cells at the periphery of individual clones. Clones with CD-sensitive morphology were picked and transferred to 25-cm^2 flasks for growth in αNS to large volume. The putative CD-sensitive revertant populations were subcloned and inspected again in the manner just outlined. The twice-purified CD-sensitive subclonal lines then were tested for drug resistance and other phenotypic characteristics as described.

Cloning efficiency tests for quantitation of drug resistance. For each experiment, cells near confluency in 25-cm^2 flasks were harvested with trypsin, counted with a model ZBI Coulter electronic particle counter, and diluted. As a control, five 25-cm^2 flasks were inoculated with 100 cells per flask in 5 ml of αNS. For each variant tested, five 25-cm^2 flasks were each inoculated with 100 cells and 5 ml of selective medium containing various concentrations of drug. After 7 to 14 days, the clones were stained with 0.5% crystal

violet and counted. In some instances (e.g., as the cloning efficiency decreased in higher concentrations of drug), greater numbers of cells were used as inocula (10^3 to 10^6). Cloning efficiency is always expressed as a percentage of the control (an identical inoculum plated in nonselective growth medium).

Chromosome analysis. Chromosomes were prepared by standard techniques and Giemsa-trypsin banded by the method of Seabright (34). Unbanded chromosome preparations were stained with either acetic orcein or Giemsa.

Analysis of monolayers for percentage of polynucleate cells. Cells in monolayer were stained with Giemsa, maintained in the original flask under a small quantity of buffer, and examined by bright-field microscopy without further dehydration or fixation. The percentage of polynucleates was determined by visual inspection of 1,000 cells in each sample.

Determination of culture doubling time. The doubling time (Td) of the parental and variant cell lines was calculated after determination of cell number increase in populations of at least two replicate cultures. The cell number was ascertained either by electronic particle counting or by in situ counting. For in situ counting, six areas were chosen at random and permanently marked on the back of the culture flask; the cell number in these areas was followed daily by microscopic inspection with an ocular grid previously calibrated at a specific magnification (×160). The size of each of the six areas counted was 0.563 mm^2, and the minimum number of cells counted at the beginning of a growth curve was 500. Growth curves derived from in situ data accurately duplicate growth curves based on Coulter counting.

Radiolabeling of membrane proteins. Cells were plated on 100-mm plastic tissue culture dishes in 10 to 15 ml of alpha minimal essential medium, with or without 1 μg of CD per ml, and were kept in a 37°C humidified incubator in a 5% CO_2 atmosphere for at least 24 h before labeling. Cell surface proteins were labeled with ^{125}I by a modification of the method of Hynes (16) as described by Pauw and David (29).

External labeling of cell surface galactosyl and galactosylaminyl residues by galactose oxidase and tritiated borohydride was performed as described by Gahmberg et al. (12).

Metabolic labeling of glycoproteins was performed by incubating cultures with [^{3}H]fucose (2.0 μCi/ml) for 24 h in alpha minimal essential medium plus 10% dialyzed fetal calf serum.

Membrane preparation. Labeled cells were harvested with a rubber policeman in 5 ml of cold 0.25 M sucrose–0.1 mM triethanolamine (pH 7.2) buffer and homogenized by 30 to 40 strokes with a tight (A) pestle of a 7-ml Dounce homogenizer (Vitro). The homogenate was centrifuged at 2,200 × *g* for 10 min at 4°C, and the resulting supernatant was then centrifuged at 20,000 rpm for 1 to 3 h in an SW39 rotor (108,000 × *g*). Radioactivity of the resulting pellet was determined in a Nuclear Chicago gamma counter. Pellets were stored at −20°C until electrophoresis.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gels were prepared by the method of Laemmli (20). Slabs were 1.5-mm thick and were either linear gradients of 10 to 15% polyacrylamide with 5% stacking gels or 7% polyacrylamide with 4% stacking gels. Protein molecular weight standards (myosin, 200,000; β-galactosidase, 116,250; phosphorylase *b*, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400) were used to estimate the molecular weights of electrophoretically separated proteins. For the determination of molecular weight, band positions were measured on densitometer scans.

Membrane pellets were solubilized by boiling for 2 min in sample buffer consisting of 2% sodium dodecyl sulfate, 10% glycerol, 6.25% mercaptoethanol, and 61 mM Tris (pH 6.8). Volumes of samples containing equal total disintegrations per minute were loaded onto each lane in the slab, and electrophoresis was performed at a constant current of 20 mA per gel for 4 to 5 h. Gels were fixed in a solution of 50% methanol–7% acetic acid and dried. Dried gels were placed in direct contact with Kodak X-Omat AR film opposite a Cronex Lightning Plus Intensifying Screen (Du Pont Co.) and incubated at −95°C for 1 to 30 days. Gels containing ^{3}H-labeled samples were prepared for fluorography by the method of Bonner and Laskey (8) before drying. Films were scanned at 550 nm by using a Gilford (model 250) spectrophotometer equipped with a linear transport.

[^{3}H]CB-binding assay. CD-displaceable binding of [^{3}H]CB was measured by the method of Magargal and Lin (W. W. Magargal and S. Lin, J. Cell Biol. **87**:215a, 1980; manuscript in preparation). Briefly, cells were grown to confluency in 60-mm plastic tissue culture dishes and then were washed three times in serum-free L-15 medium (GIBCO). [^{3}H]CB in L-15 medium was added to the culture dishes (final concentrations, 10^{-9} to 10^{-6} M), and the cells were incubated for 20 min at 37°C, either with an excess of unlabeled CD (4×10^{-6} M) as a control for nonspecific binding or without CD. The medium was removed, and the amount of [^{3}H]CB binding to whole cells in monolayer was determined. For cell extracts, cells were grown to confluency in 150-mm plastic tissue culture dishes, washed three times with cold phosphate-buffered saline, and then scraped off gently with a rubber policeman. The cells were centrifuged, resuspended in buffer A (10 mM Tris-hydrochloride [pH 7.8], 0.1 mM $CaCl_2$, 0.5 mM ATP, 0.01 mM phenylmethylsulfonyl fluoride, 0.1 mM β-mercaptoethanol), and homogenized by 50 strokes with the tight (A) pestle of a 7-ml Dounce homogenizer (Vitro). The homogenate was centrifuged at 200,000 × *g* for 30 min, and the supernatant was removed and saved. The pellet was homogenized in buffer A and centrifuged as before. The binding of [^{3}H]CB to the combined supernatants (soluble-extract fraction) and pellet (membrane-particulate fraction) was determined both in the presence and absence of excess unlabeled CD by the acid precipitation assay of Lin and Lin (22).

RESULTS

Isolation of CD-resistant variant lines. Cytochalasins, fungal metabolites first discovered in 1964, have a number of effects on cells in culture (35). These effects include inhibition of cell division, cell motility, and cytoplasmic streaming, and promotion of both nuclear extrusion and a significantly altered morphology. It has been repeatedly observed that the effects of cytochalasin are readily reversible (13).

CD-resistant variant lines were successfully obtained by cycling cell populations through several 4- to 6-day pulses of drug. This enrichment method relies upon the fact that one of the major effects of cytochalasin on cultured cells is the inhibition of cytokinesis but not of karyokinesis. The net result of application of CD over time is, therefore, the accumulation of large polynucleate cells in the population. In our laboratory, 1 μg of CD per ml produces 90% polynucleates in 30 h and more than 99% in 6 days. When a CD-treated population was transferred to drug-free medium, the large multinucleate cells failed to divide. Conversely, cells that escaped CD-induced multinucleation divided rapidly and outgrew the polynucleates. Several rounds of CD treatment, followed by outgrowth of morphologically normal cells that were resistant to the CD effects, produced a population greatly enriched for this cell type. Before using this enrichment method, several unsuccessful attempts to isolate CD-resistant variants after mutagenesis, followed by direct selection in 1 μg of drug per ml, had been made (10^8 cells tested).

In the entire multistep selection procedure, there were 16 successive cycles of drug treatment and outgrowth over 181 days. Populations in the drug-treatment phase of each round were examined by phase-contrast microscopy in attempts to identify cells as drug resistant on the basis of morphological criteria (Fig. 1). The criteria were: (i) retention of spindle-shaped, mononucleate morphology, and (ii) the apparent ability of cells to divide, as evidenced by areas of clonal growth (Fig. 1F). Drug-sensitive parental AK412 cells, on the other hand, showed a number of distinct abnormalities at the light-microscope level when exposed to CD. These changes included: (i) immediate formation of large blebs on the cell surface, which later aggregate at one or two sites on the cell, (ii) cytoplasmic contraction, (iii) formation of retraction fibers, which were formerly cell-cell and cell-substrate contacts, and (iv) nuclear protrusion and extrusion. Some of these changes are illustrated in Fig. 1C and in the drug-sensitive cell background of Fig. 1E and 1F.

Cells with drug-resistant morphology were not easily detected in the earliest cycles of selection, but by rounds six through eight, occasional cells of this type were observable. In round 12, many cells with evidently normal morphology could be seen, some occurring in pairs suggestive of cell division (Fig. 1E). In round 13, 7 to 10 cell clones were observed (Fig. 1F). The isolation, subcloning, and testing of resistant clonal lines is described above.

Phenotype of resistant lines. A number of major differences between the Cyd^R variants and the AK412 parental cell line are summarized in Table 1. The Cyd^R variants had a Td of 24 to 39 h and a cloning efficiency of 86 to 92% in medium containing 1 μg of CD per ml, whereas cells of the parental line failed to divide at all in this concentration of drug. The Td of the variants was, however, longer than that of the parent line during growth on nonselective medium; these doubling times are 18, 19, and 23 h for the variants, as opposed to 14 h for the parent.

One of the striking features of the Cyd^R variant phenotype is that 5 to 10% of the cells in frequently transferred stock populations are multinucleate (Fig. 1B). Some of the multinucleate cells in each transfer may possibly have been carried over from the previous subculture. However, most originate de novo because there is a constantly recurring high percentage of multinucleate cells in fully CD-resistant populations even though multinucleate cells usually do not divide. It has also been determined by daily cytological examination that the percentage of polynucleates increases more than twofold in 7 days.

Multinucleate cells originating in Cyd^R variant populations could arise from a defect in cytokinesis, an increase in the rate of spontaneous cell fusion, or micronucleation. Although it is difficult to prove conclusively that Cyd^R variant cells do not have a heightened ability to fuse, two initial experiments suggest that this is not the cause of increased multinucleation. First, each of the Cyd^R variants ($HPRT^-$, *glyA*), as well as the AK412 parent ($HPRT^-$, *glyA*), were cocultivated (37) with the CHO cell line 51-11 (HPRT, $glyA^-$), and the number of spontaneous hybrids on selective medium (hypoxanthine-aminopterin-thymidine, minus glycine) was observed. Hybrid clones occurred at a frequency

TABLE 1. Initial characterization of Cyd^R variant phenotype

Cell line	Population T_d[a]		Relative cloning efficiency in αS-1 CD[b]	Multinucleate cells in αNS[c] (%)	Modal chromosome number[d]
	αNS	αS-1 CD			
AK412	14	0	0	1.0	20
Cyd^R-8	18	29	92	9.2	20
Cyd^R-10	19	36	88	8.9	20
Cyd^R-20	20	38	86	10.1	20

[a] Tested within 33 cell generations after transfer to nonselective medium.

[b] Tested within 14 generations after isolation.

[c] At 24 h after routine culture transfer in nonselective medium 31 generations after isolation.

[d] At least 50 metaphases were examined for each determination.

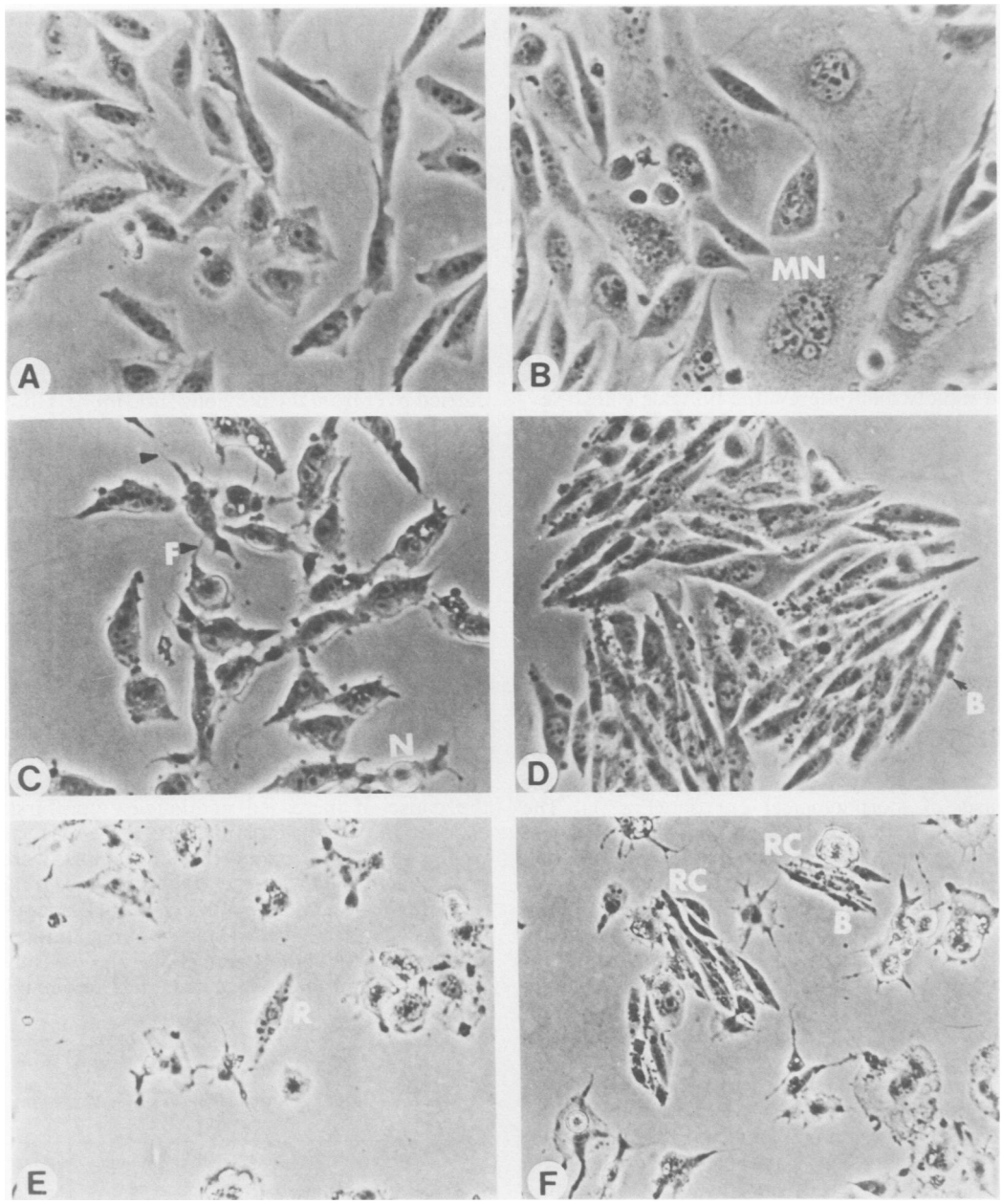

FIG. 1. Light micrographs of AK412 wild-type and Cyd^R variant cells. (A) AK412 in αNS (magnification, ca. ×530). (B) Cyd^R-10 in αNS. Note large multinucleate cells (MN) (ca ×500). (C) AK412 in αS-1 CD for 72 h. Retraction fibers (F), protruding nuclei (N), blebs, binucleate, and multinucleate cells are evident (ca. ×530). (D) Cyd^R-10 grown in αS-1 CD for many weeks. Note that nuclei are not protruding and that the cells are primarily mononucleate and bipolar. Blebs (B) are evident (ca. ×540). (E) A resistant cell (R) among a background of sensitive cells after 11 rounds of selection. The cells have been in 1 μg of CD per ml for 24 h (ca. ×285). (F) Clonal areas (RC) of resistant cells in round 13. The cells have been in 1 μg of CD per ml for 72 h (ca. ×260).

of 1.4×10^{-5} in mixtures of AK412 with cell line 51-11 and at frequencies of 7.8×10^{-6} to 2.3×10^{-5} in mixtures of Cyd^R-8, -10 and -20 with cell line 51-11; these results clearly show no increased interlineal fusion of the Cyd^R variants with cells of a closely related line. Second, the intralineal cell fusion reponse of the Cyd^R variants is indistinguishable from that of the parent

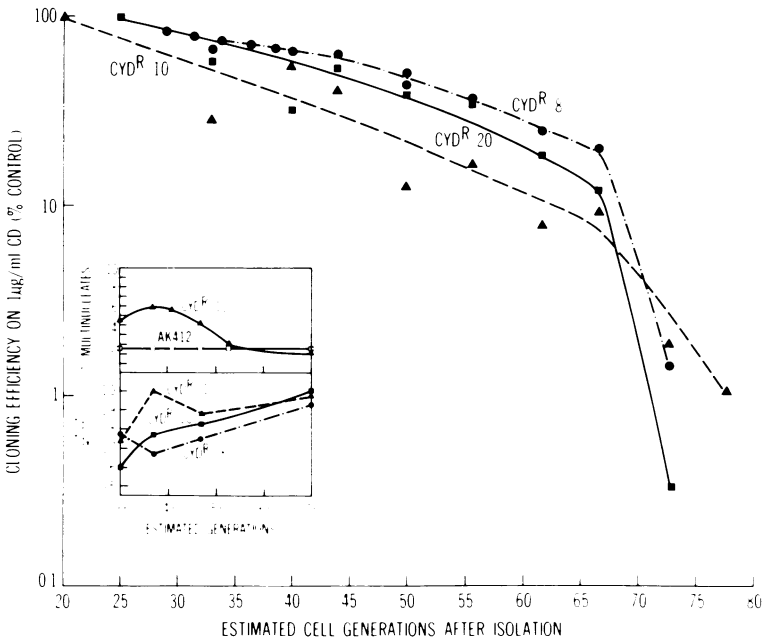

FIG. 2. Cloning efficiency on αS-1 CD of Cyd^R variant sublines grown in nonselective medium for 80 generations after isolation. Cloning efficiency in αS-1 CD was determined weekly. Cyd^R-8 (● · – · ●); Cyd^R-10 (▲ - - ▲); Cyd^R-20 (■—■). Insert: upper, frequency of multinucleate cells in a Cyd^R-10 population grown in nonselective medium; lower, decrease in Td for three Cyd^R variant sublines grown in nonselective medium. TdC/TdV is the ratio of the Td in the parental control line (TdC) AK412 to the Td of the variant (TdV). This ratio approaches unity as the variant growth rate increases.

after induction with 50% polyethylene glycol 1000 (10). Also, these multinucleate cells are not the result of micronucleation, a phenomenon commonly associated with unstable drug resistance (33), inasmuch as nuclei are always of normal size in a variant polynucleate cell (Fig. 1B).

Distinct morphological differences between parental sensitive cells and the Cyd^R variants have been observed with both light and scanning electron microscopes; these will be described elsewhere (S. H. Grund and J. K. Stadler, manuscript in preparation).

Stability of the Cyd^R resistance phenotype. For tests of phenotypic stability, the phenotype with full drug resistance was simply defined as the ability of all cells in Cyd^R variant subpopulations to undergo cytokinesis and form colonies in the presence of 1 μg of CD per ml. Cyd^R-8, -10, and -20 were subcultured continuously on nonselective medium, and cloning efficiencies on αS-1 CD were determined at weekly intervals for more than 75 generations (Fig. 2). The cloning efficiency of these variant lines plated on 1 μg of CD per ml was 85 to 100% for 20 to 25 cell generations after isolation and purification. Then, a logarithmic 50% decrease in CD resistance was observed every 10 to 15 cell generations up to cell generations 63 to 67. After cell generation 67, however, there was a much more rapid loss of resistant cells in the population so that the cloning efficiency declined from 10 to 20% to less than 1% in eight additional generations.

A loss of the CD resistance phenotype in populations grown on nonselective medium could be due to segregation of fully CD-resistant and fully drug-sensitive cells or could, alternatively, result from the gradual loss of cytochalasin resistance in many cells of the population over time. In the latter case, one might expect to see intermediate levels of resistance in derivative clones of the original resistant population after periods of culture in nonselective medium. Intermediate levels of CD resistance would be demonstrated by increased population cloning efficiency in lower concentrations of drug. However, no intermediate CD resistance phenotypes were detected in an experiment that showed that the cloning efficiencies of a population of Cyd^R-20, grown for 60 cell generations on nonselective medium, were nearly identical over a drug concentration range of 0.4 to 1.0 μg of CD per ml (1 μg/ml, 19.6%; 0.8 μg/ml, 15.8%; 0.6 μg/ml,

15.4%; and 0.4 μg/ml, 19.9%). Several other experiments also have indicated that the population loss of the CD resistance phenotype results from generation of sensitive revertants in the resistant line, followed by selective overgrowth of the CD-sensitive subtype in competition with fully CD-resistant cells of the kind first isolated (data not shown).

The rapidity of loss of the CD resistance phenotype under nonselective conditions probably is dependent upon at least two events: (i) the frequency of occurrence of sensitive segregants, and (ii) the strength of the positive selection for the drug sensitivity cell phenotype. Selection for CD-sensitive segregants and against the CD-resistant parental cells may occur because of the longer Td and the larger number of multinucleate cells in the variant lines. When the percentage of resistant cells in the population declines, the number of multinucleate cells decreases, and the culture Td decreases. It has been previously reported that there is a selective disadvantage for unstable drug-resistant cells in nonselective medium when the resistance is due to gene amplification (33). The instability and probable poor fitness of the Cyd^R phenotype must be taken into account in all experiments. Each test of variant characteristics is always performed with populations from frozen samples of the original isolates or with newly isolated subclones.

Cross-resistance to other drugs. Because pleiotropic drug cross-resistance had been demonstrated for unstable drug-resistant mutants in other laboratories (4, 7), we tested growth resistance of the Cyd^R variants to a number of other drugs with different structures and sites of action. Some of these experiments are summarized in Table 2. In this table, the results are expressed in terms of relative cross-resistance, which is defined as D_{50} variant/D_{50} AK412. D_{50} is the drug concentration that reduces cell survival, as measured by plating efficiency, 50%. It was determined that the Cyd^R variants are cross-resistant to colcemid (8- to 9.6-fold over wild type), actinomycin D (6- to 9-fold), and CB (1.6- to 2.2-fold). The Cyd^R variants also show increased sensitivity to procaine (approximately a 5.7-fold reduction in resistance). Additional preliminary cloning efficiency tests with vinblastine have been repeated twice, although D_{50} values have not yet been sufficiently defined. Cyd^R-8 and Cyd^R-10 clone 33- and 52-fold more efficiently than AK412 in medium with 0.04 μg of vinblastine per ml.

Cytology. The CD-resistant variants have some of the phenotypic characteristics of gene-amplified drug-resistant mutants, including unstable expression of the drug resistance phenotype (7). Because it has been well demonstrated that gene amplification is usually, or always, correlated with the presence of DMs or HSRs, Cyd^R-8 and Cyd^R-10 were carefully examined cytologically by using both Giemsa-trypsin banded and unbanded chromosome preparations.

TABLE 2. Relative cross-resistance[a] of Cyd^R variants to other drugs

Cell line	CD	CB	Colcemid	Actinomycin D	Procaine
AK412	1.0[b]	1.0	1.0	1.0	1.0
Cyd^R-8	18.2	1.9	9.6	6.0	0.22
Cyd^R-10	15.0	2.2	8.4	9.0	0.15
Cyd^R-20	ND[c]	1.6	8.0	8.0	0.15

[a] Relative cross-resistance is expressed as the ratio of D_{50} variant/D_{50} AK412.
[b] Five determinations were made for each data point in duplicate dose-response curves.
[c] ND, Not determined.

Analysis of original isolates of these variants, as well as four drug-resistant subclonal isolates, revealed the presence of an HSR in the distal third of the long arm of chromosome 1 (Table 3 and Fig. 3). This HSR represents up to 7% of the elongated variant chromosome and is absent in AK412. It is unique in that, for at least 50% of the metaphase cells examined, there are variable numbers of bands distal to the HSR, which seem to show a reverse repeat of the banding pattern preceding the HSR region. This can be demonstrated by cutting close to the middle of a photographed HSR and then reversing the distal portion so that the HSR halves are side by side (Fig. 3D). A one-to-one band correspondence is then clearly suggested.

The number of DMs in the drug-resistant and the parental lines also was examined, and the average number per cell was found to be quite small (1 to 2.5) (Table 3). In no instance in the variant lines did we find a correlation of numbers of DMs per cell with either drug resistance of sensitivity.

CD-displaceable binding of [^{3}H]CB. The multiple-drug cross-resistance phenotype of the Cyd^R variants strongly suggests that the underlying molecular lesion results in some generalized alteration of the level of membrane permeability (4). Shin Lin and Wells Magargal generously allowed one of us (S.H.G.) to come to their laboratory and measure the CD-displaceable binding of [^{3}H]CB in variant Cyd^R-10. Binding to intact cells and cell extracts was analyzed by the graphical procedure of Scatchard (31) (Fig. 4 and Table 4). In intact cells (Fig. 4B), there is a fourfold reduction in the number of binding sites in Cyd^R-10 compared with AK412 (6 and 24

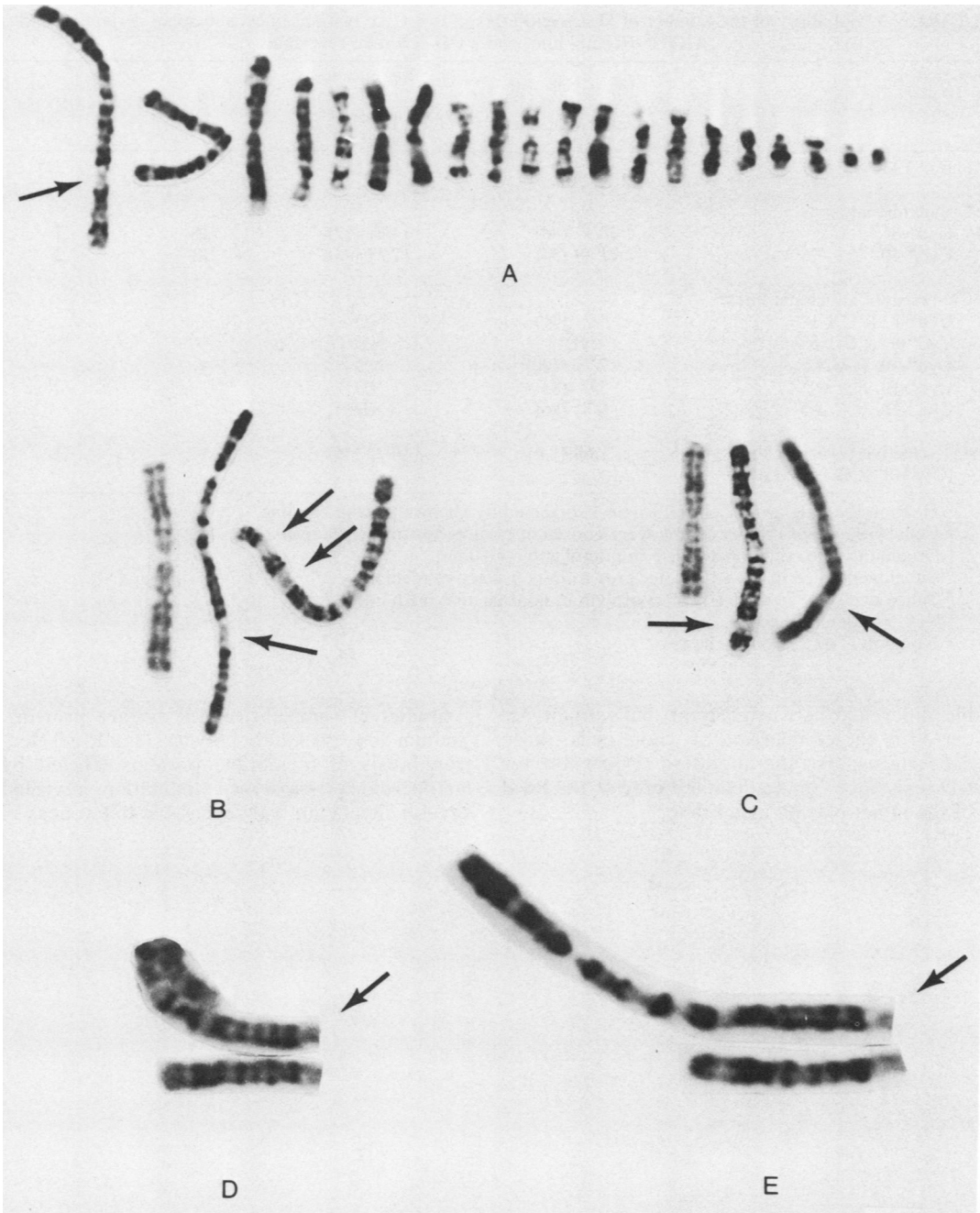

FIG. 3. Trypsin-giemsa banded chromosomes from preparations of Cyd^R-8. Arrows indicate HSR regions. (A) Karyotype of Cyd^R-8 (×400). (B and C) AK412 parental chromosome (left) compared with 2 additional Cyd^R-8 chromosomes 1 (ca. ×540 and ×385, respectively). (D and E) Banding patterns distal to the HSR look like inverted repetitions of banding patterns between HSR and the centromere. Cyd^R-8 chromosomes 1 were cut at the arrow (mid-HSR) and inverted to show concordance of bands on both sides of the HSR (×800 and ×960).

pmol per mg of protein, respectively), although both lines have a similar K_d. However, in both soluble extracts and isolated membrane of AK412 (Fig. 4A), there is no difference in the number of binding sites of K_d when compared with Cyd^R-10. Inasmuch as the only demonstra-

TABLE 3. Summary of the number of HSRs and DMs in two Cyd^R variant lines, subclonal derivatives, the AK412 parental line, and a CD-sensitive revertant

Cell line	No. of cells with HSR[a]/total examined[b] (%)	No. of cells with DMs/total metaphase cells examined (%)	Total no. of DMs	Avg. no. of DMs per cell
Parent (AK412)	0/33	4/25 (16)	10	2.5
Original isolates[c]				
Cyd^R-8	30/34 (88)	14/21 (67)	18	1
Cyd^R-10	21/39 (54)	17/37 (46)	32	2
CD-resistant subclonal lines[d]				
Cyd^R-8, R.C.1-1	5/7 (71)	ND[f]		
Cyd^R-8, R.C.1-5	ND	ND		
Cyd^R-10, R.C.3[e]	7/7 (100)	ND		
Cyd^R-10, R.C.3-3	5/6 (83)	ND		
Cyd^R-10, R.C.3-5	6/7 (86)	ND		
CD-sensitive revertant line[g] (Cyd^R-8, R.C.1, S.C.5)	1/69 (1.4)	10/22 (45)	14	1

[a] HSR on the long arm of chromosome 1, detected by Giemsa-trypsin banding.
[b] Cells with reasonable spreading were chosen at random for further analysis.
[c] Original isolate, subcloned twice in nonselective medium.
[d] Subcloned twice in αS-1 CD after growth in nonselective medium.
[e] Subcloned once in αS-1 CD after growth in nonselective medium.
[f] ND, Not determined.
[g] Isolation is described in the text.

ble difference between parent and variant occurred in the comparison of whole cells, these data suggest that the alteration responsible for CD resistance can be detected only at the level of the intact plasma membrane.

Studies of radiolabeled cell surface proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane proteins labeled by lactoperoxidase-catalyzed iodination revealed several significant and repeatable differences in

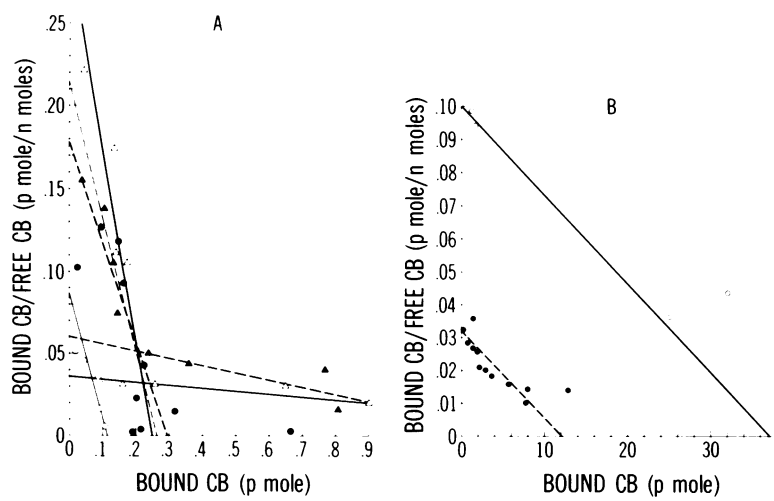

FIG. 4. Scatchard plot analysis of the binding of [^{3}H]CB to CD-displaceable sites. (A) binding of [^{3}H]CB to soluble extracts of Cyd^R-10 (△—△) and AK412 (▲ - - - ▲), and to membrane pellets of Cyd^R-10 (○—○) and AK412 (● - - - ●). The nearly horizontal lines might be due to low affinity binding of [^{3}H]CB to G-actin. (B) binding of [^{3}H]CB to intact cells of Cyd^R-10 (●) and AK412 (○). The number of binding sites is determined by dividing the X intercept by the amount of protein used in the assay (see Table 4). The concentrations of [^{3}H]CB and unlabeled CD are described in the text.

TABLE 4. CD-displaceable binding of [^{3}H]CB to intact cells and cell extracts

Cell line	Whole cell binding		Fractionated cells			
			Membrane-particulate fraction		Soluble-extract fraction	
	K_d	No. of binding sites[a]	K_d	No. of binding sites[a]	K_d	No. of binding sites[a]
AK412	3.8×10^{-7}	24	2.4×10^{-8}	2.4		ND[b]
	ND	ND	1.6×10^{-8}	2.8	1.2×10^{-8}	1.3
Cyd^R-10	3.7×10^{-7}	6	2.9×10^{-8}	2.9		ND
	3.2×10^{-7}	5.1	1.5×10^{-8}	1.6	0.9×10^{-8}	1.7

[a] Picomoles per milligram of protein.
[b] ND, Not determined.

band migration between the parent and the Cyd^R variants. Figure 5 shows an autoradiogram of a 7% gel (5A) and densitometer scans of the autoradiogram (5B). The variants (5A; b, c, d) produced virtually identical patterns, including the decreased migration (increased apparent molecular weight) of numerous bands. The most pronounced shifts are in bands migrating with

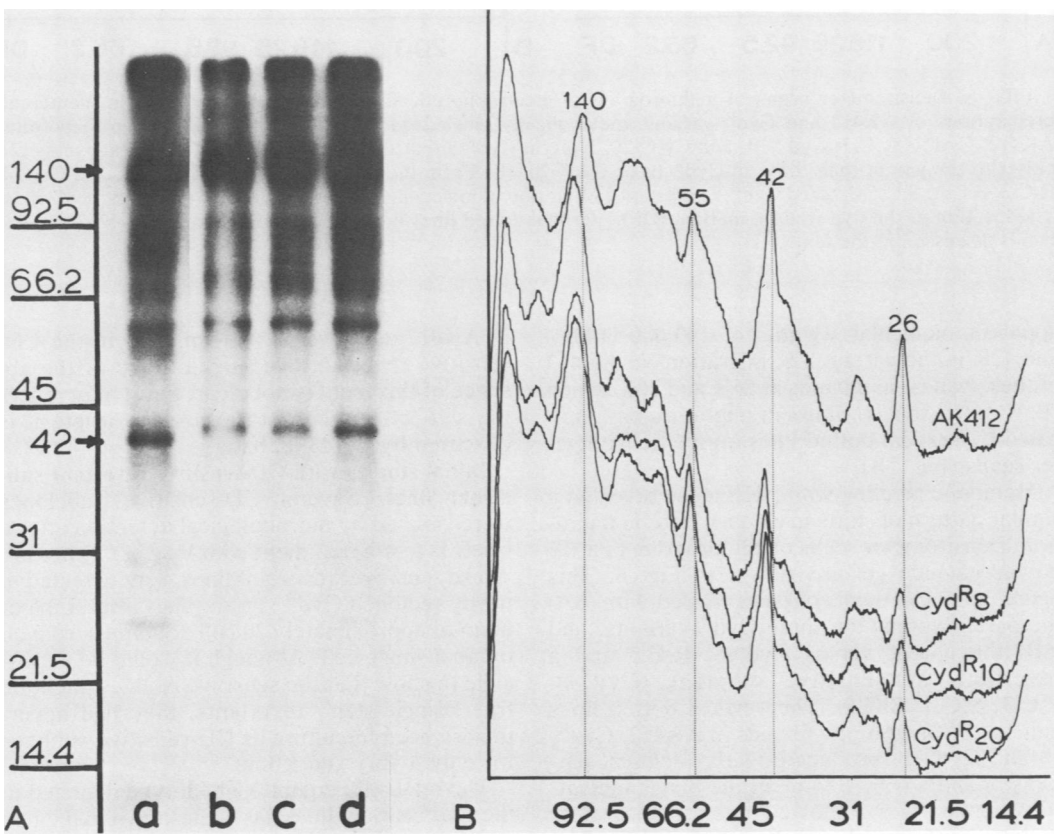

FIG. 5. Autoradiogram (A) and densitometer scans of an autoradiogram (B) of electrophoretically separated surface proteins of AK412 and Cyd^R variants labeled with ^{125}I by lactoperoxidase-catalyzed iodination. (A) (a) AK412; (b) Cyd^R-8; (c) Cyd^R-10; and (d) Cyd^R-20. Bars at the left indicate migration of molecular weight standards (molecular weight $\times 10^{-3}$). Two bands (140K and 42K), which migrate faster in AK412 than in the variants, are noted by arrows. (B) Densitometer scans of the autoradiogram in (A). Migration of molecular weight standards is noted on the X-axis. Vertical dotted lines mark the migration of several proteins in AK412 (see text for further explanation).

FIG. 6. Densitometer scans of a fluorogram of electrophoretically separated glycoproteins in membrane preparations of AK412 and Cyd^R variants metabolically labeled with [^{3}H]fucose. (A) From top to bottom: AK412; Cyd^R-8 R.C.3; Cyd^R-10; and Cyd^R-20. Note in Cyd^R-8 that the 139K, 119K, and 111K bands migrate closer to the parent than those in Cyd^R-10 or Cyd^R-20. (B) From the top: AK412; Cyd^R-8 R.C.3; and Cyd^R-8 R.C.3, S.C.4, S.C.5 (labeled Cyd^R-8 revertant). Migration of molecular weight standards is noted on the X-axes. The location of the dye front is marked (DF). Vertical dotted lines indicate the migration in AK412 of several bands discussed in the text.

apparent molecular weights of 140,000 (140K) and 42K in the parent. The migrations in AK412 of these bands, and bands at 55K and 26K which show much smaller shifts in migration, are indicated by vertical dotted lines on the densitometer scans (Fig. 5B).

Metabolic labeling with [^{3}H]fucose revealed a similar pattern of shifts in electrophoretic migration. Densitometer scans of a fluorogram of a 7% polyacrylamide gel are shown in Fig. 6. This figure compares scans of lanes loaded with (6A) the parent versus the three Cyd^R variants, and (6B) the parent versus Cyd^R-8, R.C.3 and a revertant, CD-sensitive subclone (Cyd^R-8, R.C.3, S.C.4, S.C.5). The vertical dotted lines indicate the migration of bands in AK412. Cyd^R-10 and Cyd^R-20 produced nearly identical patterns of shifts (Fig. 6A) in all the bands indicated, whereas Cyd^R-8 showed much smaller shifts in three of the bands (139K, 199K, 111K). The same pattern was seen in the CD-sensitive revertant of Cyd^R-8 except that two bands (191K and 219K), which showed large shifts (to higher apparent molecular weights) in Cyd^R-8, migrated much closer to the corresponding bands in AK412. Another band, migrating at about 130K in AK412 and Cyd^R-8, was missing in the CD-sensitive revertant. The significance of the absence of this band is not clear, but it may reflect the shift of this band to a position where it is obscured by the 119K band.

Initial studies with CD-sensitive revertant subclonal lines. Several CD-sensitive subclones were isolated by morphological detection after a brief, 1- to 4-h treatment with 1 μg of CD per ml. These sensitive, revertant lines were detected in newly recloned Cyd^R variant lines after a maximum of approximately 20 cell doublings from an original single cell. Although it would be impossible to show that the sensitive isolates might be true "single-step" revertants, they had nevertheless been in culture as CD-sensitive sublines for only a very short time.

The relatively complex phenotype detected in the Cyd^R variant lines has been shown to include multiple-drug cross-resistance, long Td, increased multinucleation in nonselective medium, the presence of a chromosome 1 HSR, and multiple differences in cell surface protein and glycoprotein band migrations. Because the Cyd^R variants were isolated in long-term selection, it is possible that these various aspects of the

phenotype may have arisen as independent, unrelated events. Alternatively, they could be the pleiotropic expression of one major basic alteration. Concurrent disappearance in the CD-sensitive revertants of the diverse Cyd^R phenotypic characteristics would argue for the latter situation.

One sensitive subclonal line from Cyd^R-8 has been studied most extensively so far. This line was designated Cyd^R-8, R.C.3, S.C.4, S.C.5 (Cyd^R-8, resistant clone 3, sensitive clone 4, sensitive clone 5) to indicate the order of subclonal isolations. In the following description, it will be referred to as S.C.5 and will be compared with its direct CD-resistant parent, Cyd^R-8, R.C.3, as well as AK412.

Experiments show that S.C.5 has regained sensitivity to all drugs tested so far; these include CD, CB, colcemid, and vinblastine. In each case, the ratio of D_{50} for S.C.5 to D_{50} for AK412 is 1.0. Also, the S.C.5 culture Td has been reduced to 14.5 h, which is quite similar to the Td at 14 h for AK412, but which is unlike the 18-h Td of the direct CD-resistant parent. The amount of multinucleation at 48 h is 3% for S.C.5, 1% for AK412, and 8% for Cyd^R-8 R.C.3. Cytology performed on a related subclone (Cyd^R-8, R.C.1, S.C.5) revealed only 1 HSR in 69 cells, as opposed to 30 HSRs in 34 cells of Cyd^R-8. These data collectively suggest a concomitant loss of the multiple phenotype; i.e., pleiotropy. Other sensitive-revertant clonal lines have been examined in only some of these tests; all, however, so far show the same pattern of return to the parental AK412 phenotype (data not given).

A further example of simultaneous phenotypic reversion in S.C.5 is provided by the previously described electrophoretic analysis of metabolically labeled glycoproteins. When the glycoprotein profile of S.C.5 is compared to those of AK412 and Cyd^R-8 R.C.3 (Fig. 6B), it is apparent that the largest migration shifts seen in Cyd^R-8, R.C.3 (bands 191K and 219K) are substantially reduced in the revertant.

DISCUSSION

We have isolated CHO variants that are resistant to 1 μg of CD per ml. This concentration of CD causes complete inhibition of cytokinesis, but not karyokinesis, in the parental line AK412. The variants were obtained through an enrichment procedure which used repeated pulses of 1 μg of CD per ml. In each round of this selection, the population was treated with cytochalasin to cause multinucleation and subsequently was placed in nonselective medium to allow for the outgrowth of any potentially CD-resistant cells that may have remained mononucleate. The Cyd^R variants, isolated after 16 selection rounds, are 20-fold more resistant to CD than the parental line, twofold more resistant to CB, cross-resistant to three structurally and functionally unrelated drugs, actinomycin D, colcemid, and vinblastine, and more sensitive to procaine.

Although a number of drug-resistant variants have been isolated by enrichment selection, the selection protocol described here differs from conventional methods in that the cells were exposed to repeated pulses of only one minimally toxic drug concentration. Other enrichment selection protocols commonly maintain steady selection pressure by continuous growth in gradually increasing, and eventually quite large, concentrations of drug (e.g., reference 4). This latter method of drug administration can be used in treatment of cancers, and it has been proposed that this protocol may allow the selection of resistant tumors, a common problem arising during the chemotherapy (27). We demonstrate that an alternate treatment regimen, relying only on repeated applications of low drug concentration followed by periods without drug, also can result in the production of drug-resistant cell lines; this may also prove true for the development of drug-resistant cancer cells in vivo.

Multiple-drug cross-resistant mutants in CHO have been isolated in other laboratories. Some of these have a stable phenotypic expression (11), whereas others are phenotypically unstable during periods of prolonged growth in medium without drugs (7). The complex Cyd^R variant phenotype described here is progressively lost during a period of approximately 70 cell generations of growth in nonselective medium.

The Cyd^R variant lines contain an HSR in the long arm of chromosome 1. The presence of this HSR correlates with the observed multiple Cyd^R phenotype that includes a longer culture Td and increased multinucleation in nonselective medium (probably resulting from a defect in cell division). Both HSR and the other characteristics just described are absent in almost all cells of the CD-sensitive revertant lines examined, suggesting that these aspects of Cyd^R phenotype may be dependent upon amplified DNA in the region of the chromosome 1 HSR. No gene product(s) has yet been associated with this HSR, but amplification of determinants controlling drug response may certainly be inferred because of analogies with previous work on HSRs at other loci (7).

The chromosome 1 HSR reported here has a variable number of distal bands; these appear to be an inverted repeat of the banding pattern on the centromeric side of the HSR. It is probable that these banding inversions arose as a result of sister chromatid exchange, followed by sister

union in the region of the HSR. Sister unions of this type are known to yield dicentric chromosomes (15), whose breakage would, in turn, yield inverted banding patterns around the HSR in some derivative cells.

A number of observations indicate that the Cyd^R lines are drug-uptake variants: (i) The CD-displaceable [^{3}H]CB-binding assay data for Cyd^R-10 show that the fourfold reduction in binding of CB requires intact cells and intact plasma membranes; these experiments, therefore, suggest that observed cytochalasin resistance is a result of the decrease in net uptake of CD (and CB). (ii) The Cyd^R variants also exhibit multiple-drug cross-resistance. Such patterns of cross-resistance are commonly observed in other drug-uptake mutants (4, 7). Also, consistent with observations on other uptake mutants, the Cyd^R cell lines are two-fold more sensitive to the anesthetic procaine (23). (iii) Experiments with Tween-80 (data not presented) show that the addition of 100 μg/ml, a concentration that is not toxic for AK412, reduced the CD-resistance of Cyd^R-8 to near parental levels. These data considered together strongly imply that the Cyd^R variants have a reduced influx of CD (reduced transport or permeability, or both) as opposed to increased efflux or catabolism (4).

Electrophoretic analysis of Cyd^R-8, -10, and -20 show multiple band migration differences after labeling either with lactoperoxidase-catalyzed iodination or with [^{3}H]fucose. In each case noted, the proteins of the variants migrated more slowly than the corresponding proteins of the CD-sensitive parent. These results are in contrast to the previously reported single 170,000-dalton plasma membrane glycoprotein difference associated with a stable drug impermeability phenotype in CHO exhibiting some similarity to the unstable Cyd^R phenotype (11). Also, an analysis of unstable vincristine-resistant Chinese hamster lung cells with an HSR on chromosome 9 has shown that the major difference in plasma membrane glycoproteins is a relative increase of a 150K and a decrease of a 100K species in the variant (7).

Our electrophoretic studies of plasma membrane so far do not allow us to make any conclusions regarding the relationship of the observed protein differences and the Cyd^R phenotype, even though the HSR-lacking, CD-sensitive, phenotypic revertant S.C.5 displayed a more parental-type migration of several glycoproteins than did Cyd^R-8. Because these Cyd^R variants were selected by a multiple-step procedure, it is not clear that the multiple migration differences are attributable to a single genetic defect.

Unstable methotrexate resistance has been studied more exhaustively in cell lines other than Chinese hamster and has, in most instances, been found to correlate with the presence of DMs (4, 14). A recent careful examination of amplification and instability of dihydrofolate reductase genes in CHO cells failed, however, to reveal a significant presence of DMs (18). The authors of that study suggest that the DMs simply may not have been detectable or that unstable amplified genes may exist in a chromosome.

Results presented in this paper and elsewhere tend to support the latter interpretation. Unstable drug resistance previously has been shown to be correlated with a chromosomal HSR in Chinese hamster cell lines in instances of both methotrexate resistance (6) and vincristine resistance (7). Our work, also with Chinese hamster cell lines, provides another instance of HSR association with a unique, unstable drug resistance phenotype. These combined results suggest that an unstable gene amplification within the chromosome which eventually results in the appearance of an HSR may be the characteristic cellular response of this species to selective pressure from growth in media with toxic drugs. A species difference of this nature implies, in turn, that there is strict genetic control of the style of permitted chromosomal gene amplification response.

ACKNOWLEDGMENTS

We are indebted to Shin Lin for allowing S. Grund to visit his laboratory and to Wells Magargal and L. Magargal for instruction in the [^{3}H]CB-binding assay. We thank Mary Sand and Karen Biagi for technical assistance.

Our work was supported by Public Health Service grant GM25728 from the National Institutes of Health.

LITERATURE CITED

1. **Alt, F. W., R. E. Kellems, J. R. Bertino, and R. T. Schimke.** 1978. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. J. Biol. Chem. **253**:1357–1370.
2. **Andrulis, I. L., and L. Siminovitch.** 1982. Amplification of the gene for asparagine synthetase, p. 75–80. *In* R. T. Schimke (ed.), Gene amplification. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
3. **Balaban-Malenbaum, G., and F. Gilbert.** 1977. Double minute chromosomes and the homogeneously staining regions in chromosomes of a human neuroblastoma cell line. Science **198**:739–741.
4. **Baskin, F., R. N. Rosenberg, and V. Dev.** 1981. Correlation of double-minute chromosomes with unstable multidrug cross resistance in uptake mutants of neuroblastoma cells. Proc. Natl. Acad. Sci. U.S.A. **78**:3654–3658.
5. **Beach, L. R., and R. D. Palmiter.** 1981. Amplification of the metallothionein-I gene in cadmium resistant mouse cells. Proc. Natl. Acad. Sci. U.S.A. **78**:2110–2114.
6. **Biedler, J. L., P. W. Melera, and B. A. Spengler.** 1980. Specifically altered metaphase chromosomes in antifolate-resistant Chinese hamster cells that overproduce dihydrofolate reductase. Cancer Genet. Cytogenet. **2**:47–60.
7. **Biedler, J. L., and R. H. F. Peterson.** 1981. Altered plasma membrane glycoconjugates of Chinese hamster cells with acquired resistance to actinomycin D, daunorubicin, and vincristine, p.453–482. *In* A. C. Sartorelli, J. S. Lazo, and

J. R. Bertino (ed.), Molecular actions and targets for cancer chemotherapeutic agents. Academic Press, Inc., Ltd., London.
8. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
9. **Chasin, L. A.** 1973. The effect of ploidy on chemical mutagenesis in cultured Chinese hamster cells. J. Cell Physiol. **82**:299–308.
10. **Davidson, R. L., and P. S. Gerald.** 1976. Improved techniques for the induction of mammalian cell hybridization by polyethylene glycol. Somatic Cell Genet. **2**:165–176.
11. **Debenham, P. G., N. Kartner, L. Siminovitch, J. R. Riordan, and V. Ling.** 1982. DNA-mediated transfer of multiple drug resistance and plasma membrane glycoprotein expression. Mol. Cell. Biol. **2**:881–889.
12. **Gahmberg, C. G., K. Itaya, and S.-I. Hakomori.** 1976. External labeling of cell surface carbohydrates, p. 179–210. *In* E. D. Korn (ed.), Methods in membrane biology, vol. 9. Plenum Publishing Corp., New York.
13. **Godman, G. C., and A. F. Miranda.** 1978. Cellular contractility and the visible effects of cytochalasin, p. 277–430. *In* S. W. Tannenbaum (ed.), Cytochalasins: biochemical and cell biological aspects. Elsevier/North-Holland Publishing Co., Amsterdam.
14. **Haber, D. A., and R. T. Schimke.** 1981. Unstable amplification of an altered dihydrofolate reductase gene associated with double-minute chromosomes. Cell **26**:355–362.
15. **Hamerton, J. L.** 1971. Human cytogenetics, p. 240–241. General Cytogenetics, vol. 1. Academic Press, Inc., New York and London.
16. **Hynes, R. O.** 1973. Alteration of cell surface proteins by viral transformation and by proteolysis. Proc. Natl. Acad. Sci. U.S.A. **70**:3170–3174.
17. **Kaufman, R. J., P. C. Brown, and R. T. Schimke.** 1979. Amplified dihydrofolate reductase genes in unstably methotrexate-resistant cells are associated with double minute chromosomes. Proc. Natl. Acad. Sci. U.S.A. **76**:5669–5673.
18. **Kaufman, R. J., and R. T. Schimke.** 1981. Amplification and loss of dihydrofolate reductase genes in a Chinese hamster ovary cell line. Mol. Cell. Biol. **1**:1069–1076.
19. **Kopnin, B. P.** 1981. Specific karyotypic alterations in colchicine-resistant cells. Cytogenet. Cell Genet. **30**:11–14.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lewis, W. H., B. A. Kuzks, and J. A. Wright.** 1978. Assay of ribonucleotide reduction in nucleotide-permeable hamster cells. J. Cell Physiol. **94**:287–298.
22. **Lin, D. C., and S. Lin.** 1980. A rapid assay for actin-associated high affinity cytochalasin binding-sites based on isoelectric precipitation of soluble-protein. Anal. Biochem. **103**:316–322.
23. **Ling, V., and L. H. Thompson.** 1974. Reduced permeability in CHO cells as a mechanism of resistance to colchine. J. Cell Physiol. **83**:103–116.
24. **Masters, J., B. Keeley, H. Gag, and G. Attardi.** 1982. Variable content of double minute chromosomes is not correlated with degree of phenotype instability in methotrexate-resistant human cell lines. Mol. Cell. Biol. **2**:498–507.
25. **Melera, P. W., J. A. Lewis, J. L. Biedler, and C. Hession.** 1980. Antifolate-resistant Chinese hamster cells: evidence for dihydrofolate reductase gene amplification among independently derived sublines overproducing different dihydrofolate reductases. J. Biol. Chem. **255**:7024–7028.
26. **Meuth, M., and H. Green.** 1974. Alterations leading to increased ribonucleotide reductase in cells selected for resistance to deoxynucleotides. Cell **3**:367–374.
27. **Mihich, E. (ed.).** 1973. Drug resistance and selectivity. Biochemical and cellular basis. Academic Press, Inc., New York.
28. **Nunberg, J. H., R. J. Kaufman, R. T. Schimke, G. Urlaub, and L. A. Chasin.** 1978. Amplified dihydrofolate reductase genes are localized to a homogeneously staining region of a single chromosome in a methotrexate-resistant Chinese hamster ovary cell line. Proc. Natl. Acad. Sci. U.S.A. **75**:5553–5556.
29. **Pauw, P. G., and J. D. David.** 1979. Alterations in surface proteins during myogenesis of a rat myoblast cell line. Dev. Biol. **70**:27–38.
30. **Ryan, J., E. C. Hardeman, A. Endo, and R. D. Simoni.** 1981. Isolation and characterization of cells resistant to ML236B (compactin) with increased levels of 3-hydroxy-3-methy 1-glutary 1 coenzyme A reductase. J. Biol. Chem. **256**:6762–6768.
31. **Scatchard, G.** 1949. The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. **51**:660–666.
32. **Schimke, R. T. (ed.).** 1982. Gene amplification. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
33. **Schimke, R. T., P. C. Brown, R. J. Kaufman, M. McGrogand, and D. L. Slate.** 1981. Chromosomal and extra chromosomal localization of amplified dihydrofolate reductase genes in cultured mammalian cells. Cold Spring Harbor Symp. Quant. Biol. **45**:785–797.
34. **Seabright, M.** 1971. A rapid banding technique for human chromosomes. Lancet **ii**:971–972.
35. **Tanenbaum, S. W. (ed.).** 1978. Cytochalasins: biochemical and cell biological aspects. Elsevier/North-Holland Publishing Co., Amsterdam.
36. **Wahl, G. W., R. A. Padgett, and G. R. Stark.** 1979. Gene amplification causes overproduction of the first three enzymes of UMP synthesis in N-(phosphonacetyl)-L-aspartate-resistant hamster cells. J. Biol. Chem. **254**:8679–8689.
37. **Wright, C. E., and T. B. Shows.** 1979. Genetics of cell fusion: human chromosome 10 assignment of a gene (FUSE) that promotes polykaryocyte formation. Somatic Cell Genet. **5**:503–517.