Somatic Cell Genetics, Vol. 4, No. 6, 1978, pp. 699–713

Inverse Relationship between Galactokinase Activity and 2-Deoxygalactose Resistance in Chinese Hamster Ovary Cells

Carolyn D. Whitfield,[1] Bruce Buchsbaum,[2] Richard Bostedor,[1] and Ernest H. Y. Chu[2]

Departments of Biological Chemistry[1] and Human Genetics,[2] University of Michigan, Ann Arbor, Michigan 48109

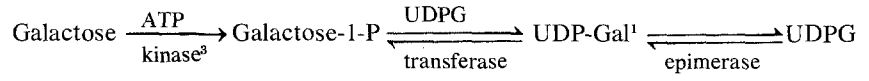
Received May 22, 1978—Final July 24, 1978

Abstract—*Galactokinase activity is reduced in 12 independent clones of Chinese hamster ovary cells resistant to 2-deoxygalactose. The frequency of resistant colonies is increased with chemical mutagens. The resistant phenotype is stable in the absence of selection. There is an inverse correlation between the levels of galactokinase activity and the cloning efficiency in deoxygalactose. Cells with high resistance have 1% or less of the enzyme activity observed in the parental cells; while cells with low resistance have 10–30% galactokinase activity. Studies with tetraploid hybrid cells reveal that resistance to deoxygalactose is a recessive trait and that cells with high resistance do not complement those with low resistance. In cell lines with low resistance, the* K_m *for galactose,* K_i *for deoxygalactose,* K_m *for ATP, and thermolability were not significantly altered compared to sensitive parental cells. Although the possibility of mutation at the structural gene locus has not been ruled out, the reduced enzyme activity may also be due to mutation at a regulatory site which affects the number of galactokinase molecules per cell.*

INTRODUCTION

Chinese hamster ovary (CHO) cells which are resistant to 2-deoxygalactose (DGA) have been isolated in order to obtain cells with a defect in galactose transport or metabolism (Leloir pathway).

0098-0366/78/1100-0699$05.00/0



Growth of CHO K1 is inhibited by the galactose analog 2-deoxygalactose in the presence of glucose. This inhibition is reversed by galactose, indicating that the action of the analog is specific for galactose-metabolizing enzymes.

Alper and Ames (1) have used DGA to select for mutants in *Salmonella typhimurium* which are deleted for galactokinase, UDPG epimerase, and galactose-1-phosphate uridylyltransferase. DGA has also been used to select for galactose permease mutants in *S. typhimurium* (2). While this work was in progress, Thirion et al. (3) reported using DGA to isolate Chinese hamster lung cells with a defect in galactokinase. In this paper, we describe the isolation, and genetic and biochemical characterization of deoxygalactose-resistant cells (Dga^R) from CHO K1. All resistant clones have reduced levels of galactokinase, which also catalyzes the ATP phosphorylation of deoxygalactose to deoxygalactose-1-phosphate (DGA-1-P) (3). A preliminary report of this work has appeared (4).

MATERIALS AND METHODS

Cell Lines and Cell Culture. Cells were grown in modified Eagle's minimum essential medium (5) (Gibco or K.C.) containing Earle's salts, hypoxanthine (H), 37 μM; thymidine (T), 21 μM; uridine (U), 21 μM; 1 mM pyruvate; 1.5 times the normal levels of vitamins and essential amino acids; and 2 times nonessential amino acids, with 5% fetal calf serum (Gibco or K.C.) for monolayer growth and 10% serum for suspension culture. For cell culture 17.9 mM $NaHCO_3$ and 8.3 mM NaCl were added to the growth media; for plating experiments, 11.9 mM $NaHCO_3$ and 14.3 mM NaCl were added. Serum dialyzed by ultrafiltration with an Amicon UM 10 filter was used in medium lacking proline, medium containing 6-thioguanine, or in medium where 14 mM galactose was substituted for glucose. HAT medium contained 3.2 μM aminopterin (A), hypoxanthine and thymidine as above. Cells were grown at 37°C in a humid atmosphere of 5% CO_2 in air.

CHO K1 (American Type Culture Collection), which is auxotrophic for proline (Pro^- Gat^+ Dga^S TG^S), and a subclone, CHO K1-T4, were

[3]Abbreviations used: Uridine diphosphate glucose (UDPG); uridine diphosphate galactose (UDP-Gal). Enzymes: galactokinase (EC 2.7.1.6); UDP-glucose: galactose-1-phosphate uridylyltransferase (EC 2.7.7.12); UDP-glucose 4'-epimerase (EC 5.1.3.2).

used to isolate Dga^R Pro^- Gat^+ TG^S cells. Gat^- Pro^+ Dga^S, referred to as Gat^-, was kindly sent to us by Dr. Larry Thompson. Gat^-, originally isolated from CHO-S by McBurney and Whitmore (6) and called AUX $B1^-$, is auxotrophic for glycine, adenosine, and thymidine and lacks folylpolyglutamate synthetase (7). Dga^R 27 TG^R Pro^+ cells were derived from Dga^R 27 without mutagenesis after successively selecting for thioguanine resistance (TG^R) in 60 μM thioguanine (no HUT) and for Pro^+ in medium without proline at 10^5 cells/100-mm petri dish.

Mutagenesis and Plating Efficiency. Cells growing exponentially in monolayer or suspension culture were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 1 μg/ml, for 5 h or with ethyl methanesulfonate (EMS), 100–400 μg/ml for 16 h. After treatment, cells were washed, grown, and transferred when necessary in complete media for 6–11 days to allow for expression of the phenotype (reviewed in 8). Resistant cells were selected in complete media containing 30 or 60 mM DGA after replating 10^5 cells/60 mm dish or 15 mM DGA at 10^4 cells/dish for 10–14 days. Resistant cell lines Dga^R 1 to 26 were selected using 2-deoxygalactose purchased from Sigma which contained traces of galactose. Dga^R 27 to 40 were isolated using the purer Aldrich DGA.

The relative plating efficiency in DGA expressed as a percent is 100 times the number of colonies or cells in deoxygalactose divided by the number of colonies or cells without deoxygalactose. Colonies were fixed with 12% formaldehyde and stained with 0.1% crystal violet.

Cell Hybridization. Dga^R Gat^+ Pro^- cells, 5×10^5 cells/35-mm plate, were fused with Dga^S Gat^- Pro^+ cells, 5×10^5 cells/35-mm plate, using 45% polyethylene glycol (9). As a control, two sensitive cell lines, Dga^S Gat^- Pro^+ and CHO K1 (Dga^S Gat^+ Pro^-), were also fused. After 19–24 h in nonselective medium, fused cells were replated at 10^3 and 10^4 cells/60-mm dish in selective medium lacking proline, adenosine, and thymidine. Only hybrid clones (denoted as K1 $\times$ Gat^- or Dga^R __ $\times$ Gat^-) which were Gat^+ Pro^+ could grow in the selective medium. Dga^R 27 Pro^+ TG^R cells were fused with Dga^R 24 Pro^- TG^S or with Dga^R 23 Pro^- TG^S, and the resulting hybrid cells (TG^S Pro^+, denoted as Dga^R __ $\times$ Dga^R 27) were selected in HAT medium lacking proline. Resistance to thioguanine results from an X-linked recessive mutation yielding very low levels of hypoxanthine guanine phosphoribosyltransferase (10–14). TG^R cells do not grow in HAT medium while TG^R $\times$ TG^S hybrid cells and TG^S cells do (13, 14).

No revertants were observed among the unfused cell populations when 3×10^5 parental cells were plated in selective medium. Hybrid cell colonies were isolated, grown in selective medium, and five or more hybrid clones from each fusion combination were tested in medium with

and without 6 mM DGA at 200–500 cells/plate. The karyotype of each hybrid clone was determined by harvesting exponentially growing cells, washing with 0.56% KCl and then with fixative solution (acetic acid/methanol, 1:3). Slides were prepared, stained with Giemsa, and 20 representative chromosome spreads were counted.

Enzyme Assays. Cell extracts for galactokinase assays were prepared at 4°C as follows. Cells, 30×10^6, were washed with 0.9% NaCl, resuspended in 0.5 ml of buffer containing 0.01 M KP_i, pH 6.7; 1 mM EDTA; 0.5 mM dithiothreitol; sonicated at intervals for a total of 30 sec, and centrifuged at 17,400*g* for 20 min. The supernatant solution was passed through a column, 1 × 3.8 cm, of Sephadex G50 and eluted in the same buffer. After aliquots were taken for protein determination, the dithiothreitol concentration was adjusted to 2 mM. EDTA was omitted for ATP kinetic studies. For epimerase and transferase assays, the buffer was the same except the pH was 7.4 and extracts were assayed without Sephadex gel filtration. Protein was determined by the method of Lowry (15).

The procedure of Sherman and Adler (16) was modified to assay galactokinase. [1-^{14}C]Galactose was incubated with ATP and hexokinase and passed over a column of DEAE 32 to remove any contaminating hexoses before use in the assay (17). In assay A, which was used for kinetic studies, the reaction mixture contained 0.2 M Tris-Cl, pH 7.8, 22°C; 5 mM $MgCl_2$; 5 mM NaF; 6 mM ATP; 5 mM dithiothreitol; [1-^{14}C]D-galactose (New England Nuclear), 0.54 μCi/ml; 0.4 mM galactose. Assay B was the same except for the concentrations of $MgCl_2$, 2 mM; ATP, 2 mM; galactose, 0.2 mM; and [1-^{14}C]galactose, 1 μCi/ml. The reaction mixture was incubated for 30 min at 37°C and terminated by spotting three 25- or 30-μl aliquots on 1.5-cm squares of DEAE paper (Whatman DE 81) and dropping immediately into 300 ml of water. Papers were washed three times with water, dried, and counted in toluene containing 0.1 g POPOP and 5 g PPO per liter. The assay was linear with time and enzyme concentration at all galactose and ATP concentrations used.

UDPG epimerase was assayed at 37°C as described previously (17), except that 0.1 M glycylglycine, pH 8.7, and 1 mM UDP-Gal were used. Galactose-1-phosphate uridylyltransferase was assayed at 37°C by a procedure similar to that of Mayes and Hansen (18). The reference and sample cuvettes contained 0.1 M glycylglycine, pH 8.7; 1 mM $MgCl_2$; 5 mM dithiothreitol; 1 mM NADP; 6 μM glucose-1,6-diphosphate; 0.5 mM UDPG; 0.1 units of phosphoglucomutase (Sigma); 0.1 units of glucose-6-phosphate dehydrogenase (Type VII, Sigma); 0.005 units of 6-phosphogluconic acid dehydrogenase (Sigma); and extract in a final volume of 1 ml. The reaction was intiated by adding galactose-1-phosphate, 0.65 mM,

to the sample cuvette. The increase at 340 nm was followed. The blank reaction without extract was subtracted.

RESULTS

The frequency of colonies resistant to 2-deoxygalactose was increased by treatment with chemical mutagens (Table 1). The average spontaneous frequency of resistant colonies was 0.6×10^6, whereas the frequency induced by MNNG was 8.7×10^6. As the concentration of EMS was increased, the frequency of resistant colonies increased from 4 to 38×10^6. The resistant phenotype was stable after repeated cloning and continuous growth in medium without deoxygalactose for seven months. These results suggest that the resistant clones arose from gene mutation and not adaptation.

The concentration of DGA needed to inhibit growth of CHO K1 varied with the number of cells plated (Fig. 1). For example, 6 mM DGA completely inhibited growth of CHO K1 when a low density of cells were plated (200–500 cells/60-mm plate). However, the relative number of cells in DGA compared to media without DGA was 1% when 10^5 cells were plated and 13% when 8×10^5 cells were plated in 6 mM DGA. For this reason, plating experiments with sensitive and resistant cell lines in DGA were performed at low cell density.

When CHO K1 was plated at 200 cells/60-mm dish in 6 mM DGA, 2 mM galactose reversed the plating efficiency in DGA from 0% to 90% of that in media without the analog. CHO K1 plated with an efficiency of only 5–7% in 6 mM DGA when 55 mM glucose, mannose, or fructose were added. Similarly, uridine, 0.12 mM, did not increase the plating efficiency in DGA.

Among 19 independently isolated clones, high and low levels of resistance to DGA were observed (Fig. 1). Most clones, such as Dga^R 3,

Table 1. Induction of Mutants Resistant to 2-Deoxygalactose in CHO Cells

Experiment	Mutagen	Observed colonies/ cells plated	Mutation frequency per 10^6 survivors[a]
A	None	$5/6.4 \times 10^6$	1.04
	MNNG (1 μg/ml)	$19/3.8 \times 10^6$	8.7
	EMS (400 μg/ml)	$54/3.7 \times 10^6$	30.1
B	None	$1/4 \times 10^6$	0.26
	EMS (100 μg/ml)	$2/1 \times 10^6$	4.0
	EMS (200 μg/ml)	$19/1 \times 10^6$	38.0
	EMS (400 μg/ml)	$14/1 \times 10^6$	28.0

[a] Corrected for plating efficiency without DGA.

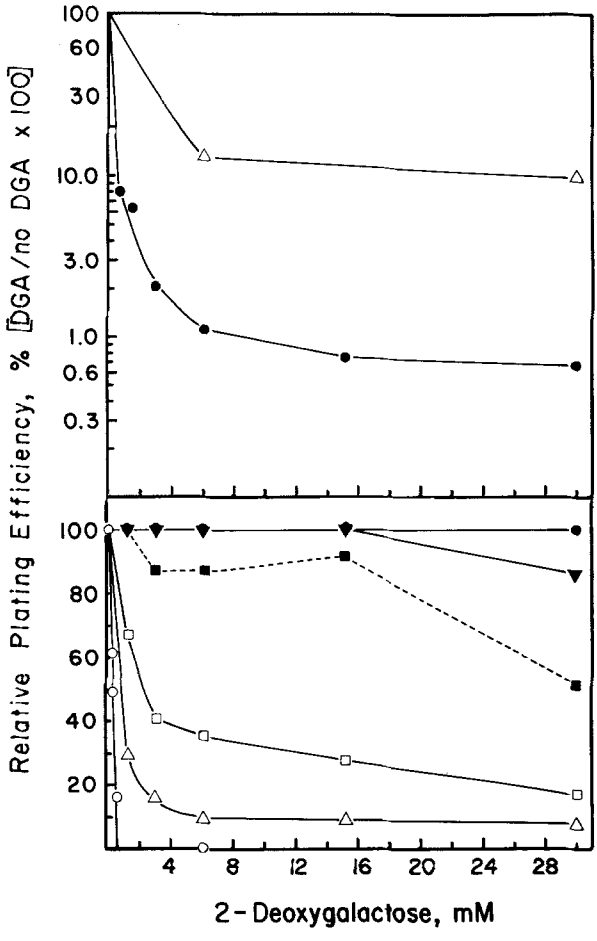


Fig. 1. Plating efficiency of sensitive and resistant cell lines in different concentrations of deoxygalactose. Upper panel (high cell density): CHO K1 was inoculated at 8×10^5 cells/60-mm plate, △; and at 1×10^5 cells/60-mm plate, ●. In media without DGA, cells were removed with trypsin and counted at confluency (3–5 days); in media with DGA, cells were counted at 6–7 days. These numbers were used to calculate the relative plating efficiency. Lower panel (low cell density): cells were plated at 200–500 cells/60-mm plate and colonies were stained and counted after 8–12 days. ○, CHO K1; △, Dga^R 26; □, Dga^R 24; ■, Dga^R 6; ▼, Dga^R 3; ●, Dga^R 8.

6, 27, were highly resistant to DGA and did not grow on galactose as a sole carbon source (Table 2). The plating efficiency of these clones in 6 mM DGA was 87–100% of that without DGA compared to 0% for CHO K1. Three clones, Dga^R 23, 24, 26, were isolated which had low resistance to DGA and grew on galactose. Dga^R 23, 24, 26 plated in 6 mM DGA with an efficiency of 10–30% even after successive recloning.

Table 2. Properties of Deoxygalactose-Sensitive and -Resistant CHO Cells

Cell line	Mutagen	Relative plating Efficiency[a] (%)	Growth in galactose[b]	Specific activity[c]		
				Kinase	Epimerase	Transferase
CHO KI	none	<0.13	+	4.17	7.14	3.14
CHO KI-T4	none	<0.08	+	4.47	7.33	4.33
DG 3-1	MNNG	100	−	0	6.90	3.29
DG 6-1	MNNG	87	−	0	7.16	2.44
DG 8-1	MNNG	100	−	0.02	7.58	1.62
DG 23-1-1	EMS	14	+	1.07	6.80	3.04
DG 24-4-3	EMS	30	+	1.40	6.80	3.18
DG 26-1	EMS	10	+	0.32	6.95	3.44
DG 27-1	none	97	−	0	6.35	3.75
DG 30-1	EMS	89	−	0	7.33	3.79
DG 31-1	MNNG	91	−	0	8.61	4.07
DG 32-1	EMS	100	−	0	10.59	4.69

[a] Plating in (6 mM DGA/0 mM DGA) × 100; 200–500 cells/60-mm dish; 3–4 plates each DGA concentration.
[b] 1×10^5 cells/60-mm plate in 15 mM galactose.
[c] Specific activity in nmol/min/mg protein. Galactokinase was determined with assay A.

Galactokinase specific activity is reduced in all deoxygalactose-resistant cell lines (Table 2). The highly resistant cells had less than 1% of the activity observed in CHO K1. Cell lines with low resistance had 7–31% of the galactokinase activity present in the sensitive parental cells. When sensitive and resistant cell extracts were mixed, the galactokinase activity of the sensitive cells was not inhibited. Removal of small molecules by Sephadex G-50 gel filtration did not restore activity in resistant cell extracts. Thus the lack of galactokinase activity in the resistant cells was not due to the presence of an inhibitor. UDPG epimerase activity in resistant cell lines is nearly the same or slighly higher than in sensitive cells. Galactose-1-P uridylyltransferase activity is also not significantly different in Dga^R cells from that of sensitive cells. Dga^R 6 and Dga^R 8 have somewhat reduced levels of transferase activity which may be due to clonal variation.

In order to determine if the galactokinase protein is altered in cells with low resistance, the kinetic and thermosensitivity properties of the enzyme were examined in cell free extracts after Sephadex G-50 gel filtration. Dga^R 24, Dga^R 26, and CHO K1 showed the same loss of enzyme activity when heat inactivated at 44–51°C for up to 10 min and then assayed at 37°C.

The K_m for galactose and ATP, K_i for deoxygalactose, and V_{max} per mg of protein were determined from Lineweaver-Burk plots where the galactose concentration was varied in the presence of a constant amount of the inhibitor analog DGA (Fig. 2). Three of the 4 lines intersect at a

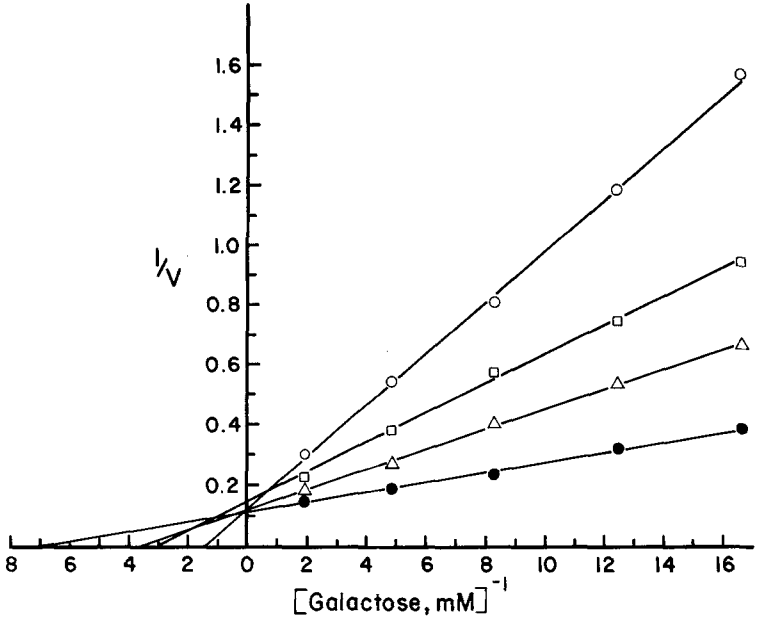


Fig. 2. Lineweaver-Burk plot of galactokinase activity in CHO K1 cells. The concentration of galactose was varied in the presence of a constant concentration of deoxygalactose (Aldrich). •, 0 mM DGA; △, 5 mM DGA; □, 10 mM DGA; ○, 20 mM DGA. Lines were drawn from regression analysis.

single point on the y axis as expected for a pure competitive inhibitor of galactose. The small deviation in the fourth line is within the variation of the radioactive assay. The K_m and V_{max} were calculated from the x and y intercepts, respectively. The K_i for deoxygalactose was calculated from the x intercept in a secondary plot of the slopes from the Lineweaver-Burk plot versus the inhibitor concentration (19) (Fig. 3). Similar data were obtained for Dga^R 23, 24, and 26 and are summarized in Table 3. The V_{max} per mg of protein is 24% of the average value for CHO K1 and T4 with Dga^R 23; 31% with Dga^R 24; 7% with Dga^R 26. However, the K_m for galactose, K_i for deoxygalactose, and K_m for ATP were not significantly altered in the resistant cells compared to the sensitive cells.

Deoxygalactose resistant cells were fused with sensitive cells to determine if resistance is a recessive or dominant trait. If resistance is recessive, these tetraploid hybrid cells are expected to be sensitive to DGA. As a control, two sensitive cell lines, K1 and Gat^-, were also fused. Most K1 × Gat^- clones showed a plating efficiency in 6 mM DGA from 0 to 5% of that with no DGA; one unusual hybrid K1 × Gat^- clone 12 exhibited an even higher plating of 18.6% (Table 4). Hybrid clones formed from sensitive cells and cells with low resistance, Dga^R 24 × Gat^- and

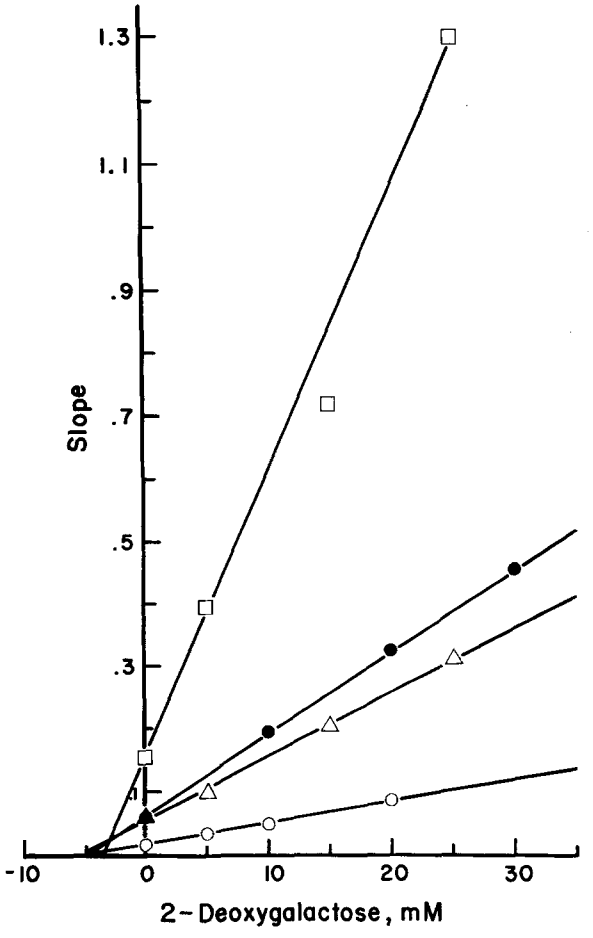


Fig. 3. Secondary plot of the data from Fig. 2 and other data not shown. DGA concentration is plotted versus the slope of the Lineweaver-Burk curves where galactose concentration was varied in the presence of a constant concentration of DGA. ○, CHO K1; △, Dga^R 23-1-1; ●, Dga^R 24-4-3; □, Dga^R 26-1.

Dga^R 23 × Gat^-, plated with an efficiency of 0–10% in 6 mM DGA. With one exception hybrid clones formed between highly resistant cells and sensitive cells, Dga^R 27 × Gat^-, exhibited a plating efficiency of 3–20% in 6 mM DGA. On the basis of colony size and plating efficiency in different concentrations of DGA (Fig. 4), hybrids Dga^R 23 × Gat^-, Dga^R 24 × Gat^-, and Dga^R 27 × Gat^- were more similar to the sensitive control hybrid, K1 × Gat^-, than to their resistant diploid parental cells. Therefore we conclude that DGA resistance is a recessive trait.

The higher plating efficiency of Dga^R 23 × Gat^-, Dga^R 24 × Gat^-,

Table 3. Kinetic Properties of 2-Deoxygalactose-Sensitive and -Resistant CHO Cells

Cell line	V_{max}/protein[a] (nmoles/min/mg)	Galactose K_m (mM)	Deoxygalactose K_i (mM)	ATP K_m (mM)
CHO Kl	5.66	0.147	4.49	0.084
CHO Kl-T4	6.43	0.180	5.08	N.D.[b]
DG 23-1-1	1.44	0.143	5.28	N.D.
DG 24-4-3	1.86	0.134	4.73	0.094
DG 26-1	0.42	0.126	3.52	0.067

[a] Assay A was used for the kinetic studies. V_{max} was determined from varying galactose concentrations.
[b] N.D. = not determined.

Dga^R 27 × Gat^-, and K1 × Gat^- clone 12 in DGA compared to the sensitive control K1 × Gat^- clone 1 (Fig. 4) is a reflection of lower galactokinase activity in these hybrids relative to K1 × Gat^- clone 1 (Table 4). Galactokinase specific activity of hybrids formed between Dga^R and Gat^- cells and K1 × Gat^- clone 12 was 48–62% of that observed with K1 × Gat^- clones 1 and 18. With the exception of K1 × Gat^- clone 12 and Dga^R 27 × Gat^- clone 4, this was the level of galactokinase activity

Table 4. Properties of Tetraploid Hybrid Cells

Cell line	Chromosome number		Relative plating efficiency[a] (%)	Galactokinase[b]	
	Mean	σ		Spec. act.	%
Gat^-	21.0	0.9	<0.05	3.9	
Kl × Gat^--1	38.2	1.3	<0.13	3.53	100
Kl × Gat^--18	38.6	2.8	2.0	3.23	100
Kl × Gat^--12	37.4	1.6	18.6	1.89	56
Dga^R 27 × Gat^--1	41.3	4.1	20.3	1.62	48
Dga^R 27 × Gat^--3	40.3	1.5	8.7	1.73	51
Dga^R 27 × Gat^--4	38.9	1.9	66.7	0.19	6
Dga^R 27 × Gat^--5	34.3	2.6	3.3	1.92	57
Dga^R 24 × Gat^--6	39.6	1.9	5.9	1.98	59
Dga^R 24 × Gat^--11	38.0	1.7	6.5	1.92	57
Dga^R 23 × Gat^--7	39.9	3.1	0.6	2.10	62
Dga^R 23 × Gat^--11	40.9	2.1	3.1	2.09	62
Dga^R 24 × Dga^R 27-6	39.2	2.9	67.0	0.72	21
Dga^R 24 × Dga^R 27-8	40.7	2.9	61.3	0.47	14
Dga^R 23 × Dga^R 27-18	38.3	1.6	61.7	0.60	18
Dga^R 23 × Dga^R 27-20	40.7	2.0	54.2	0.65	19

[a] Plating in (6 mM DGA/0 mM DGA) × 100; 200–500 cells/60-mm dish; 3–4 plates each DGA concentration.
[b] Specific activity in nmol/min/mg protein using assay B. The average value of the first two hybrid clones in the table was taken as 100%.

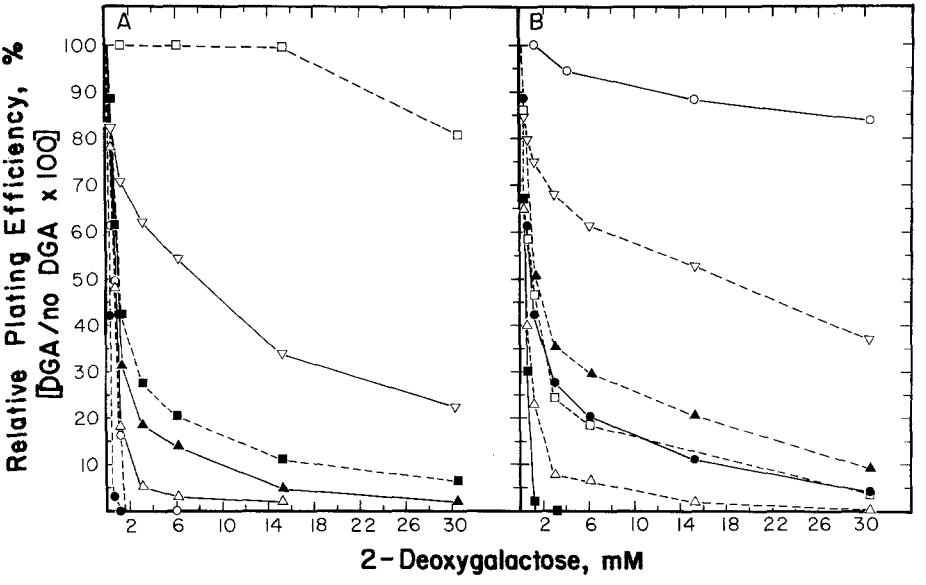


Fig. 4. Plating of representative diploid and tetraploid clones in DGA at 200–500 cells/60-mm dish, 4 plates each DGA concentration. Panel A: □, Dga^R 27-1 TG^R Pro^+; ▽, Dga^R 23-1-1 × Dga^R 27-1 clone 20; ■, Dga^R 27-1 × Gat^- clone 1; ▲, Dga^R 23-1-1; △, Dga^R 23-1-1 × Gat^- clone 11; ○, K1; ●, K1 × Gat^- clone 1. Panel B: ○, Dga^R 27-1; ▽, Dga^R 24-4-3 × Dga^R 27-1 clone 8; ▲, Dga^R 24-4-3; □, K1 × Gat^- clone 12; ●, Dga^R 27-1 × Gat^- clone 1; △, Dga^R 24-4-3 × Gat^- clone 11; ■, Gat^-.

expected for coexpression of enzyme activity considering the activity in the diploid parental cells.

Two atypical clones, Dga^R 27 × Gat^- clone 4 and K1 × Gat^- clone 12 clearly have a much higher plating efficiency in DGA than other clones from these crosses. In both cases, the higher plating corresponded with decreased galactokinase activity. It is possible that these particular hybrids have lost a functional wild-type (structural or regulatory) gene for galactokinase soon after cell fusion since the selected markers were Gat^+ Pro^+. Chromosomal loss, rearrangement, and alteration is increased in tetraploid CHO cells (20). Since the frequency of their occurrence is high (8–20%), it is unlikely that these unusual clones arose by mutation. If the galactokinase gene is functionally hemizygous (see Discussion) in DGA-sensitive CHO cells and one wild-type allele is lost or inactivated in the tetraploid cell, one would expect K1 × Gat^- clone 12 to have about 50% of the enzyme activity observed in K1 × Gat^- clone 1 and Dga^R 27 × Gat^- clone 4, about 0%. The observed values of 56% and 6%, respectively, are in good agrement with the predicted ones.

The second series of hybridization experiments involved fusion between cells with high DGA resistance and those with low resistance to

determine if they complemented each other. If complementation occurs, hybrid cells, Dga^R 23 × Dga^R 27 or Dga^R 24 × Dga^R 27, would be sensitive to DGA and plating in DGA would be intermediate between that of hybrids formed from the respective resistant cells and sensitive cells (Dga^R 23 × Gat^- and Dga^R 27 × Gat^- or Dga^R 24 × Gat^- and Dga^R 27 × Gat^-). On the other hand, if complementation does not take place, hybrid cells, Dga^R 23 × Dga^R 27 or Dga^R 24 × Dga^R 27, would be resistant to DGA and plating in DGA would be intermediate between that of the respective resistant parents (Dga^R 23 and Dga^R 27 or Dga^R 24 and Dga^R 27). Dga^R 23 × Dga^R 27 and Dga^R 24 × Dga^R 27 gave large colonies in medium containing DGA with a high plating efficiency of 50–80% (Table 4). Plating of these hybrids at different concentrations of DGA was intermediate between that of the respective resistant parental cells (Fig. 4). Galactokinase activity in Dga^R 23 × Dga^R 27 and Dga^R 24 × Dga^R 27 was 14–21% of that observed in the sensitive control hybrids, K1 × Gat^- clones 1 and 18 (Table 4). From the high plating efficiency in DGA and the low galactokinase activity in Dga^R 23 × Dga^R 27 and Dga^R 24 × Dga^R 27, we conclude that cells with high resistance did not complement those with low resistance with respect to DGA resistance of in vitro galactokinase activity.

DISCUSSION

The increased number of resistant cells after treatment with chemical mutagens and the stability in the absence of selection suggest that DGA resistance arises from gene mutation. Although CHO cells in culture are diploid in terms of DNA content, extensive chromosomal rearrangement has occurred compared to the Chinese hamster (20). Since so many autosomal recessive mutants have been isolated in CHO, Siminovitch (21) has suggested that CHO may be functionally hemizygous for many autosomal gene loci due to chromosomal rearrangement. This may be the case with DGA resistance. Considering that resistance is recessive, the spontaneous frequency, 0.6×10^6, is consistent with a functionally hemizygous state in CHO due to X linkage or autosomal rearrangement (22). The galactokinase structural gene has been mapped on autosomal chromosomes in humans (23), primates (24), and mice (25).

Galactokinase activity is reduced in all DGA-resistant cell lines derived from CHO K1. Thus, formation of deoxygalactose-1-phosphate is implicated in growth inhibition by DGA. In rat ascites hepatoma cells, DGA-1-P represents 99% of the total metabolites of DGA (26). In rat liver, UDP-deoxyhexoses are also formed from DGA (27). These studies suggest that DGA might inhibit growth by depletion of the intracellular pools

of inorganic phosphate or uridine. If DGA inhibits cell growth by trapping uridine, then uridine or other hexoses, such as glucose, fructose, or mannose, which are metabolized to UDPG and UDPGal might reverse the inhibition. We found that galactose but not glucose, fructose, mannose, or uridine reversed the DGA inhibition of growth of CHO cells. Therefore, it is likely that DGA primarily inhibits growth because it accumulates as DGA-1-P in a reaction catalyzed by galactokinase. This results in the trapping of inorganic phosphate which is required for energy production by glycolysis and oxidative phosphorylation. There are several possible reasons why all of our resistant cell lines are defective in galactokinase activity and none deficient at other steps: (1) DGA is not metabolized further than DGA-1-P; (2) a double mutation is required to significantly affect activity at other loci; (3) we have examined too few resistant cell lines, or (4) our selective conditions are not optimal to detect other types of mutations.

Most interestingly, there is a rough inverse relationship between the level of galactokinase activity and the plating efficiency in DGA. With one exception (Dga^R 26), sensitive and resistant cell lines fall into four groups. The first group is represented by sensitive cell lines as CHO K1, T4, Gat^-, K1 × Gat^- clones 1 and 18, which plate with an efficiency of 0–3% in DGA and have 65–100% galactokinase activity. The second group (e.g., Dga^R 23 × Gat^-, Dga^R 24 × Gat^-, Dga^R 27 × Gat^-, K1 × Gat^- clone 12) is very slightly resistant with a plating efficiency of 1–20% in DGA and enzyme activity of 48–62%. The third group is composed of cells with low resistance, as Dga^R 23 and Dga^R 24, which have a plating efficiency of 14–30% in DGA and 24–31% galactokinase activity. The last group (e.g., Dga^R 27, Dga^R 23 × Dga^R 27, Dga^R 24 × Dga^R 27, Dga^R 27 × Gat^- clone 4) is highly resistant with 54–100% plating in DGA and 0–21% enzyme activity. It is noteworthy that two atypical hybrid clones, K1 × Gat^- clone 12 and Dga^R 27 × Gat^- clone 4, picked at random for traits other than resistance, exhibited a higher plating efficiency in DGA than other clones from these crosses and simultaneously showed a decreased galactokinase activity. This further confirms the inverse relationship between DGA resistance and the level of galactokinase activity.

Analysis of DGA resistance and in vitro galactokinase activity in Dga^R × Dga^S hybrid cells show that resistance is a recessive mutation and that galactokinase levels are those expected for coexpression of the enzyme activity. Cells with high resistance do not complement those with low resistance with respect to plating in DGA or galactokinase activity in extracts. The lack of complementation indicates that cell lines with high and low resistance have mutations in the same cistron affecting galactokinase activity. Since galactokinase is a simple monomeric protein (28), dif-

ferent mutations in the structural gene for this enzyme clearly would not complement one another. However, mutation at a regulatory locus affecting transcription or translation of galactokinase is also possible.

The reduced activity of galactokinase in the Dga^R cells could be due to a mutation in a regulatory gene or structural gene. A regulatory mutation affecting transcription, posttranscription, or translation will not modify the thermolability or kinetic properties of the galactokinase enzyme in the resistant cells compared to the sensitive parental cells. These properties might be altered in a structural gene mutation (11) or mutation affecting posttranslational modification of the protein. Although the V_{max} per mg of protein was reduced in cells with low resistance, the K_m for galactose, K_i for deoxygalactose, K_m for ATP, and thermolability were not significantly altered. Hence, it is possible that the reduced enzyme activity is due to a regulatory gene mutation which decreases the number of protein molecules per cell. However, a structural gene mutation which decreased the activity of the protein is not completely ruled out.

ACKNOWLEDGMENTS

We greatly appreciate the many helpful discussions with Drs. Dennis Yep and Harvey Whitfield. We would like to thank Drs. Miriam Meisler and Elaine Lewis for reading the manuscript. This research was supported by grants GM-20608 and AM-19602 from the U.S. Public Health Service. This work was done in part during the tenure of an Established Investigatorship from the American Heart Association awarded to C.W.

LITERATURE CITED

1. Alper, M. D., and Ames, B. N. (1975). *J. Bacteriol.* **121**:259–266.
2. Nagelkerke, F., and Postma, P. W. (1978). *J. Bacteriol.* **133**:607–613.
3. Thirion, J.-P., Banville, D., and Noel, H. (1976). *Genetics* **83**:137–147.
4. Whitfield, C. D., and Chu, E. H. Y. (1976). *Experta Med. Int. Congr. Ser.* **397**:55.
5. Eagle, H. (1959). *Science* **130**:432–437.
6. McBurney, M. W., and Whitmore, G. F. (1976). *Cell* **2**:173–182.
7. Taylor, R. T., and Hanna, M. L. (1977). *Arch. Biochem. Biophys.* **181**:331–344.
8. Chu, E. H. Y., and Powell, S. S. (1976). In *Adv. Hum. Genet, Vol. 7* (ed. by Harris, H., and Hirschhorn, K.) (Plenum Press, New York), pp. 189–258.
9. Davidson, R. L., O'Malley, K. A., and Wheeler, T. B. (1976) *Somat. Cell Genet.* **2**:271–280.
10. Stutts, P., and Brockman, R. W. (1963). *Biochem. Pharmacol.* **12**:97–104.
11. Sharp, J. D., Capecchi, N. E., and Capecchi, M. R. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70**:3145–3149.
12. Farrell, S. A., and Worton, R. G. (1977). *Somat. Cell Genet.* **3**:539–551.
13. Littlefield, J. W. (1964). *Science* **145**:709.
14. Chu, E. H. Y., Brimer, P., Jacobson, K. B., and Merriam, E. V. (1969). *Genetics* **62**:359–377.

15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193:**265–275.
16. Sherman, J. R., and Adler, J. (1963). *J. Biol. Chem.* **238:**873–878.
17. Beutler, E. (1975). *Red Cell Metabolish,* 2nd ed. (Grune & Stratton, New York), pp. 97–102.
18. Mayes, J. S. and Hansen, R. G. (1966). In *Methods in Enzymology, Vol. 9* (ed. by Wood, W. A.) (Academic Press, New York), pp. 708–713.
19. Segel, I. H. (1975). *Enzyme Kinetics* (J. Wiley, New York), pp. 108–109.
20. Worton, R. G., Ho, C. C., and Duff, C. (1977). *Somat. Cell Genet.* **3:**27–45.
21. Siminovitch, L. (1976). *Cell* **7:1**–11.
22. Chasin, L. A. (1973). *J. Cell Physiol.* **82:**299–308.
23. Elsevier, S. M., Kucherlapati, R. S., Nichols, E. A., Creagan, R. P., Giles, R. E., Ruddle, F. H., Willecke, K., and McDougall, J. K. (1974). *Nature* **251:**633–635.
24. Orkwiszewski, K. G., Tedesco, T. A., Mellman, W. J., and Croce, C. M. (1976). *Somat. Cell Genet.* **2:**21–26.
25. Kozak, C. A., and Ruddle, F. H. (1977). *Somat. Cell Genet.* **3:**121–133.
26. Smith, D. F., and Keppler, D. O. R. (1977). *Eur. J. Biochem.* **73:**83–92.
27. Starling, J. J., and Keppler, D. O. R. (1977). *Eur. J. Biochem.* **80:**373–379.
28. Wilson, D. B., and Hogness, D. S. (1966). In *Methods in Enzymology, Vol. 8* (eds.) Neufeld, E. F., and Ginsburg, V. (Academic Press, New York), pp. 229–240.