*GENETICS OF SOMATIC MAMMALIAN CELLS, VI. USE OF AN ANTIMETABOLITE IN ANALYSIS OF GENE MULTIPLICITY**

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Study of biochemical genetics in mammalian cells requires the availability of large numbers of stable, well-characterized genetic markers, delineation of their associated biochemical actions, localization of the genes involved on their respective chromosomes, and mapping of the genes within the chromosomes. While recent developments of *in vitro* methodologies promise to help achieve these ends, the difficulty involved in study of single gene phenomena in diploid cells in which simple recombination techniques are not available remains a deep-seated problem. In a preceding paper, properties of a mutational marker in a Chinese hamster cell involving an absolute requirement of proline for growth were described.[1] Evidence was presented suggesting that the deficient mutant, which possessed only 21 instead of 22 chromosomes, had lost one of the critical genes as a result of the loss of some of its chromatin and had undergone spontaneous mutation in the sister gene on the homologous chromosome so that a complete proline deficiency resulted. Revertants were selected from this clone, and these exhibited properties consistent with those of a hemizygote, as expected from this hypothesis. With the aid of this stable genetic marker, a method for isolating nutritionally deficient mutants of mammalian cells utilizing the combined effects of 5-bromodeoxyuridine (BUdR) incorporation and illumination with near-visible light was developed.[2] This method has been used to obtain mutants with other nutritional deficiencies.[3]

The present paper describes experiments designed to test whether an antimetabolite can be used to differentiate between cells with different multiplicities of a specific gene. If simple dosage relationships are obeyed, different concentrations of the appropriate antimetabolite might be required to produce growth inhibition of cells with such different gene multiplicities. It should then be possible to effect various genetic operations like the isolation and characterization of heterozygous cells. The experiments that follow describe the behavior of Chinese hamster cells containing different multiplicities for the proline gene when treated with the proline antimetabolite, L-azetidine-2-carboxylic acid (AZCA).

Materials and Methods.—Cell cultures: The following cell cultures, most of which have been described previously, were employed: CHO/Pro$^-$ ($\sim 2n$): a 21-chromosome Chinese hamster ovary cell with an absolute growth requirement for proline;[1] CHO/Pro$^+$ ($\sim 2n$): clones of spontaneous revertants to proline independence, selected from the CHO/Pro$^-$ ($\sim 2n$) culture and possessed of the same chromosomal constitution as the proline-requiring cell;[1] CHO/Pro$^+$ ($\sim 4n$): a near-tetraploid form arising from one of the CHO/Pro$^+$ ($\sim 2n$) cultures; CHL ($\sim 2n$): an almost diploid (modal chromosome number of 23) Chinese hamster lung cell derived from a different animal and exhibiting no growth requirement whatever for proline;[1] CHL ($\sim 4n$): a tetraploid form derived from the CHL ($\sim 2n$) cell. As has been previously described,[4] Chinese hamster cell cultures retain a near-euploid karyotype but contain about 10% of cells with chromosome

numbers differing by ±1, 2, or 3 from the modal number. Thus the designations ~2*n* and ~4*n* are employed, because of these small deviations from euploidy. Where the ploidy of a cell is not specified, it will be understood to be the near-diploid state.

The proline requirement has been shown to involve loss of the ability to convert glutamic acid to its γ-semialdehyde in the proline-synthetic pathway.[1] Since other steps in the pathway never seem to be limiting in the cell cultures studied here, the presence or absence of this gene alone appears to determine the extent of proline synthesis, and hence it will be referred to as the proline gene.

Titrations: Two hundred single cells were deposited in Petri dishes in the standard medium consisting of F12 solution[5] supplemented with 5 mg/ml of the macromolecular fraction of fetal calf serum. The presence of proline and AZCA were adjusted as indicated in each experiment. Plates were incubated for 7 days in controlled atmosphere as previously described,[6] then fixed, stained, and scored for colonies. AZCA, a specific antimetabolite for proline, which can be incorporated into proteins in place of the latter,[7] was obtained from Calbiochem, Los Angeles.

Measurement of proline synthesis: Experiments were conducted to compare the capacity for proline synthesis by various cell cultures employed in these studies. C^{14}-L-glutamic acid (sp. act. 200 mc/mmole, Schwarz BioResearch, Inc.) was added at a final concentration of 2 μc/ml to each of a series of plates containing actively growing cells, which were placed in fresh, protein-supplemented F12 medium, modified as follows: nonradioactive glutamic acid was removed from the F12 medium to prevent dilution of the radioactivity, and the proline concentration was reduced to $3 \times 10^{-5} M$, a concentration which permits maximum rate of growth of the proline-deficient cells but which produces no depression of proline synthesis by the proline-independent cells. After 3 days of incubation the supernatant liquid was harvested, applied to Whatman's *O*-(diethylaminoethyl) cellulose (DEAE-cellulose) anion exchange paper, and chromatographed for 16 hr at room temperature in a 3:1 mixture of *n*-valeric acid and water. The peak due to C^{14}-proline is readily identifiable and the radioactivity was counted in a Beckman low-Beta counter.

Experimental Results.—(1) *Evidence that CHO/Pro$^+$ revertant behaves like a hetero- or hemizygote:* Since the CHO/Pro$^+$ revertant is used as a model system in these studies, their validity depends upon its actually behaving like a hetero- or hemizygote with respect to the proline gene. Previous evidence that this cell's synthetic capacity for proline is intermediate between the fully deficient form and the near-diploid cell, CHL, is as follows: (*a*) all of the revertant clones (approximately 65) isolated from CHO/Pro$^-$ culture grow in the absence of proline, but at a reduced rate which can be raised to the maximum value if proline is added to the medium; (*b*) unlike the near-diploid CHL cells, this revertant shows a very poor ability to act as a feeder layer for CHO/Pro$^-$ cells in a medium deficient in proline; and (*c*) this revertant is obtained from the CHO/Pro$^-$ cell by a spontaneous reverse mutation which has a frequency of approximately 10^{-6} per cell generation, a value which approximates that of single gene mutations in other organisms.[1]

Additional evidence was sought for the haploid-like state of the proline gene in these revertant clones. The amount of L-proline synthesized by these cells was compared with that produced by the near-diploid, proline-independent CHL cells by chromatographic isolation and measurement of the amount of C^{14}-L-proline produced after feeding of C^{14}-L-glutamic acid to the cultures, as described in the section on *Materials and Methods*. The data are presented in Figure 1. The CHO/Pro$^-$ cell revealed no detectable proline synthesis, while all the revertant CHO/Pro$^+$ cells synthesized approximately half as much

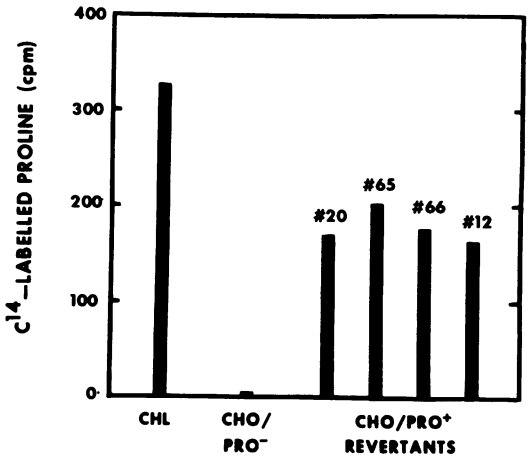

FIG. 1.—Comparison of the amount of C^{14}-proline formed from C^{14}-glutamic acid by the presumed diploid cell (CHL); the proline-deficient form (CHO/Pro$^-$); and four spontaneous revertants of the latter, selected at random. The extent of proline synthesis in a standard aliquot is maximum in the CHL cell, zero in the CHO/Pro$^-$ culture, and approximately half maximum in each of the four revertants tested.

proline as the near-diploid cell with no chromosomal deficiencies. Thus proline production by the revertant cell is intermediate between that of the cell with complete proline deficiency and that of the presumed fully diploid cell, CHL, whose growth is maximal in the absence of exogenous proline.

In another type of experiment, two near-tetraploid clones were isolated from a CHO/Pro$^+$ revertant by means of the colcemide method described elsewhere.[8] If simple gene dosage effects are indeed operative, such tetraploid cells might well display maximum growth rate without any added proline, despite their origins in a cell whose growth rate is proline-limited, since they should contain two proline genes.

Figure 2 shows qualitatively that the growth rates exhibit the expected dependence on proline in the CHO/Pro$^+$ ($\sim 2n$) but not in the tetraploid clone derived from it, even though both clones have the same plating efficiency in the presence and absence of proline. Measurement of the average number of cells per colony after seven days of incubation permits estimation of the average doubling time of the CHO/Pro$^+$ ($\sim 2n$) as 18.2 $\pm$ 2.1 hours, in the absence of proline. In the presence of added proline this cell exhibits a doubling time of 13.4 $\pm$ 0.5 hours, which approximates that of the tetraploid both in the presence or absence of proline, and of the value for the maximum growth rate of any of the Chinese hamster cells used in these studies.[9]

The fact that these different results all demonstrate the expected systematic

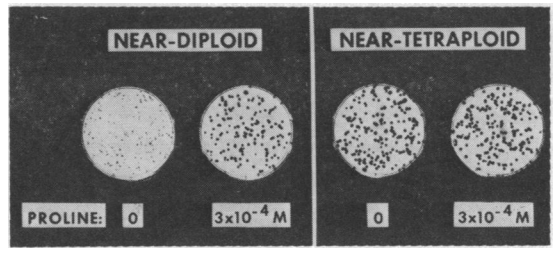

FIG. 2.—Demonstration that, while the near-diploid revertant CHO/Pro$^+$ grows at a reduced rate unless proline is added to the medium, the tetraploid cell isolated from it, and which presumably has two proline genes, grows at a maximum rate either in the presence or absence of proline. The plating efficiency is the same, however, in all plates.

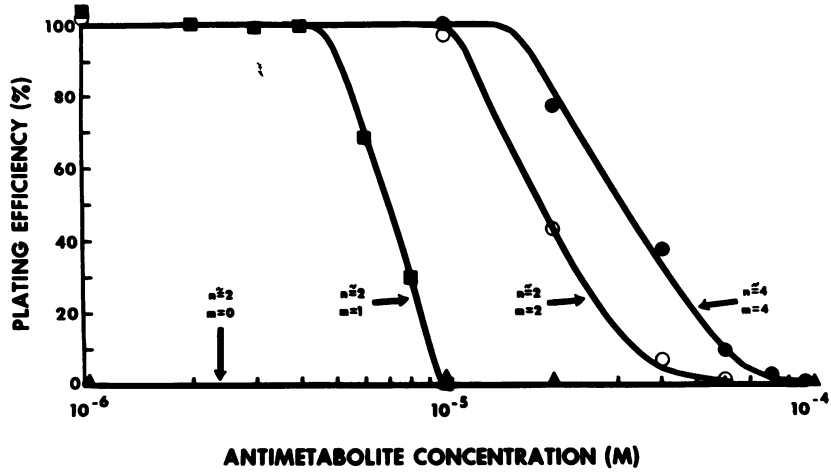

FIG. 3.—Titration of the effect of AZCA on the relative plating efficiency of four Chinese hamster clones: CHO/Pro$^-$ ($\sim 2n$), $m = 0$; CHO/Pro$^+$ ($\sim 2n$) revertant, $m = 1$; CHL ($\sim 2n$), $m = 2$; and CHL ($\sim 4n$), $m = 4$. Each point represents the mean derived from two to four experiments, each involving replicate determinations. The points have been expressed relative to the average of the plating efficiencies at AZCA concentrations so low as not to be significantly different from the control. The average standard deviation of individual points in replicate experiments was $\pm$ 26% of their mean.

variations in the proline-synthetic capacity of the different cell cultures employed allows the provisional assignment of a multiplicity (m) for the proline gene of 1 and 2, respectively, in CHO/Pro$^+$ and CHL, and the corresponding doubling of those numbers in their tetraploid forms.

(2) *Titration with AZCA:* Tests were carried out to determine whether this competitive antagonist for proline would discriminate between cells with different multiplicities of the proline gene. Four different cell types varying in their over-all chromosomal ploidy and the presumed multiplicity of the gene responsible for proline synthesis were titrated for their susceptibility to growth inhibition by AZCA. Figure 3 shows the results which illustrate the following points: (*a*) As expected, the deficient mutant CHO/Pro$^-$ ($n \cong 2$; $m = 0$) has zero plating efficiency at any concentration of AZCA, since this mutant will not even grow in the basal medium which lacks proline. (*b*) The revertant CHO/Pro$^+$ ($n \cong 2$; $m = 1$), which is presumably hemizygous, displays the expected S-shaped sensitivity curve. (*c*) The near-diploid, CHL cell ($n \cong 2$; $m = 2$), displays a higher resistance to AZCA. (*d*) Finally, the near-tetraploid cell ($n \cong 4$; $m = 4$), derived from the diploid CHL, has a still higher resistance to the antimetabolite. The concentrations of AZCA required to lower the relative plating efficiency to 50 per cent, for the clones of presumed proline gene multiplicity of 1, 2, and 4, are 0.69×10^{-5} M, 1.8×10^{-5} M, and 3.1×10^{-5} M, respectively, a relative progression to be expected on the basis of a simple competitive model (*Appendix*).

(3) *Specificity of the AZCA action:* To demonstrate that the inhibitory action of this antimetabolite is indeed proline-specific, AZCA was added to each of the various cell cultures studied, in a concentration just great enough to lower the plating efficiency to zero. A series of different proline concentrations was

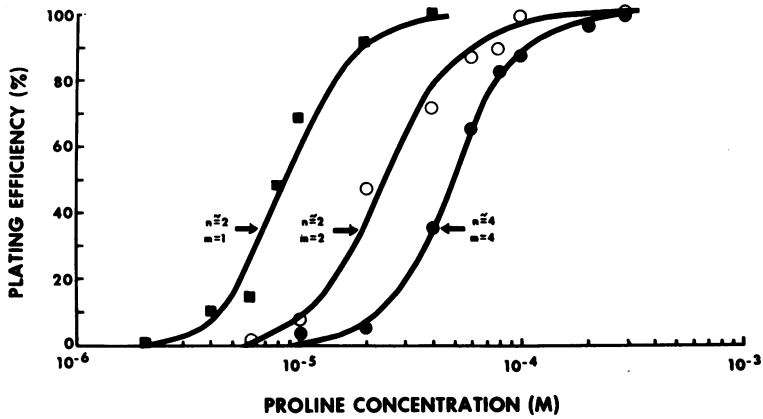

FIG. 4.—Demonstration that proline completely reverses the inhibitory action of AZCA, and that a parallel family of curves is obtained for the various cell types studied, in accordance with the behavior to be expected if the action of the drug is indeed confined to proline competition in every case. In these titrations, AZCA was added to each plate in the following concentrations as determined from Fig. 3, which just sufficed to reduce the plating efficiency of the cells to zero: CHO/Pro$^+$ revertant ($\sim 2n$), $m = 1, 2 \times 10^{-5} M$; CHL/Pro$^+$-($\sim 2n$), $m = 2, 6 \times 10^{-5} M$; and CHL/Pro$^+$ ($\sim 4n$), $m = 4, 10 \times 10^{-5} M$. Various concentrations of proline were added to the plates and the titrations carried out as in Fig. 3. For convenience the points at the highest proline concentrations were normalized to 100% in a manner similar to that of Fig. 3.

then added to such plates, and the colony counts were determined after incubation, in order to ascertain whether proline addition alone would completely restore the original plating efficiency of each culture. The experimental results are shown in Figure 4. They demonstrate that in every case proline completely restored the growth capacity of the cells; that a parallel family of curves is obtained from the different cultures; and that the concentration of proline required to restore the plating efficiency to 50 per cent in each case obeys the relationships postulated in the simple model (*Appendix*).

(4) *Single cell growth and the reversibility of AZCA action:* The principal usefulness of these relationships in genetic studies would lie in the identification of gene multiplicity of selected cell clones and the isolation of clones with increased and with decreased resistance to the action of the antimetabolite, as compared to the wild-type cells. In preliminary tests, artificial mixtures of cells with multiplicities of the proline gene of 1, 2, and 4 were prepared and treated with various concentrations of AZCA ranging from 3×10^{-6} to 6×10^{-5} M. The plating efficiencies agreed closely with that expected on the basis of the calculated values obtained from the survival curves for the individual cell types. In order for this procedure to be maximally useful in genetic studies, it is also necessary that AZCA inhibition be sufficiently reversible to permit use of the BUdR near-visible light technique previously described,[2] for isolation of the clones less resistant than the wild type. Consequently, experiments were carried out to test whether cells inhibited from reproduction by AZCA for periods of up to four days could be restored to colony formation by removal of the

TABLE 1. *Demonstration of the reversibility of AZCA action on representative cells.*

Cell culture	AZCA concentration (M)	Plating efficiency (%) after 7 days in AZCA-containing medium*	Plating efficiency (%) after incubation in AZCA-containing medium for no. days shown plus 5–7 days in standard growth medium		
			Two days	Three days	Four days
CHO/Pro$^+$ revertant ($n \cong 2, m = 1$)	5×10^{-6}	70.0	98.0	73.2	79.0
	1×10^{-5}	0	79.0	64.2	76.5
	2×10^{-5}	0	63.0	25.2	3.8
CHL ($n \cong 2, m = 2$)	2×10^{-5}	37.5	86.0	77.0	91.8
	4×10^{-5}	8.3	84.0	71.4	84.9
	6×10^{-5}	0	80.0	76.2	57.8

Two hundred cells were plated and incubated in Petri dishes in the standard medium together with AZCA in the concentrations indicated.

At various time intervals as noted, the medium was replaced with standard growth medium free of the antimetabolite, and the incubation continued for an additional 5–7 days, after which all plates were fixed and the colonies were scored.

* Without transfer to standard growth medium.

inhibitor. The data presented in Table 1 demonstrate that the action of the inhibitor under these conditions is satisfactorily reversible.

Discussion.—These findings suggest that the following operations may be possible: (*a*) Enrichment of polyploid cells in a culture: Since AZCA can be used to eliminate cells with low proline gene ploidy, the survivors will contain the polyploid cells. It should then be possible to select from among these clonal cell lines of various higher ploidies. (*b*) Isolation of cells with extra members of the specific chromosome containing the proline gene: Treatment of a cell population with AZCA should enrich the survivors in all cells containing extra members of the chromosome carrying the proline gene. Therefore, in addition to polyploid cells, cells trisomic for the proline chromosome should be enriched in the survivors. Methods for producing nondisjunction in one or a few mammalian cell chromosomes have now been described.[8, 10] Selection of AZCA-resistant cells from such a random population and examination of their chromosomes might reveal the presence of a common extra chromosome which may be the carrier of the proline gene. (*c*) Isolation of cells with missing chromosomes: Spontaneous or induced nondisjunction may also result in the production of cells lacking one or a few chromosomes. The use of AZCA plus the BUdR near-visible light technique should make possible isolation of those cells with a ploidy smaller than that of the starting culture for the chromosome carrying the proline gene.

This system should also lend itself to study of mutagenesis in the direction leading to proline gene inactivation. If a diploid homozygous cell population is treated with a mutagen, the great majority of the mutants produced will, of course, be heterozygotes because of the extreme improbability of obtaining a viable cell in which two sister genes have been simultaneously inactivated. However, the addition of appropriate amounts of AZCA might cause the heterozygote to behave like a completely deficient cell and therefore to become isolatable by means of the BUdR-visible light methodology. Similarly, in the

presence of AZCA, rates of spontaneous and induced reversion of heterozygotes to homozygote-competent forms should be measurable.

The present experiments have dealt with a single metabolic antagonist whose action has been shown to obey simple dosage relationships. It would appear possible that similar experiments could be carried out with specific antagonists for other metabolites, provided, of course, that gene dosage effects prevail. If this hope should materialize, the range of genetic biochemical experiments that can be carried out on mammalian cells *in vitro* will be greatly extended. Experiments described elsewhere have shown that the gene for galactose transferase in mammalian cells also obeys simple dosage effects.[11]

For genes which do not obey dosage effects, experiments with appropriate antimetabolites will yield results different from those obtained with AZCA as shown in Figure 3. However, such behavior would yield valuable information about the gene in question. Finally, the use of specific metabolic antagonists may be useful in cell-plating experiments on cells derived from various tissue biopsies, for determining the presence of heterozygotes for selected gene defects in various mammalian populations.

Summary.—L-azetidine-2-carboxylic acid, a specific antimetabolite for proline, inhibits growth of Chinese hamster cells *in vitro*. The growth inhibition is progressively greater with decreasing multiplicity of the proline-synthesizing gene set in the cell. Use of such antimetabolites is suggested for isolation and identification of cells with different multiplicities of genes for which dosage relationships are fulfilled and for other types of genetic experiments.

Appendix.—Let $[M]$ = the concentration in the cell of the normal metabolite, proline, and $[A]$ = the concentration of the antimetabolite AZCA which is incorporated in place or proline to form a toxic protein. Since both M and A compete for the same critical enzyme, E (which may be the proline-activating enzyme),

$$\frac{[ME]}{[M]\ [E]} = K_1 \quad \text{and} \quad \frac{[AE]}{[A]\ [E]} = K_2.$$

We shall assume as a first approximation that the plating efficiency, P, is proportional to the ratio of normal to toxic protein synthesized, which is determined by the ratio $[ME]/[AE]$. Therefore,

$$P = K_3 \frac{[ME]}{[AE]} = K_3 \left(\frac{K_1}{K_2}\right) \frac{[M]}{[A]}.$$

In the presence of appreciable amounts of the antimetabolite A, the amount of M bound to the enzyme decreases. Hence, in the region of concentrations where significant competition is occurring (i.e., in the region of 50% plating efficiency), the values for $[A]$ and $[M]$ in the preceding equation may be respectively approximated by the concentration of A added and the steady-state concentration of M produced by the cell. Therefore, $[A]_{1/2}$, the point where the plating efficiency has been reduced to 0.50 by addition of A, is given by

$$[A]_{1/2} = 2\, K_3 \frac{K_1}{K_2} [M].$$

If gene dosage conditions are obeyed, the steady-state $[M]$ will be proportional to the gene ploidy so that $[A]_{1/2}$ will exhibit a similar proportionality, a situation reasonably well approximated by the data of Figure 3.

In the curves of Figure 4, where an initial amount of AZCA had been added just sufficient to reduce the plating efficiency to zero and titration with proline was carried out, the model predicts that at $[M]_{1/2}$, the point of 50% plating efficiency, the ratio of $[M]_{1/2}/[A]$ should be constant. The value of $[M]_{1/2}$ for each of the cells indicated in Figure 4, where $m = 1$, 2, and 4, is 0.85×10^{-5}, 2.50×10^{-5}, and 5.0×10^{-5}, respectively, and the corresponding ratios of $[M]_{1/2}/[A]$ are 0.42, 0.42, and 0.50.

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