Somatic Cell Genetics, Vol. 8, No. 2, 1982, pp. 207–222

Bromodeoxyuridine Resistance in CHO Cells Occurs in Three Discrete Steps

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Received 11 August 1981—Final 5 November 1981

Abstract—*Four independent mutants were isolated from mutagenized cultures of CHO cells by sib selection on the basis of resistance to a low concentration (2.6×10^{-5}M) of BrdU. All four lines were stable, but all had about 100% of the wild-type (WT) specific activity of thymidine kinase (TK). None of the four yielded derivatives resistant to a high level of BrdU (2×10^{-4}M) in one step even after mutagenesis, but variants resistant to 4–6 $\times 10^{-5}$M BrdU could be isolated at frequencies of about 2×10^{-5}/cell. At frequencies of 10^{-4}–10^{-5}, the second-step mutants gave colonies resistant to 2×10^{-4} M BrdU. The second and third steps of resistance were correlated with partial and complete reduction, respectively, in the specific activity of TK, suggesting that the variants may be genotypically heterozygous and homozygous-negative at the tk locus. The first step of BrdU resistance was dominant and appeared to result from a mutation in the gene for ribonucleotide reductase, since in vitro assays on partially purified preparations showed that the reductase activity in mutant cells was less sensitive to BrdUTP than that in WT cells.*

INTRODUCTION

Recessive drug resistance in cultured somatic cells has frequently been shown to correlate with the loss of a specific enzymatic activity. In cases where this resistance is both stable and acquired at a detectable, but low, frequency which is increased by mutagenesis, the conclusion generally drawn is that a mutation has occurred at the gentic locus specifying either the structural enzyme affected or a regulatory function controlling this locus. It is also generally concluded that the locus is present in only one functional copy. In the case of diploid loci, two-step acquisition of full resistance to one drug, 8-azaadenine (AAd), has been demonstrated (1, 2), with the frequencies at

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each step being approximately the value expected for single genetic change ($\sim 10^{-5}$). In the intermediate stage, the specific activity of the affected enzyme, adenine phosphoribosyltransferase (APRT), was about one half of the wild-type (WT) activity, and the conclusion drawn was that in this state the cell was heterozygous at the *aprt* locus.

At another often-studied diploid locus, thymidine kinase (*tk*), the intermediate cell type with about 50% of the WT enzyme activity has only been demonstrated indirectly, by isolation of revertants of the homozygous-negative mutant (3–5). This system is somewhat complicated by the fact that the selective agent usually used, 5-bromodeoxyuridine (BrdU), has several toxic effects on mammalian cells. Of particular interest, in addition to cell death resulting from incorporation of this drug into DNA, is the effect BrdU in the triphosphate form is known to have on ribonucleotide reductase. This enzyme controls deoxyribonucleotide triphosphate pool sizes, and both dTTP and its analog BrdUTP exert strong negative-feedback control on the conversion of CDP to dCDP (6). At sufficiently high concentrations, these effectors induce a "deoxycytidineless" state and cell death (6). In addition, an extensive study on the mechanism of BrdU mutagenesis (7–10) has shown that perturbations in the dCTP pool size play a key role, again suggesting an important effect of BrdU on the reductase enzyme.

It is perhaps not surprising, then, that direct isolation of a $tk^{+/-}$ heterozygote in BrdU has not yet been reported. Although such a cell may maintain a lower rate of phosphorylation of BrdU than the WT, the multiplicity of effects of the drug may result in irreversible damage before the partially resistant cell type can be removed from the selective medium. Indeed, in preliminary attempts to isolate $tk^{+/-}$ cells in a single step (unpublished data), all candidate colonies seen in BrdU consisted of unhealthy-looking cells which, when plated in nonselective medium, were not viable. Following the reasoning above, we decided that a better procedure for isolating these cells would be sib selection (11, 12) in which genetically identical siblings of drug-resistant mutants can be isolated without exposure to the selective medium. By using this method, we have succeeded in isolating BrdU-resistant mutants in a direct stepwise fashion, but full resistance was attained in three steps instead of two. The first level of resistance was not due to a mutation at the *tk* locus, but appears to result from an altered ribonucleotide reductase with decreased BrdUTP sensitivity. The second and third levels apparently corresponded to $tk^{+/-}$ and $tk^{-/-}$ genotypes.

MATERIALS AND METHODS

Cells and Media. The wild-type CHO cell line was the gift of C. Stanners. Cells were grown as previously described (13), in alpha medium

(14) without DNA or RNA precursors and supplemented with 10% fetal calf serum. Two cell lines used, G1441 and D416, are variants already containing other markers; G1441 was derived from the WT CHO line by three successive sib-selection procedures (12): first for glucose-6-phosphate dehydrogenase (G6PD) deficiency (12), then hypoxanthine phosphoribosyltransferase deficiency, and finally partial APRT deficiency (Bradley and Letovanec, in press). D416 was isolated by sib selection for partial APRT deficiency from WT CHO cells provided by M. Meuth (Bradley and Letovanec, in press). All cell lines were Mycoplasma free as assayed by [^{3}H]thymidine labeling followed by autoradiography (15). Variants of CHO and G1441 resistant to 2.6×10^{-5}M BrdU were isolated at 38.5°C to maximize the chance of isolating temperature-sensitive mutants; however, none of the mutants isolated had this property. The temperature of all other experiments was 37°. BrdU was obtained from Sigma and deoxycytidine (dC) from PL Biochemicals.

Mutagenesis. Mutagenesis was carried out by incubating 5×10^6 cells in a 10-cm plastic dish in the presence of 150 μg/ml ethyl methanesulfonate (EMS; Sigma) in medium for 24 h. This routinely resulted in a survival frequency of 45–50% as measured by colony-forming ability immediately after treatment. After 4–9 days growth in alpha medium (with subculturing as necessary) to permit expression of the mutant phenotype, the cells were trypsinized and subjected to selection, as described in the Results.

Thymidine Kinase Assay. Thymidine kinase in crude cell extracts was assayed as was previously described (13). Briefly, cells were scraped into a sonication buffer (50 mM Tris, pH 7.4, with 10 mM β-mercaptoethanol) and sonicated for 5 sec and the suspensions were centrifuged at 4°C at 18,000 rpm for 30 min. An aliquot of the supernatant was taken for a Lowry protein assay (16); the rest served as crude extract. The reaction mixture contained 0.2 M Tris (pH 8.0), 5 mM $MgCl_2$, 5 mM ATP, 10^{-5} M methyl [^{14}C]dT (55 mCi/mmol; New England Nuclear), and 50–100 μg of crude extract protein. After incubation at 33°C, thymidine monophosphate was separated from unreacted dT by electrophoresis, and the spots detected by autoradiography, cut out, and counted in a scintillation counter.

Cell–Cell Hybridization. Each of the lines to be tested for dominance or recessivity of BrdU resistance was fused with 8-3TG, a thioguanine-resistant derivative of the ouabain-resistant CHO strain C-Al kindly provided by Dr. R.M. Baker (17). Equal numbers of each parental cell line (10^5 cells) were mixed in a large (2-ml capacity) Linbro well in normal medium. The next day the medium was removed and replaced with a solution of 50% polyethylene glycol (PEG) in alpha medium without serum for 60 sec. The PEG was then removed, and the well was washed four times with alpha medium without serum. The cells were then covered with normal medium and, after 24 hr at

37°C, were trypsinized and seeded in 10-cm dishes containing alpha medium with 3 mM ouabain and HAT [10^{-4} M hypoxanthine (Sigma), 1.6×10^{-5} M thymidine (Sigma), and 5.5×10^{-6} M methotrexate (Lederle)]. Colonies appearing in 8–10 days were picked, recloned, and tested for the hybrid karyotype (13).

Determination of dCTP and dTTP Pools. The deoxyribonucleoside triphosphates were extracted from log-phase cells with 0.5 M perchloric acid and determined after neutralizing the extract by the limit reaction of DNA polymerase, as described by Skoog (18) and Lindberg and Skoog (19). Polymerase and poly d[A-T] and poly d[G-C] templates were obtained from Boehringer-Mannheim. Deoxyribonucleoside triphosphates were purchased from PL Biochemicals

Preparation of Partially Purified CDP Reductase. Cells were grown in suspension to a cell density of $5–7 \times 10^5$ cells/ml. The cells were then washed with phosphate-buffered saline and were resuspended in two volumes of buffer A (50 mM HEPES (pH 7.8), 1mM $MgCl_2$, and 2 mM dithioerythritol). The cells were disrupted by sonication, and the extract was centrifuged at 10,000*g* for 10 min. The supernatant solution was then treated for 20 min at 4°C with 1% streptomycin sulfate, and the precipitate was removed by centrifugation at 10,000*g*. Ammonium sulfate was added to the supernatant to 60% of saturation and the supernatant was stirred at 4°C for 30 min. After centrifugation at 10,000*g* for 20 min, the pellet was dissolved in buffer A. After dialysis overnight against the same buffer the solution was clarified by centrifugation and stored at −80°C.

CDP Reductase Assay. The reaction mixture contained in a total volume of 60 μl: 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 6 mM ATP, 6 mM dithioerythritol, 10 mM NaF, 0.054 mM [^{14}C]CDP (0.06 μCi/mmol; Amersham Corp.), and 200–400 mg of protein of the partially purified extract. The mixtures were incubated for 45 min at 37°C and then boiled for 4 min. After treatment for 2½ h with 0.8 mg/assay of *Crotalus adamenteus* venom (Sigma Chemical), the deoxycytidine formed was measured by thin-layer chromatography on polyethyleneimine cellulose sheets which had been converted to the borate form as described by Jacobsen and Kirchner (20). Chromatograms were developed at room temperature in a solution of 50 ml 0.02 M ammonium formate (pH 4.7) and 50 ml ethanol. The areas corresponding to cytidine and deoxycytidine were cut out and their radioactivity determined by scintillation counting. The radioactivity associated with deoxycytidine was expressed as percentage of the total radioactivity in the two spots, and from this was calculated the reaction rate. The reaction rate was linear for more than 60 min. In a total of 11 separate assays, the specific activity of CDP reductase in WT cells was 6.8 ± 2.9 (SD) nmol/h/mg protein.

RESULTS

Isolation of BrdU-Resistant Mutants by Sib Selection. The principle of sib selection is multistep enrichment for a variant in a population. At each step the population is subdivided and, after a few generations of growth in nonselective medium, each subpopulation is sampled to determine which has the highest proportion of the variant in question. Two independently cloned populations of CHO, C24 and C25, were mutagenized and, after a period of growth in alpha medium, 6.4×10^4 cells of each were divided into 16 two-ml-capacity Linbro wells (Table 1). The first test of growth potential in BrdU was not done for 20 days, during which time the subpopulations were passaged as necessary. On day 20, 10^4 cells from each well were placed in each of one 60-mm dish with 3×10^{-5} M BrdU and one fresh Linbro well with alpha medium. After 9 days some colonies could be clearly distinguished in a few BrdU dishes, and the wells corresponding to the dishes with the most healthly-looking colonies were trypsinized and counted. On the assumption that the colonies in BrdU were descendants of a single variant present among the 4×10^3 cells of the original population, enough cells were distributed among 24 fresh Linbro wells in alpha medium to assure that at least one well would contain siblings of the BrdU-resistant cells (Table 1). Four days later the contents of each well were trypsinized and about 80% of each placed in one 60-mm dish with 3×10^{-5} M BrdU. In each case populations containing BrdU-resistant cells were identified, and the enrichment procedure was repeated two more times. The details of each step are provided in Table 1. After 4 cycles the enriched populations were plated in alpha medium at very low density and several colonies were isolated, grown up in alpha medium, and subsequently tested by plating a known number of cells in $2.6–3.0 \times 10^{-5}$ M BrdU. Two cultures with a substantially higher cloning efficiency (CE) in BrdU than the parental CHO cells were recloned in alpha medium and named B210 and B211, descended from C24 and C25, respectively (Table 1).

Two further variants have been isolated from parental cells already carrying other markers, G1441 and D416. In these experiments, mutagenized cells were distributed into large wells as indicated in Table 1 and, after 4 days, 60–80% of the cells in each well were transferred to a 60-mm dish (or a 100-mm dish for D416) with medium containing 2.6×10^{-5} M BrdU. As before, colonies appeared after 8–10 days in BrdU, and the sister culture of the dish with the most healthy-looking BrdU-resistant colonies was trypsinized for the second cycle. The procedure was repeated until colonies with increased BrdU resistance were isolated (Table 1) and recloned as above. These were called G144121 and D41621, descended from G1441 and D416, respectively.

The killing curves for the parental and variant cell types in BrdU are

Table 1. Sib Selection of CHO Variants Partially Resistant to BrdU

Parent	CHO clone 24	CHO clone 25	G1441[a]	D416[a]
1st cycle[b]				
Total no. of cells/no. of subpopulations	$6.4 \times 10^4/16$	$6.4 \times 10^4/16$	$4 \times 10^4/24$	$2.0 \times 10^5/48$
No. of subpopulations with BrdU^r colonies (no. of colonies)	2 (4;2)	1 (1)	2 (3;1)	2(2;5)
2nd cycle				
Total no. of cells/no. of subpopulations	$2 \times 10^4/24$	$2 \times 10^4/24$	$4 \times 10^3/24$	$1.5 \times 10^4/24$
No. of subpopulations with BrdU^r colonies (no. of colonies)	2 (4;2)	2 (1;2)	2 (4;4)	2 (9;7)
3rd cycle				
Total no. of cells/no. of subpopulations	$3.5 \times 10^3/24$	$2 \times 10^3/24$	600/24	$1.2 \times 10^3/24$
No. of subpopulations with BrdU^r colonies (no. of colonies)	3 (39;9;7)[c]	1 (1)	3[d]	5[d]
4th cycle				
Total no. of cells/no. of subpopulations	350/24	350/24		
No. of subpopulations with BrdU^r colonies	2	3		
No. of colonies picked/no. of colonies BrdU^r			18/1	72/1
5th cycle				
No. of colonies picked/no. of colonies BrdU^r	10/6	16/2		
Name of cloned BrdU^r cell line	B210	B211	G144121	D41621

[a] G1441 is a derivative of CHO which is G6PD and HPRT deficient, and partially APRT deficient; D416 is a derivative of CHO which is partially APRT deficient (Bradley and Letovanec, in press).
[b] Each cycle consisted of a period of growth (usually 4–5 days) in alpha medium to generate many descendants of each original cell and, after splitting each subpopulation in the proportion of about 1:4, a period of exposure of the major fraction of each to 2.6 or 3.0×10^{-5} M BrdU. Resistant colonies were visible after 8–11 days.
[c] Due to the statistical nature of both the sampling and the CE of a cell in BrdU, considerable variation in the number of resistant colonies was possible, as was seen in this case.
[d] Number of colonies not recorded.

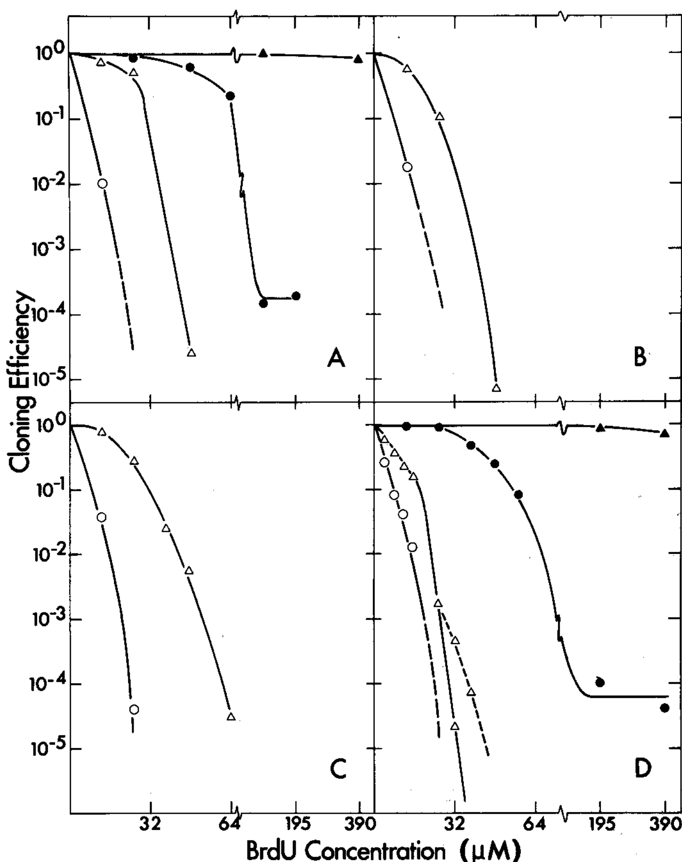

Fig. 1. Cloning efficiencies of CHO, G1441, D416, and BrdU-resistant variants in BrdU. (A) ○, CHO; △, B210; ●, B212; ▲, B212B60. (B) ○, CHO; △, B211. (C) ○, D416; △, D41621. (D) ○, G1441; △, G144121; ●, G144125; ▲, G144125B60. Derivations of the cell lines are described in the footnotes to Tables 2 and 3.

shown in Fig. 1. The extent of resistance to BrdU varied somewhat, between extremes of D41621 with LD_{10} (drug concentration giving a 90% reduction in CE) of 3.1×10^{-5} M and G144121 at 2.0×10^{-5}, but all values were about threefold higher than the LD_{10} for their respective parental cell lines.

Derivation and Characterization of Mutants of Higher BrdU Resistance. The partial BrdU resistance of these mutant lines did not correspond to a significant decrease in cytosol TK activity, since TK levels were near 100% of the WT TK specific activity (Table 2). Further, these cell lines were not capable of forming colonies in $>2 \times 10^{-4}$ M BrdU (full BrdU resistance) in a single step even after mutagenesis (Fig. 1), so they did not fit these

Table 2. Characteristics of BrdU-Resistant Mutants

Cell line	Parent	TK specific activity		Apparent *tk* genotype	Karyotype[b]
		absolute[a]	% of parent		
CHO		0.92		+/+	20
B210	CHO C24	1.04	113	+/+	19
B212[c]	B210	0.51	49	+/−	18
B212B60[d]	B212	<0.02	<4	−/−	ND
B211	CHO C25	1.04	113	+/+	19
21BU1[c]	B211	0.56	54	+/−	20
21BU1.9-2[d]	21BU1	<0.02	<2	−/−	20
D416		1.00		+/+	20
D41621	D416	0.94	94	+/+	20
1EB[c]	D41621	0.30	30	+/−	ND
G1441		1.04		+/+	20
G144121	G1441	1.01	97	+/+	19
G144125[c]	G144121	0.38	0.38	+/−	20
G144125B60[d]	G144125	<0.02	<2	−/−	20

[a] Units are nmol/min/mg protein.
[b] Value given is the mode of 30 metaphase spreads.
[c] B212 and G144125 were isolated from mutagenized cultures of B210 seeded in 5×10^{-5} M BrdU, and G144121, seeded in 3.3×10^{-5} M BrdU, respectively. 1EB was isolated from a mutagenized culture of D41621 seeded in 6×10^{-5} M BrdU. 21BU1 was isolated from B211 by sibselection (see Table 3).
[d] B212B60 and G144125B60 are third-step resistant cell lines isolated from mutagenized cultures of, respectively, B212 and G144125 plated in 2.0×10^{-4} M BrdU. 21Bu1.9-2 is a spontaneous derivative of 21BU1 isolated in 4×10^{-4} M BrdU.

criteria of heterozygosity at the *tk* locus (1, 21). However, when mutagenized cultures were seeded at a low density ($<5 \times 10^4$/10-cm dish) in medium containing twice the LD_{10} concentration of BrdU, small colonies appeared after about 10 days from all four variants at frequencies of about 3×10^{-5}.

Some of these colonies were picked, grown up in alpha medium and characterized. B212 and 1EB, derived from B210 and B41621, respectively, each had about half the WT TK specific activity (Table 2) and in one step yielded stable mutants resistant to 2×10^{-4} M BrdU (data for B212 shown in Fig. 1A), which were phenotypically TK^- (Table 2). Similarly G144125, a line of intermediate BrdU resistance derived from G144121, also had a reduced level of TK and also yielded stable TK^- mutants at frequencies of about 10^{-4} when plated in 2×10^{-4} M BrdU (Fig. 1D). Finally, the cell line 21BU1, a derivative of B211 resistant to 6×10^{-5} M BrdU and isolated by sib selection (Table 3) had similarly reduced TK specific activities (Table 2) and frequencies of mutation to full BrdU resistance of about 10^{-5}. These third-step derivatives were also stably TK^- (e.g., 21BU1.9-2, Table 2). It should be pointed out that the TK specific activity in the mutants of intermediate BrdU resistance ($\sim 6 \times 10^{-5}$ M) was occasionally variable from one passage to another, and sometimes these cultures yielded TK^- derivatives at frequencies $>10^{-4}$. This was so for 1EB and G144125, but not for the other mutants

Table 3. Sib Selection of 21BU1, a Derivative of B211 Resistant to 6×10^{-5} M BrdU[a]

Enrichment round	Number of cells seeded	Number of populations	Number of populations with BrdU^r colonies
1	5.9×10^5	24	6
2	5.6×10^4	24	2
3	6.8×10^3	24	2
4	880	48	2
5	24	24	3

[a]A culture of B211 cells was mutagenized, and the indicated number of viable cells was distributed into 24 2-ml-capacity Linbro wells and grown in alpha medium for 5–8 generations. Each population was trypsinized; 10% of each was placed in one 10-cm petri dish with 6×10^{-5} M BrdU, and most of the rest was kept in nonselective medium. Seven days later BrdU-resistant colonies were visible in several dishes, and the population yielding the most vigorously growing colonies was chosen for the next round of enrichment. The cells of that population which had been maintained in alpha medium were trypsinized, counted, and the indicated number were distributed into 24 2-ml-capacity Linbro wells in alpha medium. The enrichment process was repeated until the 5th round, when 24 colonies were picked from a dish of cells seeded very sparsely in alpha meidum. These colonies were tested individually for BrdU resistance, and one of the resistant colonies was recloned in alpha medium and named 21BU1.

mentioned above. This was not due to irreproducibility of the assay, but to genuine fluctuations in the TK specific activity of the cell lines concerned. This has been observed in another *tk* heterozygote (13), and we have recently shown that exposure to the selective medium appears to be responsible for this anomalous behavior (Bradley, manuscript in preparation).

To determine whether karyotypic instability was associated with the acquisition of any of the levels of BrdU resistance, the chromosome numbers in metaphase spreads of most cell lines were determined. As shown in Table 2, very little deviation from the WT CHO karyotype was observed.

These genetic and biochemical data indicate that mutation of the two alleles at the *tk* locus can occur in our cells in a manner similar to that described for the *aprt* locus by Jones and Sargent (1), but in this case the initial cell lines had first undergone a separate mutational change. If our WT parental lines were triploid at the *tk* locus, this first change could be mutation at one of the three alleles, but the biochemical data are not consistent with this (Table 2). Further, such a mutation would be expected to be recessive, whereas our first-step resistant mutants, as shown below, are dominant.

Characterization of the First Step of BrdU Resistance. We therefore concluded that TK deficiency was not involved in the acquisition of the first step of BrdU resistance. This was not unexpected in light of the known effects of BrdU on nucleotide metabolism, as discussed in the Introduction. Two hypotheses were formulated and tested to explain the nature of the change in these cell lines. First, pool sizes of dCTP and dTTP were measured to test specifically whether the mechanism of BrdU resistance may be similar to that of dT resistance observed in some other cell lines (22), that is dCTP pool

Table 4. Pool Sizes of First-Step BrdU-Resistant Cells[a]

Cell line	dTTP	dCTP
CHO	33.6	131
B210	32.0[b]	128
B211	31.0	124
G1441	24.5	117
G144121	35.0	9
D416	25.0	157[b]
D41621	19.5	72[b]

[a]Results are presented as pmol/10^6 cells, averages of three or four determinations.
[b]Average of two determinations.

expansion. The results (Table 4) showed no increase in dCTP in any of the four lines, so pool expansion is not involved in the observed increase in BrdU resistance. It is interesting to note that in two lines the dCTP pool is in fact reduced, by about half in D41621 and by about 90% in G144121. We do not understand the significance of this, but it seems reasonable that if any enzyme subject to such complex control patterns as is ribonucleotide reductase (23) is altered (as is concluded to be the case from the data presented below), some measurable metabolic perturbations may occur.

The second hypothesis tested was that the gene coding for ribonucleotide reductase had been mutated so that the enzyme was resistant to inhibition by BrdUTP. Two approaches have been used. The first took advantage of the demonstration that cell death caused by the presumed dC-less state induced by BrdU could be overcome by dC (6). We reasoned that if the reductase was altered such that BrdU no longer killed primarily by this mechanism, exogenous dC should give no protective effect on the first-step resistant cells. If any effect existed, it should not result in increasing the LD_{10} of BrdU to a concentration higher than the LD_{10} for WT cells in the presence of dC. Two of the cell lines, B210 and B211, were therefore tested for resistance to BrdU in the presence and absence of 0.1 mM dC, and the results obtained suggest that BrdU does not induce a dC-less state in these mutants (Fig. 2). The LD_{10} of BrdU for CHO was increased by a factor of about 4, much higher than the increases for either B210 or B211, which were about 1.1 and 1.2, respectively (Table 5). Moreover, the BrdU resistance of B211 in the presence of dC was no higher than that of CHO in the same medium, suggesting that the degree of resistance of B211 in the absence of dC was due to the cells' ability to at least partially overcome BrdU-induced deoxycytidine deficiency. Similar results were obtained when D416 and its BrdU-resistant derivative were tested (Table 5), except the dC-induced increase in the LD_{10} of D416 was less than that of CHO. G144121 was not tested because of the very small increase in BrdU-resistance relative to its parent.

The second approach to test this hypothesis was to directly assay and

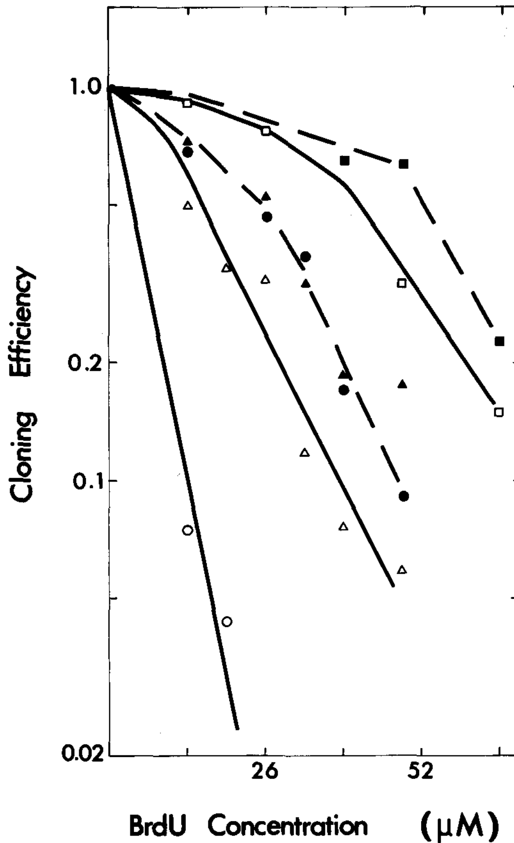

Fig. 2. Effect of deoxycytidine on BrdU toxicity in CHO, B210, and B211. Cells were plated in α+10% dialyzed fetal calf serum, with or without 0.1 mM dC. Open symbols, colony-forming ability in α+BrdU; closed symbols, in α+dC+BrdU. Circles, CHO; squares, B210; triangles, B211.

Table 5. Effect of Exogenous Deoxycytidine on BrdU Resistance

Cell line	LD_{10}[a] of BrdU ($\times 10^{-5}$ M)		Ratio $\frac{LD_{10} \text{ in } \alpha + dC}{LD_{10} \text{ in } \alpha}$
	In α	In α + dC	
CHO	1.24	4.7	3.8
B210	6.8	7.2	1.1
B211	3.8	4.7	1.2
D416	0.88	2.0	2.3
D41621	5.7	6.4	1.1

[a] LD10 (concentration of BrdU resulting in 90% reduction in colony-forming ability) was determined graphically; for CHO, B210, and B211 this was from Fig. 3.

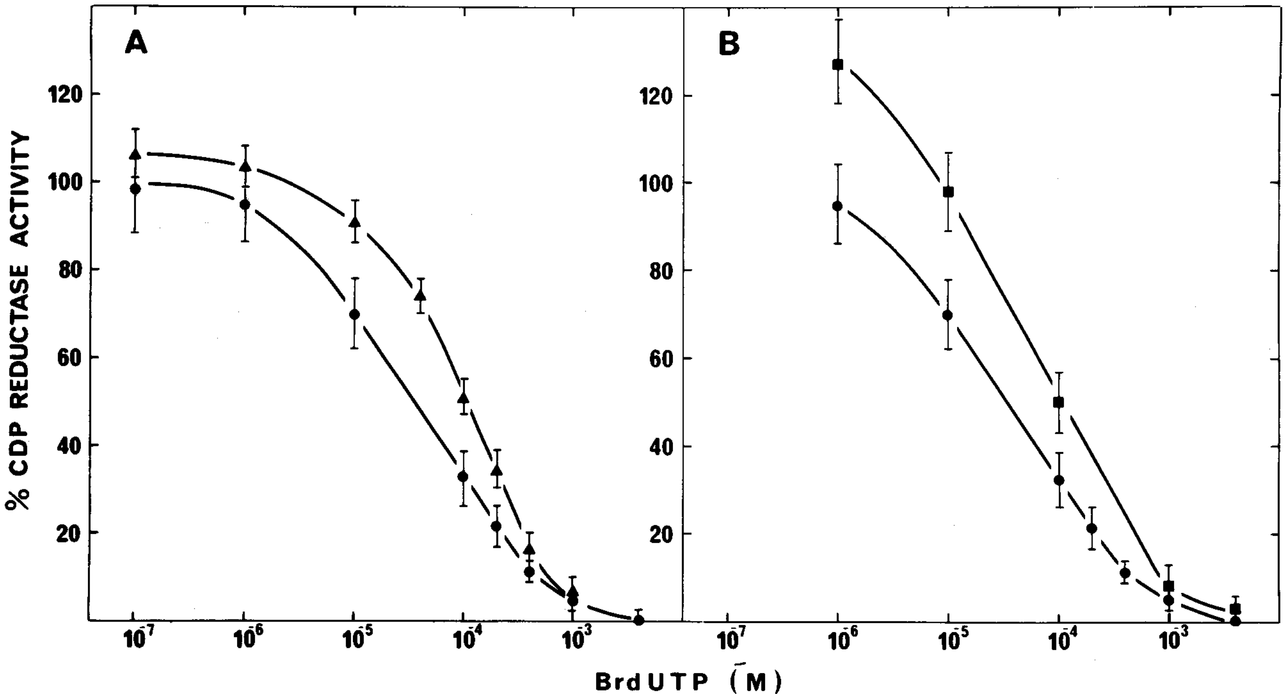

Fig. 3. Inhibition of ribonucleotide reductase by BrdUTP. Extracts of CHO and two first-step BrdU-resistant mutants were assayed for CDP-reductase activity in the presence of increasing concentrations of BrdUTP, as described in Materials and Methods (A) ●, WT; ▲, B211. (B) ●, WT; ■, D41621. Points are averages of four determinations, with standard deviation; WT curves are the averages of two determinations of CHO and two determinations of D416. Values for each extract are expressed as percent of activity of that extract in the absence of BrdUTP.

compare the in vitro reductase activities of the WT lines and two of the mutant lines in the presence of increasing concentrations of BrdUTP. The results of four experiments with each cell line are presented in Fig. 3 and show that the activity in the mutant cell lines is in fact less sensitive: inhibition of the activity requires three- to fourfold more BrdUTP in B211 and D41621 extracts than in WT extracts. This suggests that in the mutant cells a given reductase activity can be maintained at an external BrdU concentration about three- to fourfold higher than that which reduces the enzyme activity in WT cells to the same level. This, in fact seems to be the case, since the increase in in vivo drug resistance based on increases in LD_{10} (Fig. 1) was about threefold.

Dominance of BrdU Resistance. To determine whether the BrdU resistance at 2.6×10^{-5} M was dominant or recessive, about 10^5 cells of each of B210, B211, and D41621 were mixed in separate large Linbro wells with an equal number of the TG-resistant, ouabain-resistant cell line 8-3TG. After induction of fusion by treatment with PEG (Materials and Methods), hybrid colonies were selected in HAT + 3 mM ouabain. Three colonies from each of the above three fusions, as well as from the control fusion of 8-3TG with CHO, were picked, recloned, grown up, and tested for BrdU resistance and karyotype. All hybrid lines were pseudotetraploid, and in each case the killing curve in BrdU was not significantly different from that of the parental cell line tested (data for D41621 shown in Fig. 4). Thus, the BrdU resistance at about 2.6×10^{-5} M is dominant.

DISCUSSION

The original reason for choosing sib selection as the means to isolate BrdU-resistant cells was to obtain $tk^{+/-}$ mutants in a state resembling, as closely as possible, the parental CHO line. One property which in our experience (13) is often significantly altered after BrdU selection is the cell's karyotype, and it was particularly desirable for subsequent genetic studies that the mutants isolated retain the pseudodiploid karyotype of the parental lines. In this regard the approach seems to have succeeded, since chromosome numbers in all mutants were very close to those in WT cells, and recent banding experiments (data not presented) confirm no noticeable loss or gain of genetic material.

Although sib selection did not yield the *tk* heterozygotes initially expected, once cell lines with the first level of BrdU resistance had been isolated it was relatively easy to generate mutants with higher resistance. Based on the biochemical and genetic data presented, we concluded that at least some of these were $tk^{+/-}$, and even though most were isolated directly in BrdU, all were still pseudodiploid. The procedure we followed therefore

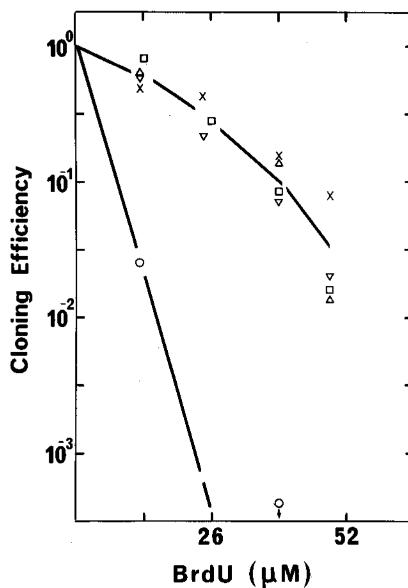

Fig. 4. Dominance of first-step resistance in D41621. Cloning efficiencies of D416, D41621, and three independent hybrids of D41621 and 8-3TG were measured in increasing concentrations of BrdU. ○, D416; □, D41621; X,Δ,∇, hybrid cell lines.

appears to be a good protocol for isolation of $tk^{+/-}$ heterozygotes with a minimum of cytogenetic aberrations.

The biochemical basis for the first step of resistance has been tentatively identified as a change in ribonucleotide reductase activity. Several previous reports exist describing drug-resistant mutants in which the involvement of the reductase was suspected or demonstrated. Recently a group of mutants of BHK cells resistant to adenosine arabinoside (araA) has been described which were shown by in vitro assays to have a reductase activity less sensitive to inhibition by araA than that of WT cells (24). These mutants were cross-resistant to araC and had the interesting property of undergoing spontaneous mutation to thioguanine resistance at an increased rate. Perhaps the best-characterized mutants involving the reductase enzyme are those resistant to the cytotoxic effects of hydroxyurea (HU; for review see 25). Three classes have been delineated; in the first (26) the drug-resistant phenotype was dominant (or codominant) and stable, and in vitro assays demonstrated a decreased sensitivity of the enzyme to HU. Mutants of the second group were also dominant but frequently unstable. The reductase activity in these cells had WT sensitivity to the drug, but its intracellular level was enhanced, typically on the order of fivefold (25, 27). The third class consists of mutants whose reductase exhibits both reduced drug sensitivity

and increased activity (28). Mutants of classes 1 and 2 are thought to arise, respectively, by structural gene mutation and enzyme overproduction, the latter perhaps as a result of gene amplification.

The properties of the first-step BrdU-resistant mutants reported here suggest they are similar to the first class of HU-resistant mutants above. The phenotype was dominant and stable, and in vitro assays of the reductase in two of the mutants showed decreased sensitivity to the drug in question. Furthermore the enzymatic specific activities in extracts of the mutants were about 50–60% of that found in WT extracts. As has been reported by others (27), this type of assay is subject to some quantitative variability, but the reproducibility we achieved (Materials and Methods) was sufficient to detect a twofold enhancement in the mutants, had it existed.

We therefore propose that our cell lines acquired BrdU resistance through a mutation in the structural gene for ribonucleotide reductase. This change was apparently sufficient to permit isolation in subsequent steps of derivatives with higher BrdU resistance resulting from sequential mutation of the *tk* genes. This is the first report describing stepwise isolation of what we believe to be $tk^{+/-}$ and $tk^{-/-}$ cells, and it may be that initial isolation of mutants with an altered reductase facilitated this. In fact, since BrdUTP is an inhibitor of reductase, one could predict that most other BrdU-resistant mutants of CHO, although characterized only as TK^-, should also have acquired either a genetically altered reductase or some other change to overcome this otherwise lethal effect of the drug. If either were the case, the cell line in question would have some unidentified difference with respect to the WT parent. For this reason we believe that the cell lines we have isolated can be used to isolate better-characterized TK-deficient mutants than have yet been reported.

ACKNOWLEDGMENTS

We wish to acknowledge the excellent technical assistance of Hélène Duguay-Brouillette, France Laviolette, and Claire Guilbault. Mark Meuth contributed helpful discussions. This work was supported by the Medical Research Council of Canada and the National Cancer Institute of Canada.

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