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High-Frequency Induction by 5-Azacytidine of Proline Independence in CHO-K1 Cells

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Abstract—*Proline independence in CHO-K1 Chinese hamster cells has in previous studies been characterized as an auxotrophic gene mutation. In the absence of direct proof, an alternative model must be considered, based on suppression of proline synthesis by DNA methylation changes at one or more loci concerned. This concept receives strong support from the present study, in which we show that treatment of CHO-K1 cells with 5-azacytidine induces a 10^5–10^6 increase in background conversion to the proline-independent state. Revertants thus obtained, as well as those arising spontaneously or after treatment with ethyl methane sulfonate, are stable phenotypically in the presence or absence of proline. Proline independence in all variants examined was correlated with increased activity of pyrroline-5-carboxylate synthase. Four of five variants induced with 5-azacytidine showed simultaneous increases in activity of ornithine aminotransferase as well. Our data suggest that epigenetic, rather than genetic changes, underlie the transitions between proline dependence and independence in CHO-K1 cells.*

INTRODUCTION

The requirement of CHO-K1 Chinese hamster cells for exogenous proline is well-known (1, 2) and has generally been regarded as a prototype for auxotrophic mutations in mammalian cells (3–6). Several properties of this naturally occurring marker are consistent with a mutational origin. Proline dependence in CHO-K1 cells is stable phenotypically, and spontaneous reversions to proline independence as measured by fluctuation tests occur at a frequency of less than 10^{-7} (7). The incidence of revertants can be increased somewhat by exposure to mutagens (8, 9), and *pro*$^-$ and *pro*$^+$ cells may be distinguished by assays for proline synthesis in cell extracts (2). On this basis Puck and others (3, 4, 7, 8, 10) have maintained that proline deficiency in the CHO-K1 line is the result of a single gene mutation and that reversion is likewise implemented by genetic change.

In the absence of direct proof, an alternative hypothesis must be considered, namely that the proline requirement in these cells represents a loss in gene expression rather than gene mutation. Such repression could stem from DNA hypermethylation at one or more loci involved in proline synthesis. Studies with 5-azacytidine (5-aza-CR) have been used to probe the association between methylation changes and stable phenotypic alterations in mammalian cells (11, 12). Activation of single loci by 5-aza-CR has been linked to decreases in DNA methylation in studies on the metallothionein gene (*MT-1*) in W7 mouse thymoma cells (13), and in reexpression of the herpes *tk* gene from an inactive

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state in mouse LTK$^-$ cells (14, 15). That these epigenetic conversions are not unique is indicated by related studies with other markers. Thus, treatment with 5-aza-CR causes massive shifts in thymidine kinase-deficient Chinese hamster (16) and mouse (17) cells to the tk^+ state, conversion of glutamine and asparagine auxotrophs to prototrophs (18, 19), and activation of prolactin synthesis in rat pituitary tumor cells (20). In the present experiments we show that proline-deficient CHO-K1 cells can similarly be converted in high frequency to proline independence by 5-aza-CR, with simultaneous increases in activity of two enzymes involved in proline biosynthesis. Our results support the suggestion of Riggs and Jones (21) that stochastic changes in DNA methylation patterns may lead to heritable epigenetic changes, which can then masquerade as gene mutations.

MATERIALS AND METHODS

Cell Lines and Culture Procedures. CHO-K1 cells used for this study were obtained originally from the American Type Culture Collection, Rockville, Maryland, as were the DON Chinese hamster cells used for comparison in enzyme studies. CHO-2 is a clonal subline of CHO-K1, isolated without selection, and V79-56 is subclone from stock V79 cells, auxotrophic for glutamine (19). Stock cells of each line were maintained as monolayers in 10% fetal calf serum plus 90% alpha modification of Eagle's minimum essential medium (10FCS-α-MEM). Ribosides and deoxyribosides were omitted, and the concentrations of glucose, bicarbonate, and glutamine adjusted to 4.5 mg/ml, 3.7 mg/ml, and 600 μg/ml, respectively. For selection of proline-independent (pro^+) variants, cells were grown in 10% dialyzed fetal calf serum plus 90% Dulbecco's modification of Eagle's nutrient (PRO$^-$ medium). The serum was dialyzed with agitation in 50-ml aliquots for three days at 4°C with seven changes of Hanks' saline, sterilized by filtration, and frozen until needed. The PRO$^-$ medium was completed by supplementation with pyruvate, L-alanine, L-asparagine, L-aspartic acid, and L-glutamic acid to the same concentrations as in α-MEM. Petri dish cultures were maintained in a humidified CO_2 incubator with fluid changes after five days, and once or twice a week subsequently as needed. When colonies were well-formed, experiments were terminated by staining the cultures for 30 min in a saturated solution of crystal violet in 0.85% NaCl, after which the dishes were washed in tap water and air dried.

Induction and Isolation of Variants. All pro^+ revertants were isolated as well-defined colonies in PRO$^-$ medium, using stock CHO-K1 populations. Variants were obtained in some cases by treatment of CHO-K1 cultures for 24 h with ethyl methane sulfonate (EMS) at 300 μg/ml in 10FCS-α-MEM. After three days of recovery in 10FCS-α-MEM the cells were assayed for colony formation in PRO$^-$ medium. In other experiments log phase cells for treated for 24 h with graded concentrations of 5-aza-CR in 10FCS-α-MEM, rinsed two times with 10FCS-α-MEM, and allowed to recover for two days in this nutrient before assays in PRO$^-$ medium. A similar protocol was followed for induction studies in 24-hour exposures of CHO-K1 cells to graded concentrations of ara-C and hydroxyurea. Proline-independent variants of all types were isolated initially in PRO$^-$ medium, after which they were transferred to 10FCS-α-MEM for serial culture, and aliquots were frozen for further study. Each pro^+ clone was also tested for normal growth and plating efficiency in PRO$^-$ medium supplemented by 50 μg/ml L-proline, a nutrient in which stock CHO-K1 auxotrophs proliferate freely. This procedure eliminates the effect of unidentified growth factors on the growth of induced or uninduced cells.

Preparation of Cell Homogenates. Log-phase cells were inoculated into 100-mm petri dishes at 2.0–3.0 $\times$ 10^6 in 10FCS-α-MEM and harvested at two days to yield 7–10 10^7

cells. The cultures were rinsed two times with cold Dulbecco's phosphate-buffered saline (PBS) and the cells gently scraped into fresh PBS and centrifuged 10 min at 1000*g* in a refrigerated centrifuge. This procedure was repeated and the final pellet redispersed gently into 1.2 ml ice-cold 0.1 M KH_2PO_4, pH 7.4. The cells were disrupted by freezing in a CO_2–methanol bath and thawing twice. Aliquots of the final homogenate were frozen for protein analysis and the remainder used for enzyme assays on the same day. Protein determinations were made by the procedure of Bradford (22), using bovine plasma gamma globulin as a standard, and reagents obtained from BioRad Laboratories.

Assays for Pyrroline-5-Carboxylate Synthase. Pyrroline-5-carboxylate synthase (P5C synthase) activity in cell homogenates was determined by the procedure of Lodato et al. (23), which incorporates corrections into the method as originally described (24). In this procedure labeled glutamate is converted to pyrroline-5-carboxylate (P5C) and linked to *O*-aminobenzaldehyde (OAB), after which the P5C–OAB complex is separated from other compounds on an ion-exchange column. All assays for P5C synthase were performed with [^{14}C]glutamate (Amersham, 280 mC/m mol), using 1.0 μCi per sample. A standard assay mixture of 250 μl contained 0.1 M KH_2PO_4, pH 7.4, 5 mM ATP, 14.5 mM creatine phosphate, 10 mM DL-isocitrate, 200 mM L-proline, 0.4 mM NADPH, 25 mM $MgCl_2$, 1.0 mM 2-mercaptoethanol, 0.125 mg *O*-aminobenzaldehyde (OAB), 2 units creatine phosphokinase, 0.9 units isocitrate dehydrogenase, and about 200–400 μg cell protein. Samples were incubated on a shaker for 60 min at 37°C, and the reaction terminated by addition of 50 μl 6 N HCl containing 30 mg/ml OAB. The tubes were incubated an additional 5 min at 37°C, sedimented in an Eppendorf centrifuge, and the pellets discarded. Fractionation of the supernatants was performed with 8-mm disposable polypropylene columns (Kontes) containing 3 ml bed volume AG50W cation-exchange resin (200–400 mesh, 8% cross-linked hydrogen form). Aliquots of 200 μl supernatant were placed on columns and washed with 25 ml 2 N HCl. The OAB–P5C condensation product was then eluted as a sharp peak with 6 ml 2 N NaOH, and fractions collected in scintillation vials. Aquasol (15 ml/vial) was added and the vials counted in a Beckman LS 7000 liquid scintillation system. All samples were assayed in duplicate and blanks were run with homogenate denatured by OAB in HCl before incubation.

Assays for Ornithine Aminotransferase. Ornithine aminotransferase (OAT) activity was determined by measuring the conversion of labeled ornithine to pyrroline-5-carboxylate (25). Standard reaction mixtures of 200 μl contained 0.1 M KH_2PO_4, pH 8.0, 0.7 mM L-ornithine, 0.7 mM α-ketoglutarate, 0.4 μg/ml pyridoxal phosphate, 0.5 μCi [^{14}C]ornithine hydrochloride (Amersham, 280 mCi/m mol), and about 200–400 μg cell protein. Samples were incubated on a shaker for 30 min at 37°C and the reaction terminated by the addition of 50 μl 6 N HCl containing 30 mg/ml OAB. After an additional incubation at 5 min at 37°C, the samples were centrifuged and the supernatants fractionated on AG50W columns as described above for P5C synthase. All samples were assayed in duplicate and blanks were run with α-ketoglutarate omitted from the reaction mixture.

RESULTS

Induction of Proline-Independent Variants. The background frequency of proline-independent variants in stock populations of CHO-K1 cells was found to be approximately 10^{-7} when stock populations were maintained in PRO$^-$ medium with adequate fluid changes (Table 1). Since this nutrient contains only one of the precursors (glutamic acid) utilized by animal cells for the biosynthesis of proline (Fig. 1), we also determined the incidence of *pro*$^+$ variants in PRO$^-$ medium supplemented

Table 1. Induction of Proline-Independent Variants from CHO-K1 Cells

Treatment[a]	Expression time (days)	Selective medium	No. of cells plated	*pro*$^+$ colonies	Plating efficiency[b]	Colonies per 100 surviving cells
None	—	PRO$^-$	17.8×10^6	5	96.7	1.2×10^{-5}
		PRO$^-$ + 1mM L-ornithine	3.6×10^6	1	101.0	2.7×10^{-5}
EMS 300 μg/ml	3	PRO$^-$	2.1×10^6	8	44.3	8.5×10^{-4}
5-aza-CR	0	PRO$^-$	60,000	12	25.6	0.078
1.0 μg/ml	1		6,000	106	32.3	5.5
	2		6,600	155	30.7	7.7
	3		6,600	140	32.8	6.5
	7		6,600	366	38.2	14.5
2.0 μg/ml	2	PRO$^-$	6,000	147	40.0	6.1
2.0 μg/ml +3.0 mM L-ornithine	2	PRO$^-$ + 3 mM L-ornithine	6,000	196	40.3	8.1

[a]Log-phase cells were inoculated as a mass population in prescription bottles for 24 h in 10FCS-α-MEM containing inductors as shown. Cultures were then rinsed 2× with 10FCS-α-MEM and incubated for various periods (expression time) before plating in selective medium.

[b]Plating efficiencies were determined by inoculating cells from each population (3–6 petri dish cultures at 100 cells) in selective medium supplemented by addition of 50 μg/ml L-proline. Values shown represent average colony counts per 100 cells.

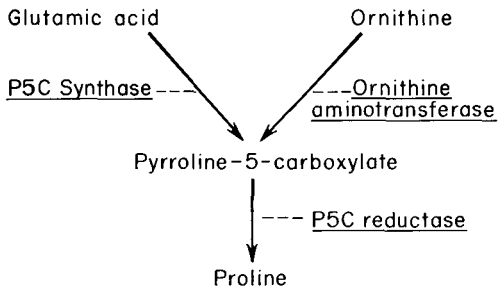

Fig. 1. Biosynthesis of proline in mammalian cells.

with 1 mM L-ornithine. As shown in Table 1, the frequency of spontaneous variants was not significantly different in the two media.

In agreement with earlier reports (8, 9) treatment of CHO-K1 cells with ethyl methane sulfonate (EMS) increased the occurrence of proline-independent variants. Thus, exposure to EMS at 300 μg/ml for 24 h resulted in a 70-fold elevation in *pro*$^+$ colonies. While this increase is clear-cut, the potential for reversion to proline independence is in fact much greater. This is shown by the high-frequency induction of *pro*$^+$ variants which takes place when CHO-K1 cultures are treated for 24 h with 5-aza-CR. Under optimal conditions, increases of 10^5–10^6 over background levels can be routinely obtained with a single exposure (Fig. 2). Other studies show that expression of proline independence after exposure to 5-aza-CR is progressive, rising sharply during the first day in culture and remaining thereafter at high levels (Table 1). The dose–response curve for a standard expression time of two days (Fig. 2) is strongly concentration dependent. The pattern obtained is essentially identical, qualitatively and quantitatively, to those previously described for induction of thymidine kinase and asparagine synthetase with 5-aza-CR (16, 18).

Induction of proline-independent variants by 5-aza-CR, as in the systems mentioned above, is associated with growth inhibition and a decline in plating efficiency. In the experiments shown in Fig. 2, the plating efficiency after two days' expression time

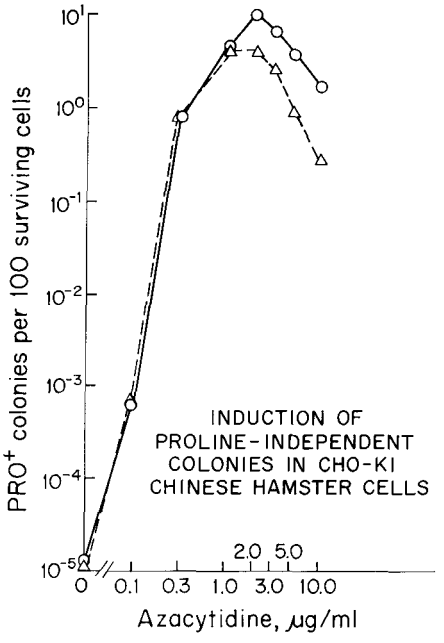

Fig. 2. Induction of proline-independent variants by 5-aza-CR in stock CHO-K1 cells, O; and in CHO-2, △, subclone of CHO-K1. All populations were exposed to 5-aza-CR for 24 h and were allowed to recover in 10FCS-α-MEM for two days before selective plating in PRO$^-$ medium. Average counts for pro$^+$ colonies are shown as a function of the number of surviving cells (three to six petri dish cultures per point).

decreased from 100% in controls to about 30% for cells exposed to 5-aza-CR at 1.0–10.0 μg/ml. The inductive action of 5-aza-CR on these cells, however, is not a generalized effect common to S-phase inhibitors. Thus, 24 hr treatment of CHO-K1 cultures with araC (0.1–1.0 μg/ml) and with hydroxyurea (3.0–30.0 μg/ml) produced similar growth inhibition and declines in plating efficiency, but failed to elevate the frequency of *pro*$^+$ colonies in survivor populations above background levels (data not shown).

Although conversion to proline independence is greatly increased by 5-aza-CR, it was not clear whether the transition was a stable one. In an initial test, five *pro*$^+$ clones induced by 5-aza-CR were isolated from single colonies in PRO$^-$ medium and were divided into paired sublines. These were maintained for two serial passages (10–12 doublings) as mass

populations in PRO$^-$ medium, with and without the addition of 50 μg/ml L-proline. The relative plating efficiency of each subline was then determined in the presence and absence of L-proline, respectively. No significant differences were observed between the two sublines (Table 2). Subsequently, all pro$^+$ clones were grown serially for 7–11 additional passages in proline-containing medium (10FCS-α-MEM) in order to conduct biochemical studies. During this period each clone retained the ability to form colonies in PRO$^-$ medium, in correlation with altered enzymatic patterns to be described (see below). Our data indicate that proline independence, once acquired, is a stable phenotypic change, unaffected by the presence or absence of proline in growth or plating media.

Enzymatic Changes in Proline-Independent Variants. Animal cells in culture are known to synthesize proline from both glutamic acid and ornithine (26, 27), although the relative contribution of these pathways varies among cell types (9). In earlier studies with CHO-K1 cells, conversion of glutamic acid to proline was found to be blocked, and although the pathway from ornithine remained open, the amount of proline thus produced was insufficient to support growth and survival (2, 9, 28). The basis for this double deficiency has never been explained. But since the addition of pyrroline-5-carboxylate to proline-deficient media was shown to restore growth and colony-forming potential in CHO-K1 cells (2), defects must reside in one or both pathways leading to this common intermediate (Fig. 1). That both may be involved is suggested by reports of proline-independent variants from CHO-K1 cells that are dependent on elevated levels of P5C synthase (24) or OAT activity (25), respectively.

Table 2. Phenotypic Stability of Proline-Independent Variants Induced by 5-Azacytidine

Cell line[a]	Maintenance medium[b]	Plating efficiency per 100 cells[c]	
		+Pro	−Pro
CHO-K1	+Pro	102.3	$<1.2 \times 10^{-5}$
CHO-2	+Pro	86.8	$<1.2 \times 10^{-5}$
1554-1	−Pro	47.3	65.0
	+Pro	66.7	58.0
1554-2	−Pro	74.0	73.3
	+Pro	87.7	89.0
1554-3	−Pro	47.0	49.0
	+Pro	57.7	51.0
1554-4	−Pro	91.3	65.0
	+Pro	103.7	102.7
1554-5	−Pro	101.0	95.0
	+Pro	100.7	108.3

[a]CHO-2 is a subclone of CHO-K1, isolated in nonselective medium. 1554-1 to 1554-5 are proline-independent clones isolated from CHO-K1 cultures after treatment with 5-aza-CR.

[b]Populations of variant cells were divided into sublines which were cultivated for two passages in the presence or absence of proline, prior to tests for plating efficiency.

[c]Plating efficiencies for 1554 sublines were determined by inoculating cells into media with or without proline (3 petri dish cultures at 100 cells per series). Values shown represent average numbers of colonies per 100 cells plated. Plating efficiencies for CHO-K1 and CHO-2 in −Pro medium are based on inoculation of 9 petris each at 10^5–10^6 cells.

As part of the present study, we examined activities of P5C synthase and OAT in stock cell lines, and in proline-independent variants arising spontaneously from CHO-K1 or by induction with EMS and 5-aza-CR. Table 3 and Figs. 3 and 4 summarize the data obtained for P5C synthase. All *pro*$^+$ variants, regardless of origin, showed enhanced levels of activity when compared to parental CHO-K1 cells. P5C synthase activity in V79-56 or DON cells, however, was substantially greater. One possible explanation is that a single allele for P5C synthase has been activated in *pro*$^+$ revertants, while both alleles are functional in wild-type cells. Alternatively, both alleles may be activated in spontaneous or induced revertants, but are not expressed at as high a rate or efficiency as in the prototrophic DON or V79 cells.

OAT activity in the same assemblage of cell types presents a more complex picture (Table 4 and Figs. 5 and 6). Valle et al. (28) reported that CHO-K1 cells have a basal OAT activity which is approximately 10% of wild-type levels, and our measurements for CHO-K1 cells are consistent with this finding.

Table 3. P5C Synthase Activity in Chinese Hamster Cell Lines

Cell line	Origin	Inducing agent[a]	Peak counts, P5C–OAB, from [^{14}C]glutamic acid (cpm/μg protein)[b]
V79-56	WT		28.4
DON			21.7
CHO-K1			1.3
1851-1	Revertant, CHO-K1	Spontaneous	11.2
1860-1			8.6
1554-1	Revertant, CHO-K1	5-aza-CR	13.9
1554-3			8.5
1554-4			11.3
1554-5			10.2
1898-1	Revertant, CHO-K1	EMS	9.8
1898-2			18.0
1898-3			12.8
1898-4			11.0

[a]Treatment with 5-aza-CR: 2.0 μg/ml for 24 h; with EMS: 300 μg/ml for 24 h. All variants isolated in PRO$^-$ medium except for 1860-1, where the selective medium was supplemented with 1.0 μg/ml L-ornithine HCl.
[b]Total counts incorporated in P5C–OAB from [^{14}C]glutamine acid, as eluted with 6 ml 2 N NaOH. All values shown are averages for two or more assays performed in duplicate.

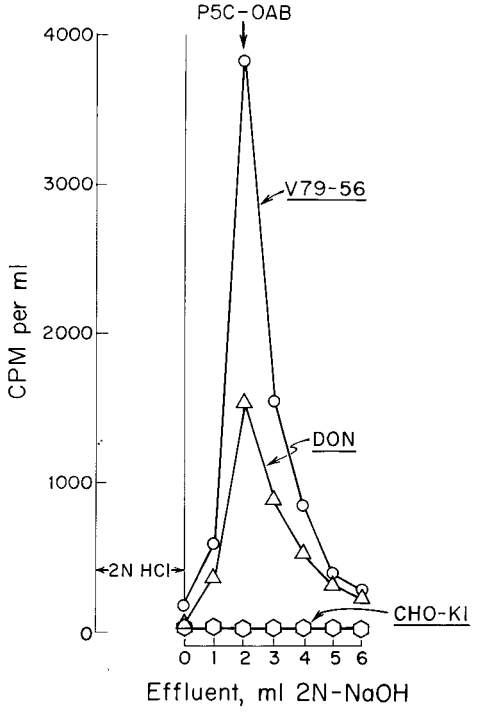

Fig. 3. P5C synthase activity (peak counts of [^{14}C]glutamic acid incorporated into P5C–OAB) in stock cell lines. V79-56, O; DON, △; CHO-K1, ○ . Protein per sample: V79-56, 415 μg; DON, 273 μg; CHO-K1, 273 μg.

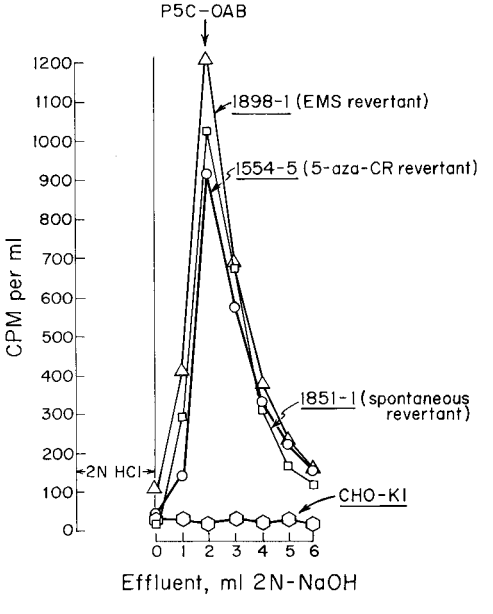

Fig. 4. P5C synthase activity (peak counts of [^{14}C]glutamic acid incorporated into P5C-OAB) for CHO-K1 and proline-independent sublines. 1851-1 (spontaneous revertant), □; 1554-5 (5-aza-CR) revertant), O; 1898-1 (EMS revertant), △. Protein per sample: 1851-1, 345 μg; 1554-5, 490 μg; 1898-1, 390 μg. CHO-K1 values (from Fig. 2) are shown for comparison.

Table 4. Ornithine Aminotransferase Activity in Chinese Hamster Cells

Cell line	Origin	Inducing agent[a]	Peak counts, P5C–OAB from [^{14}C]ornithine HCl (cpm/μg protein)[b]
V79-56	WT		177.5
DON			112.7
CHO-K1			16.5
CHO-2	Subclone of CHO-K1		21.9
1851-1	Revertant, CHO-K1	Spontaneous	17.2
1860-1			13.4
1898-1	Revertant, CHO-K1	EMS	14.6
1898-2			21.8
1898-3			21.4
1898-4			16.9
1554-1	Revertant, CHO-K1	5-aza-CR	97.0
1554-2			28.2
1554-3			57.1
1554-4			68.1
1554-5			45.3

[a]Revertants were induced by 300 μg/ml EMS for 24 h with a three-day expression time, and by 2.0 μg/ml 5-aza-CR with a two-day expression time. Revertants were isolated as clones in PRO$^-$ medium (supplemented for 1860-1 with 1.0 mM ornithine).

[b]Counts eluted with 6.0 ml 2 N NaOH from AG50W columns. Values shown are based on two or more independent determinations, each performed in duplicate.

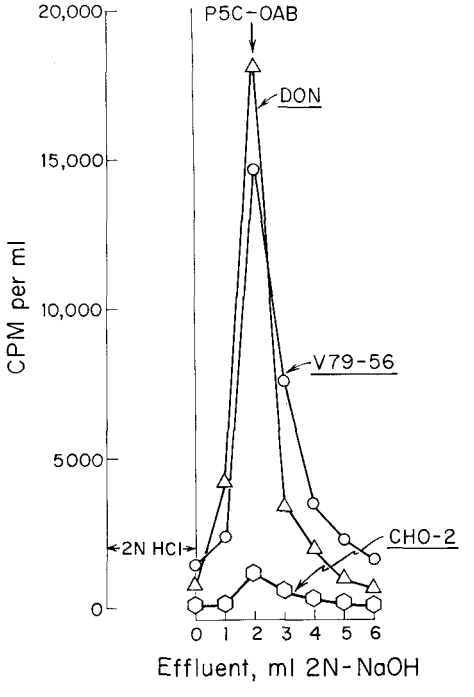

Fig. 5. Ornithine aminotransferase activity (peak counts of [^{14}C]ornithine HCl incorporated into P5C-OAB) in stock cell lines. V79-56, O; DON, △; CHO-2 (subclone of CHO-K1), ○ . Protein per sample: V79-56, 408 μg; DON, 530 μg; CHO-2, 313 μg.

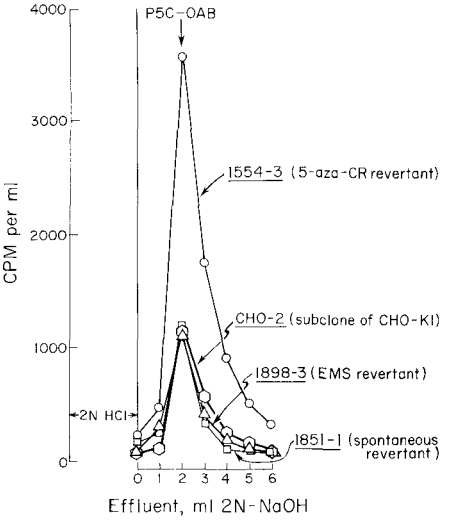

Fig. 6. Ornithine aminotransferase activity (peak counts of [^{14}C]ornithine HCl incorporated in P5C-OAB) for CHO-2 (subclone of CHO-K1) and proline-independent sublines obtained from CHO-K1. 1851-1 (spontaneous revertant), □; 1898-3 (EMS revertant), △; 1554-3 (5-aza-CR revertant), O. Protein per sample: 1851-1, 345 μg; 1898-3, 250 μg; 1554-3, 338 μg. CHO-2 values (from Fig. 4) are shown for comparison.

Values for subclone CHO-2 were essentially unchanged, indicating that the basal OAT activity in CHO-K1 cells is not to be explained by accumulation of spontaneous *pro*$^+$ revertants in stock populations. Neither of two spontaneous *pro*$^+$ revertants tested, isolated in the presence and absence of ornithine, respectively, showed any increase in OAT activity over the CHO-K1 background level. Similarly, four *pro*$^+$ clones induced with EMS displayed levels of OAT activity indistinguishable from CHO-K1 parent cells. In contrast, OAT activity was significantly enhanced in four of five *pro*$^+$ clones obtained with 5-aza-CR, with one clone unresponsive after inductive treatment. It should be emphasized that all five of the 5-aza-CR clones also exhibited elevated levels of P5C synthase activity (Table 3). Thus, simultaneous activation of these enzymes occurs frequently after 5-aza-CR treatment, a phenomenon not previously reported for *pro*$^+$ revertants in CHO-K1 cells.

DISCUSSION

A central question in this work is whether transitions between proline dependence and independence in CHO-K1 cells are based on genetic changes or on stable shifts in gene expression. Fluctuation tests (7) show that *pro*$^+$ revertants arise by spontaneous and random changes, but do not reveal the nature of the underlying process. While we have shown that 5-aza-CR induces high–frequency conversion to proline independence, it is difficult to account for this effect on a genetic basis. The data of Landolph and Jones (29) suggest, in fact, that 5-aza-CR has little if any mutagenic activity on mammalian cells in conventional test systems. On the other hand, the ability of 5-aza-CR to cause changes in gene expression is well-established (11, 21). Thus, it appears probable the action of 5-aza-CR is epigenetic, involving DNA hypomethylation at one or more control sites in loci responsible for proline biosynthesis. Low-level induction of *pro*$^+$ variants by EMS could conceivably also be mediated by methylation changes, although the intermediate stages remain to be defined. A possible link may be found in the observations of Wilson and Jones (30), which show that EMS and other alkylating agents can directly inhibit DNA methylation reactions in vitro. Alternatively, methylation changes may arise during the repair of DNA damaged by exposure to EMS. It has been shown that DNA methylation in repair patches is slow and incomplete, which raises the possibility of heritable loss of methylation at some sites in the surviving cells (31).

The requirement for proline in CHO-K1 cells has usually been attributed to a lesion in the conversion of glutamic acid to pyrroline-5-carboxylate (2–4). It has become evident, however, that reversions to proline independence may involve either the glutamic acid or ornithine pathways, or both. In epigenetic terms, these data suggest that spontaneous or induced derepression of the alleles for P5C synthase and OAT may occur at different rates, with the frequency and type of variants dependent on the probability of DNA methylation changes at the loci concerned. Evidently the probability is greatest for the activation of P5C synthase, since *pro*$^+$ variants of all types in the present study showed elevated P5C synthase activity. Within the limited number of revertants we examined, increases in OAT activity were observed only in variants induced with 5-aza-CR, and none of these showed increased OAT activity alone. However, one such variant has been described by Smith and Phang (25), obtained from CHO-K1 cells after EMS treatment. The variant cells had high OAT activity, but were unable to convert labeled glutamic acid to proline. Increased activity in both glutamic acid and ornithine pathways has been reported for a *pro*$^+$ variant obtained by Baich (9) with EMS from CHO-K1 cells. In the present study, dual increases were a conspicuous feature in four of five *pro*$^+$ variants induced by 5-aza-

CR. It remains to be seen whether simultaneous activation of P5C synthase and OAT at high frequency by 5-aza-CR can be explained on a purely stochastic basis, or whether the coordinate response, as in the reactivation of X-chromosomes (32–35), may be based on a broader pattern of inductive changes.

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