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Chromosome-Mediated Gene Transfer of Hydroxyurea Resistance and Amplification of Ribonucleotide Reductase Activity

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Metaphase chromosomes purified from a hydroxyurea-resistant Chinese hamster cell line were able to transform recipient wild-type cells to hydroxyurea resistance at a frequency of 10^{-6}. Approximately 60% of the resulting transformant clones gradually lost hydroxyurea resistance when cultivated for prolonged periods in the absence of drug. One transformant was subjected to serial selection in higher concentrations of hydroxyurea. The five cell lines generated exhibited increasing relative plating efficiency in the presence of the drug and a corresponding elevation in their cellular content of ribonucleotide reductase. The most resistant cell line had a 163-fold increase in relative plating efficiency and a 120-fold increase in enzyme activity when compared with the wild-type cell line. The highly hydroxyurea-resistant cell lines had strong electron paramagnetic resonance signals characteristic of an elevated level of the free radical present in the M2 subunit of ribonucleotide reductase. Two-dimensional electrophoresis of cell-free extracts from one of the resistant cell lines indicated that a 53,000-dalton protein was present in greatly elevated quantities when compared with the wild-type cell line. These data suggest that the hydroxyurea-resistant cell lines may contain an amplification of the gene for the M2 subunit of ribonucleotide reductase.

In mammalian cells, ribonucleoside diphosphate reductase (RDR) is the sole enzyme responsible for the conversion of the four ribonucleotides to the deoxyribonucleotides required for DNA synthesis (35). The enzyme exhibits complex and strict allosteric control by a variety of positive and negative nucleotide effectors (9). In addition, the levels of RDR are tightly controlled so that greatly increased amounts of active enzyme are present at the onset of DNA synthesis, and high levels are maintained until DNA synthesis decreases (15, 21, 24).

Despite the central role that RDR must play in mammalian cell division, almost nothing is known about how the level of enzyme activity is regulated.

Although RDR activity was first reported in mammalian cell extracts over 20 years ago (23), only recently has the enzyme been purified to near homogeneity from calf thymus (7, 34). The mammalian enzyme is very similar to the one found in *Escherichia coli* which consists of two nonidentical subunits with a total molecular weight of 240,000 (33). In *E. coli*, one subunit binds the nucleotide effectors and contains active thiol groups which are involved in the replacement of the hydroxyl group with a hydrogen at the 2′ position of ribose of the ribonucleotide (5, 37). The other subunit contains a free radical dependent upon the presence of two atoms of nonheme iron. The free radical is necessary for enzymatic activity and can be destroyed by hydroxyurea, a potent inhibitor of RDR activity and cell division (4, 17).

In mammalian systems, the iron-containing subunit M2 has not been purified to homogeneity, but recent data suggest that it is this subunit, or its free radical content, which increases during periods of RDR elevation (1, 8).

To date, studies of the fluctuation of RDR levels have been severely hindered by the difficulty of the enzyme assay and by the lack of antibody and nucleic acid sequences specific for the enzyme.

We have used a biochemical genetic approach to facilitate the study of RDR. Somatic cell mutants can be selected for resistance to hydroxyurea, and these mutants have been shown to have alterations in the level or resistance of RDR activity, or both (1, 18, 20). Since in all cases these mutations are expressed codominantly (19), we investigated whether a gene

transfer system could be developed for hydroxyurea resistance. Similar DNA-mediated gene transfer procedures have directly led to the cloning of the genes for chicken thymidine kinase (26) and hamster adenine phosphoribosyl transferase (22).

MATERIALS AND METHODS

Cell culture. All cell lines were routinely maintained in α-minimal essential medium (Flow Laboratories) supplemented with 5% horse serum and 2% fetal bovine serum (MA Bioproducts). V79 O^R-1 is a derivative of the Chinese hamster lung cell line V79/V6 (36) selected for resistance to 3 mM ouabain. Mtx^{RIII} is a Chinese hamster ovary cell line isolated in two steps for resistance to methotrexate (10).

Chromosome-mediated gene transfer. Metaphase chromosomes were purified from hydroxyurea-resistant cell lines as previously described (31). Chromosomes were precipitated with calcium phosphate (12) and added to recipient cells by a modified procedure (14, 16). After 24 h, the medium containing the chromosome-calcium phosphate precipitate was removed and replaced with fresh medium. After an additional 24 h of expression, the cells were trypsinized, counted, and plated at 5×10^5 cells per 100-mm tissue culture dish (Lux Scientific) containing 15 ml of α-minimal essential medium, 10% fetal bovine serum, and 35 μg of hydroxyurea per ml. After 12 days of incubation at 37°C, the number of colonies per dish was counted.

Assay of RDR. RDR activity was assayed by measuring the conversion of [^{14}C]CDP to [^{14}C]dCDP in cell-free extracts (18) or in intact permeabilized cells (15). For the permeabilized cell assay, the cells were plated in 100-mm culture dishes in standard medium and harvested by trypsinization at approximately 80% confluence. The cells were washed once with 80 mM KCl containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2) and suspended at 10^7 cells per ml in permeabilizing buffer consisting of 3% Tween 80, 80 mM KCl, and 50 mM HEPES (pH 7.2). After 18 min at 37°C, the permeabilized cells were dispensed at 0.75 ml per assay tube and centrifuged at 1,000 rpm for 6 min. The cell pellet was resuspended in 200 μl of permeabilizing buffer and made up to 300 μl to give a final concentration of 6 mM ATP, 6 mM dithiothreitol, 10 mM $MgCl_2$, 0.04 mM [^{14}C]CDP (60 cpm/pmol), and 50 mM HEPES (pH 7.2). The cells were incubated in the reaction mixture for 2.5 to 60 min depending upon the level of CDP reductase present in the cell line. All assay time points were chosen to be within the linear period of increasing CDP reduction with time. The quantity of [^{14}C]deoxycytidine formed was measured (32), and the level of CDP reductase was expressed as pmol of deoxycytidine formed per h per 7.5×10^6 cells.

EPR measurements. For electron paramagnetic resonance (EPR) measurements, hydroxyurea-resistant cells were grown in the absence of the drug for 7 to 8 generations, trypsinized, and then suspended in Tris-saline buffer as described previously by Åkerblom et al. (1). The cells were then pelleted in EPR tubes calibrated with diphenylpicrylhydrazyl in benzene. Sufficient cell suspension was placed in each tube to give a packed cell height of 1 inch (ca. 2.54 cm) or more. The packed cells were then frozen at 77 K, and the spectra were recorded by a Varian E-9 spectrometer. Control V79 cells were processed in the same manner. The Teflon sample support of the EPR cavity was adjusted for maximum signal in a reference sample.

Two-dimensional electrophoresis of cell extracts. Cells exponentially growing in 150-cm^2 tissue culture flasks were washed once with ice-cold sonication buffer consisting of 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris-hydrochloride (pH 6.8). The cells were scraped from the surface, pelleted at 1,000 rpm for 10 min, washed once with sonication buffer, and finally resuspended at 10^8 cells per ml in the same buffer. After three 10-s bursts of sonication, the disrupted cell preparation was centrifuged at 100,000 × *g* for 60 min, and the supernatant was adjusted to 4 mg of protein per ml. Urea was added to the extract to give a final concentration of 9.0 M, and electrophoresis was carried out as described previously by O'Farrell (25). Electrophoresis in the isoelectric-focusing dimension was carried out with a pH gradient of approximately 4.0 to 7.0, followed by sodium dodecyl sulfate electrophoresis in an 8 to 13% linear polyacrylamide gradient. Gels were stained with 0.25% Coomassie brilliant blue R-250 in 50% methanol–10% acetic acid.

RESULTS

Selection of hydroxyurea-resistant cell line. Since previously selected hydroxyurea-resistant cell lines often displayed a decreased growth rate in culture, we decided to use the V79 Chinese hamster lung cell line because of its extremely rapid doubling time. Exponentially growing V79 cells were mutagenized with 500 μg of ethyl methane sulfonate per ml for 20 h and grown for 11 days before being plated in medium containing 30 μg of hydroxyurea per ml. Hydroxyurea-resistant colonies arose at a frequency of approximately 10^{-7}. One such colony was picked, recloned, and labeled Hyd^R-4. Figure 1A confirms that the Hyd^R-4 cell line was capable of forming colonies in the presence of 10-fold higher concentrations of hydroxyurea when compared with the V79 wild-type parental line. This resistance was stable upon prolonged cultivation in the absence of drug (data not shown). When the CDP reductase activity was tested for inhibition by hudroxyurea, the enzyme activity from Hyd^R-4 exhibited increased resistance when compared with the wild-type enzyme activity (Fig. 1B). This is characteristic of one class of hydroxyurea-resistant mutants previously isolated from the Chinese hamster ovary cell line (18). Hyd^R-4 cells also contained three- to five-fold-higher levels of CDP reductase activity as measured in permeabilized cells or cell-free extracts (see Fig. 4 and Table 2).

Chromosome transfer of hydroxyurea resistance. Since previous work has shown that the alterations resulting in hydroxyurea resistance are expressed in hybrid cells formed by fusion of wild-type and hydroxyurea-resistant cell lines,

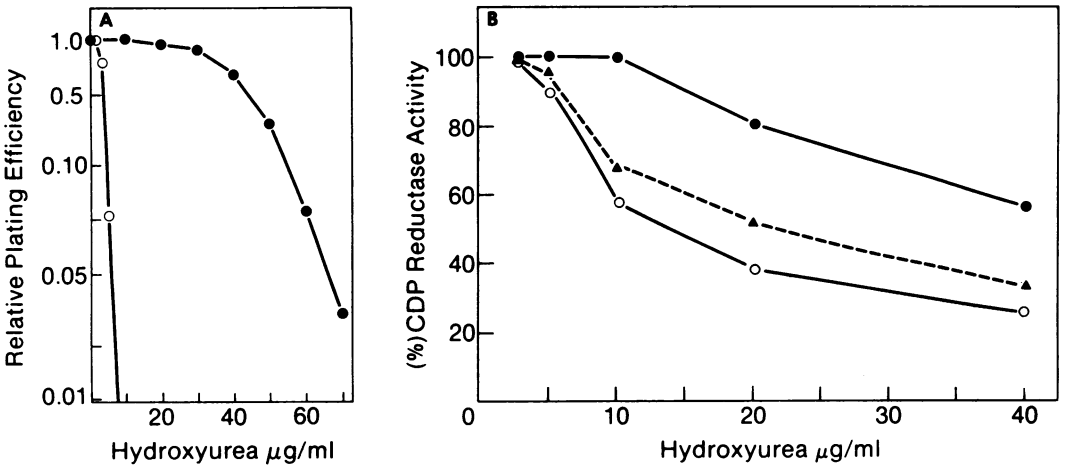

FIG. 1. (A) Relative plating efficiency of wild-type V79 (○) and Hyd^R-4 (●) cells in increasing concentrations of hydroxyurea. (B) Inhibition of CDP reductase activity by hydroxyurea. Cell-free extracts were prepared from V79 (○), Hyd^R-4 (●), and 96-V-2 (△) cells and assayed for CDP reduction in the presence of increasing concentrations of hydroxyurea.

we tested whether isolated metaphase chromosomes could transfer the drug resistance to sensitive cells. Using a calcium phosphate coprecipitation-mediated gene transfer procedure adapted for hamster cells (14, 31), we could reproducibly generate Hyd^R transformants after treatment of recipient cells with chromosomes isolated from Hyd^R-4. The results of several representative transfer experiments are given in Table 1. With both V79 and the Chinese hamster ovary recipient Mtx^{RIII}, transformant colonies arose at a frequency between 4×10^{-7} and 1×10^{-6}. Recipient cells treated with calcium phosphate alone or with control chromosomes isolated from the wild-type V79 gave no Hyd^R colonies when at least 10^7 cells were plated in the selecting concentration of 35 μg of hydroxyurea per ml. A characteristic of chromosome- or DNA-generated transformants is their relative instability when cultivated in the absence of selective pressure (11, 16, 27). A total of eight Hyd^R transformant colonies were picked from the selection plates, grown to 10^7 cells in the presence of hydroxyurea, and then passaged in drug-free culture medium for a period of 100 cell doublings (ca. 2 months). Figure 2 shows that when the cells from each clone were tested for the ability to form colonies in medium containing 30 μg of hydroxyurea per ml, the majority of the transformants gradually lost resistance to the drug. Only three of the eight clones maintained their original hydroxyurea resistance. This frequency of stable clones after chromosome-mediated gene transfer is similar to the frequency we observed previously with methotrexate-resistant transformants (16).

Cell extracts were prepared from one of the stable Hyd^R transformants, 96-V-2. CDP reductase activity from this transformant exhibited an intermediate resistance to hydroxyurea inhibi-

TABLE 1. Frequency of hydroxyurea-resistant colonies obtained after chromosome-mediated gene transfer

Donor cells	Recipient cells	Dosage ($\times 10^6$)[a]	Hyd^R colonies[b]	Colonies per 10^7 cells
V79	V79 O^R-1	8	0 (20)	<1
Hyd^R-4	V79 O^R-1	0	0 (20)	<1
	V79 O^R-1	4	7 (20)	7
	V79 O^R-1	8	11 (20), 10 (20)	11
Hyd^R-4	Mtx^{RIII}	0	0 (20)	<1
	Mtx^{RIII}	4	4 (20)	4
	Mtx^{RIII}	8	6 (19)	6
96-V-2(230)	V79 O^R-1	0	0 (10)	<2
	V79 O^R-1	4	65 (16)	81
	V79 O^R-1	8	35 (8), 50 (10)	94
96-V-2(600)	V79 O^R-1	0	0 (10)	<2
	V79 O^R-1	4	25 (10)	50
	V79 O^R-1	8	51 (8), 64 (10)	128

[a] Represents the number of cell equivalents of chromosomes added to the recipient cells as described previously (14).

[b] Represents the number of colonies which formed when chromosome-treated cells were plated in standard medium containing 35 μg of hydroxyurea per ml and incubated at 37°C for 12 days. The figures in parentheses indicate the number of 100-mm tissue culture dishes counted.

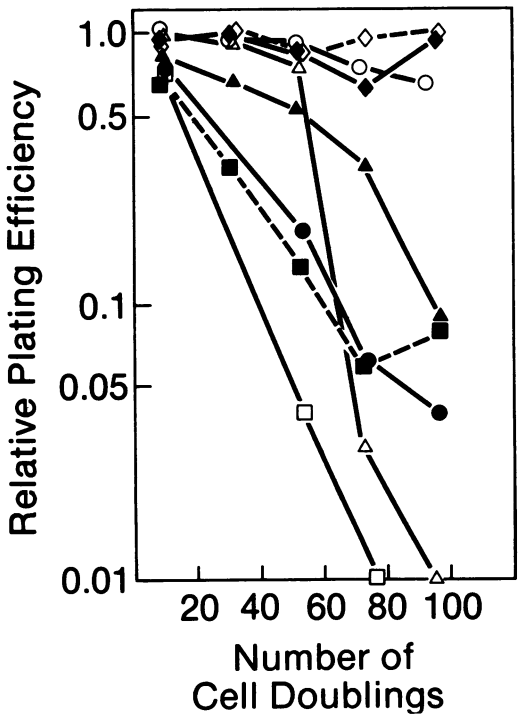

FIG. 2. Relative plating efficiency of hydroxyurea-resistant V79 transformant clones grown in the absence of drug for various periods of time. Symbols: ◆, 96-V-1; ○, 96-V-2; △, 96-V-3; □, 96-V-4; ●, 96-V-5; ◇, 96-V-6; □, 96-V-7; and ▲, 96-V-8.

tion compared with the recipient V79 enzyme activity (Figure 1B).

Generation of cell lines with increased RDR activity. Results from a number of laboratories have indicated that hydroxyurea (1, 20) or aphidicolin (28) often select for resistant cell lines containing four- to eight-fold-elevated levels of RDR. The mechanism for this increase in enzyme activity is not known but may be due to an increase only in the M2 subunit or free radical content of the enzyme (1). It is known that mammalian cell transformants selected for transfer of the dihydrofolate reductase gene or the hypoxanthine phosphoribosyl transferase gene often contain the gene product in elevated quantities (6, 16, 39). Therefore, we attempted to increase the level of ribonucleotide reductase in the 96-V-2 cell line by growing the cells stepwise in increasing concentrations of hydroxyurea. This was done by seeding 10^5 cells in 75-cm^2 tissue culture flasks in various concentrations of the drug. After 14 days of incubation, the flask with approximately 100 colonies (10^{-3} survival) was trypsinized, and the population of cells was expanded in the presence of the selecting concentration of hydroxyurea. This process was repeated with increasing concentrations of hydroxyurea, generating a set of five cell lines selected at 100, 230, 360, 600, and 700 μg of hydroxyurea per ml. The 96-V-2(700) cell population grew extremely slowly, and it was not practical to select in higher concentrations of the drug. As expected, when these cell lines were tested for their plating efficiency in hydroxyurea, the higher the "training" concentration of hydroxyurea, the more resistant the selected cell population became. Figure 3A shows the relative plating efficiencies of the five cell lines compared with those of the original 96-V-2 transformant and the wild-type recipient line V79 O^R-1. The concentration of hydroxyurea required to reduce the relative plating efficiency to 0.1 varied from 55 μg/ml for 96-V-2 to more than 800 μg/ml for 96-V-2(700). For the 96-V-2(700) cell line, this represents a 160-fold increase in resistance to hydroxyurea when compared with the wild-type recipient cell line V79 O^R-1.

RDR activity in hydroxyurea-resistant cell lines. To quantify the level of RDR in the cell lines generated during the stepwise selections, each of the cell lines was grown under identical conditions and then permeabilized with Tween 80 as described above. The amount of CDP reduced to dCDP by the permeabilized cells was measured, and the data in Fig. 3B show that for each cell line, the greater the relative plating efficiency in hydroxyurea, the higher the level per cell of CDP reductase. The cell line 96-V-2(700) had a more than 100-fold increase in CDP reductase activity when compared with V79 O^R-1. To check whether the increase in enzyme activity was due to differences in cell permeability during the assay, cell-free extracts were prepared from V79 O^R-1, Hyd^R-4, and 96-V-2(230). After passage through Pharmacia Sephadex G25 to remove small molecules, increasing volumes of the extracts were assayed for CDP reductase activity. Figure 4 shows that all three cell lines exhibited a nonlinear increase in enzyme activity with increasing quantities of protein. This is characteristic of mammalian RDR and is thought to be due to dissociation of the two subunits at low protein concentrations (15).

At 550 μg of protein per assay, the 96-V-2(230) cell extract had approximately 40-fold more CDP reductase activity than did V79 O^R-1. This value compares well with the results of the permeabilized assay presented in Fig. 3A.

EPR spectra of hydroxyurea-resistant cell lines. If the increase in ribonucleotide reductase activity is due to elevated levels of the M2 subunit of the enzyme then this should be reflected in an elevation in the free radical thought to be essential for activity. In *E. coli*, the free radical is generated on a tyrosine residue and gives a

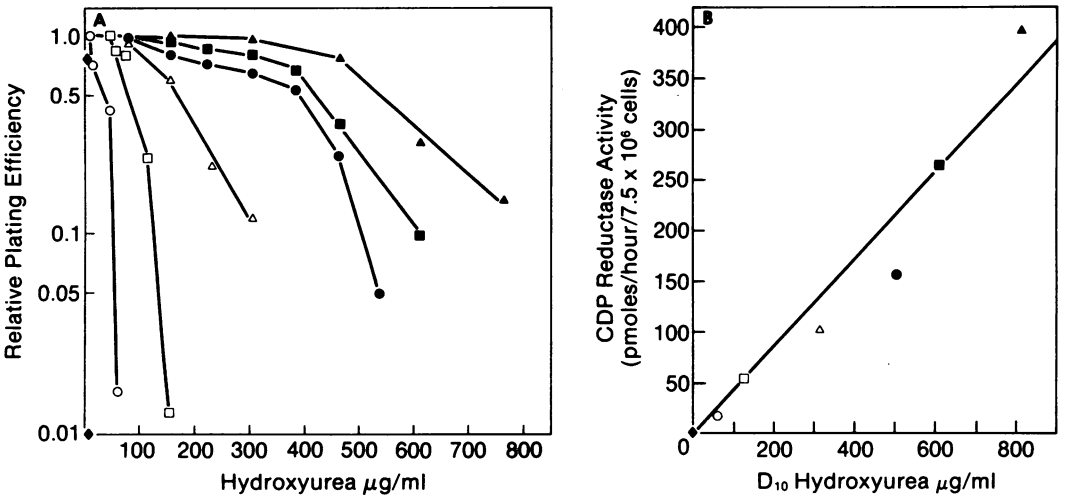

FIG. 3. (A) Relative plating efficiency of 96-V-2 cell lines grown stepwise in increasing concentrations of hydroxyurea. Symbols: ♦, V79 O^R-1; ○, 96-V-2; □, 96-V-2(100); △, 96-V-2(230); ●, 96-V-2(360); ■, 96-V-2(600); and ▲, 96-V-2(700). (B) Ribonucleotide reductase activity of the 96-V-2 cell lines plotted against the relative plating efficiency of each line as defined in the legend to Table 2. Symbols as in (A).

characteristic EPR signal (30). A similar EPR signal has been observed in hydroxyurea-resistant mouse 3T6 cells (1, 13). Figure 5A shows the detailed EPR spectrum at 77 K of pelleted 96-V-2(600) cells. The spectrum is centered at g' = 2.0040 and is similar, but not identical, in shape to the *E. coli* spectrum. Figure 5B compares the EPR spectrum of 96-V-2(600) cells with the spectrum obtained with V79 O^R-1 cells.

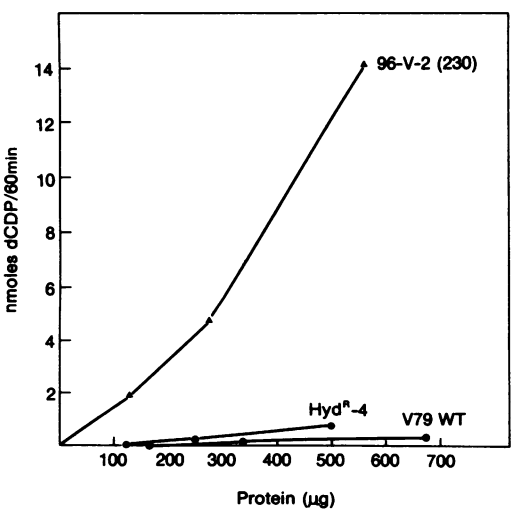

FIG. 4. Ribonucleotide reductase activity versus increasing amount of cell-free extract. WT, Wild type.

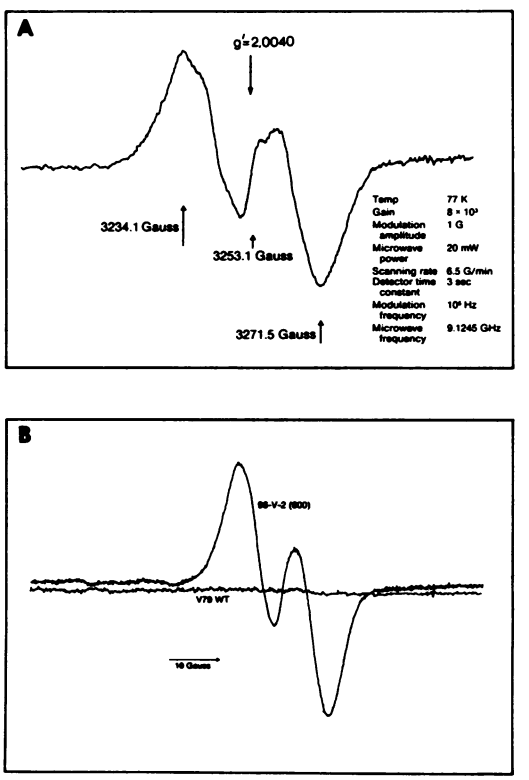

FIG. 5. (A) EPR spectrum of packed 96-V-2(600) cells at 77 K. (B) EPR spectra of packed V79 and 96-V-2(600) cells performed under identical conditions.

TABLE 2. Comparison of hydroxyurea-resistant cell lines

Cell line	Relative plating efficiency[a]	Relative CDP reductase level[b]	EPR signal[c]
V79 O^R-1	1	1	<0.05
Hyd^R-4	11	5	
96-V-2	11	5	0.44
96-V-2(100)	25	16	0.51
96-V-2(230)	63	31	0.85
96-V-2(360)	101	47	1.08
96-V-2(600)	122	80	
96-V-2(700)	163	120	1.14

[a] Represents the concentration of hydroxyurea which reduces the plating efficiency of each line to 10% of the plating efficiency in the absence of the drug, expressed relative to a value of 5.0 μg/ml for V79 O^R-1.

[b] Represents the level of CDP reductase in permeabilized cells relative to a value of 3.3 pmol/h per 7.5×10^6 V79 O^R-1 cells.

[c] Represents an arbitrary measurement of the peak-to-peak heights of the EPR signal.

We could detect no signal with the wild-type cells. Table 2 gives arbitrary peak-to-peak heights for several of the hydroxyurea-resistant cell lines. Although there is a correlation of increasing peak-to-peak height with increasing enzyme activity in the various cell lines, there seems to be a much steeper rise in the signal height with the first few stepwise selections. For example, there is at least a 10-fold elevation from the wild type to the transformant 96-V-2, but only a 2.6-fold increase from 96-V-2 to the most resistant cell line 96-V-2(700). However, it must be pointed out that these EPR measurements are only semiquantitative.

Two-dimensional gel electrophoresis of cell extracts. The two-dimensional fractionation of crude cell extracts by isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis is capable of resolving hundreds of separate protein molecules within cells (3, 25). If the resistance of our cell lines is due to the overproduction of RDR molecules, then the proteins involved may be identifiable by comparing two-dimensional gels of the wild-type and hydroxyurea-resistant cell lines. Figure 6 shows representative Coomassie blue-stained gels of V79 O^R-1 and 96-V-2(600) soluble proteins. We could reproducibly identify well over 100 proteins present in similar quantities in the extracts from both cell lines. However, four proteins were consistently present in elevated amounts in 96-V-2(600) extracts (Fig. 6, numbers 1 through 4). The most striking of these was a protein of 53,000 to 55,000 daltons (number 1) which had become the fifth most abundant soluble protein in 96-V-2(600). The corresponding area in the V79 O^R-1 gel usually contained from one to three faintly visible protein spots. Other workers have estimated the molecular weight of the M2 polypeptide to be 55,000 (7, 28), and we believe protein number 1 in our gel system corresponds to this polypeptide.

Examination of the gels in Fig. 6 does not reveal a corresponding increase in any protein close to the 84,000-molecular-weight M1 subunit (7). It is difficult to reconcile the 100-fold increase in RDR activity without a corresponding increase in the M1 protein. However, it is known that M2 is present in cell extracts in low, non-stoichiometric amounts when compared with M1 (7, 34), and cell cycle analyses indicate that the levels of M2 fluctuate and may be rate limiting for RDR activity (8). We are presently preparing antibody to the 53,000-dalton protein and to the M1 subunit purified by dATP-Sepharose affinity chromatography (7) to measure the levels of these two proteins in exponentially growing and synchronized cells.

We now believe that the proteins of molecular weights 42,000 (Fig. 6, number 2) and 47,000 (Fig. 6, number 3), seen in various but elevated amounts in 96-V-2(600) gels, are proteolytic degradation products of the 53,000-dalton protein. In the absence of protease inhibitors, these two spots became more prominent and the quantity of the 53,000-dalton protein decreased (data not shown). Furthermore, it is known that 40% ammonium sulfate will precipitate RDR activity from crude cell extracts (9). When 40% ammonium sulfate precipitates of 96-V-2(600) extracts were electrophoresed as described above, the 42,000- and 47,000-molecular-weight proteins were no longer present. In contrast, this precipitate was enriched for the 53,000- and 110,000-molecular-weight proteins numbers 1 and 4 and retained all ribonucleotide reductase activity present in the original crude extract (data not shown).

Transfer of hydroxyurea resistance with chromosomes isolated from transformants. In the well-characterized systems of methotrexate-resistant and *N*-(phosphonacetyl)-L-aspartate-resistant mammalian cell lines, the genes for the target enzymes dihydrofolate reductase and aspartate transcarbamylase, respectively, have been shown to be present at elevated copy number (2, 38). If a similar phenomenon has occurred in the highly hydroxyurea-resistant transformants, then the frequency of gene transfer mediated by genetic material isolated from these lines may also be elevated. To test this, we purified metaphase chromosomes from the 96-V-2(230) and 96-V-2(600) cell lines and tested their ability to transfer hydroxyurea resistance to recipient V79 O^R-1 cells. Table 1 shows that in

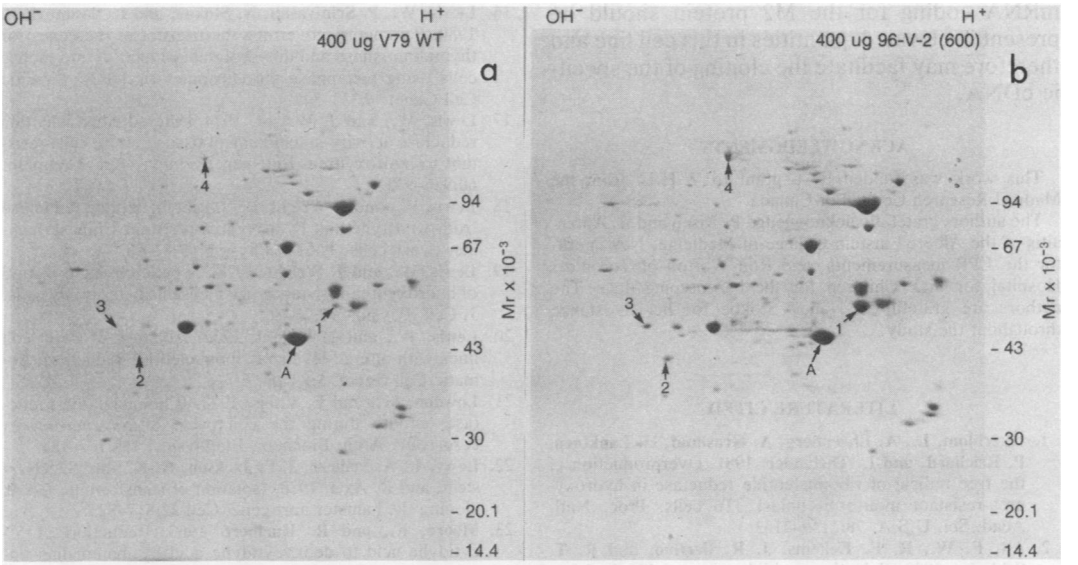

FIG. 6. Coomassie blue-stained two-dimensional gels of 400 μg of soluble proteins from V79 O^R-1 (a) or 96-V-2(600) (b) cells. A, Position of actin. WT, Wild type.

both cases the frequency of transformation was approximately 10^{-5}. This represents more than a 10-fold increase compared with the transformation mediated by chromosomes isolated from Hyd^R-4 (Table 1).

Karyotype of hydroxyurea-resistant cell lines. Others have shown that rodent cell lines resistant to high concentrations of methotrexate often exhibit chromosomal changes such as homogeneously staining regions and double minute chromosomes (29). Therefore, detailed karyotypes were determined for the cell lines V79, Hyd^R-4, and 96-V-2(600). The karyotypes of the V79 and the Hyd^R-4 cell lines were very similar, with modal chromosome numbers of 22. The highly hydroxyurea-resistant cell line 96-V-2(600) karyotype showed no evidence of homogeneously staining regions or double minute chromosomes. However, the cell line had a modal chromosome number of 23, having gained an extra chromosome 7. In addition, there were two reciprocal translocations, between chromosomes 1 and 3 and between chromosomes 2 and 6. Whether any of these changes are involved in the generation of the drug-resistant phenotype or whether they represent random chromosomal instability is not known.

DISCUSSION

Mammalian cell lines selected stepwise for increasing resistance to a variety of cytotoxic drugs often contain amplifications of the gene coding for the particular target protein (29). The highly hydroxyurea-resistant cell lines described in this paper have many properties suggesting that at least the gene for the M2 subunit of RDR has been amplified. As the cell lines became increasingly resistant to hydroxyurea, they contained a corresponding increase in RDR activity and an elevation in the EPR signal characteristic of the free radical present in M2. Extracts from the highly hydroxyurea-resistant cell line 96-V-2(600) contained a 53,000-dalton protein present in extremely high quantities when compared with the protein present in extracts of hydroxyurea-sensitive wild-type cells. Finally, chromosomes isolated from the hydroxyurea-resistant transformant cell lines were capable of generating secondary transformants at a 10-fold higher frequency when compared with the original Hyd^R-4 cell line.

We have not been successful in attempts to transfer hydroxyurea resistance to recipient cells with DNA purified from either Hyd^R-4 or the highly hydroxyurea-resistant cell line 96-V-2(600). We do not know whether this is because Chinese hamster cells are extremely poor recipients for calcium phosphate-DNA-mediated gene transfer (14, 31) or because the size of the genetic material necessary to transfer the resistance is larger than the size of the DNA fragments.

We are presently raising antibodies to the 53,000-dalton protein recovered directly from the two-dimensional gels. This antibody should be valuable for analyses of the level of M2 protein during the cell cycle. In addition, the

mRNA coding for the M2 protein should be present in elevated quantities in this cell line and therefore may facilitate the cloning of the specific cDNA.

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