GENETICS OF SOMATIC MAMMALIAN CELLS. IV. PROPERTIES OF CHINESE HAMSTER CELL MUTANTS WITH RESPECT TO THE REQUIREMENT FOR PROLINE[1]

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Received October 17, 1966

WHILE the availability of the single cell plating technique has facilitated study of some aspects of the genetic biochemistry of mammalian cells *in vitro* (PUCK and FISHER 1956; TJIO and PUCK 1958; PUCK, CIECIURA and ROBINSON 1958), others like mutagenesis and recombination have progressed slowly, largely because of the difficulty in securing adequate genetic markers. In contrast to the abundance in microorganisms of many stable markers such as those involving nutritional deficiency and virus-, drug- and radiation-resistance, the markers available for genetic studies in mammalian cells have been limited in number; their expression is often variable from day to day; and the spontaneous reversion rate is often so high as to make the resolving power of genetic experiments much less than that attainable with fungi, bacteria and bacteriophage.

A program was begun in this laboratory to attempt isolation of nutritional markers in mammalian cells cultivated *in vitro*. To this end, the molecular requirements for single-cell plating were first defined for the S3 HeLa cell (FISHER, PUCK and SATO 1959). However, the high degree of chromosomal instability and polyploidy characteristic of this heteroploid cell line made it disadvantageous for genetic experiments. Consequently, attention was turned to the Chinese hamster cells which had been introduced into *in vitro* culture in this laboratory (PUCK, CIECIURA and ROBINSON 1958). While these cells also have a small degree of variability in chromosome number presumably due to spontaneous lag or nondisjunction, the modal number is usually diploid and relatively constant, and karyotypic analysis is possible with only small uncertainty, at least as compared with the HeLa or similar cells. Moreover, the low chromosome number of Chinese hamster cells makes karyotype analysis feasible and their rapid growth (10-hour generation time) is favorable for experimentation.

A defined medium for single cell plating of Chinese hamster cells was developed (HAM 1962). One subclone derived from a Chinese hamster ovary which had

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been routinely cultivated for several years was subsequently found to require proline for growth (HAM 1963) and to be capable of spontaneous reversion to proline independence (HAM, unpublished). The present paper reports further studies of this system.

MATERIALS AND METHODS

All procedures were as described earlier (HAM and PUCK 1962) except where changes have been indicated. The basal medium for single-cell growth was HAM's F12 (HAM 1965) from which linoleic acid and proline were omitted; this was supplemented, in order to accelerate growth, with 1.5 mg/ml of the macromolecular fraction of fetal calf serum, prepared by passage over a coarse-grade, Sephadex G-50 column. (Either purified fetuin plus albumin, or total fetal calf protein can serve as the macromolecular supplement, but the latter is easier to prepare.) Plates with single cells were incubated for 5 to 7 days in electronically controlled CO_2 incubators

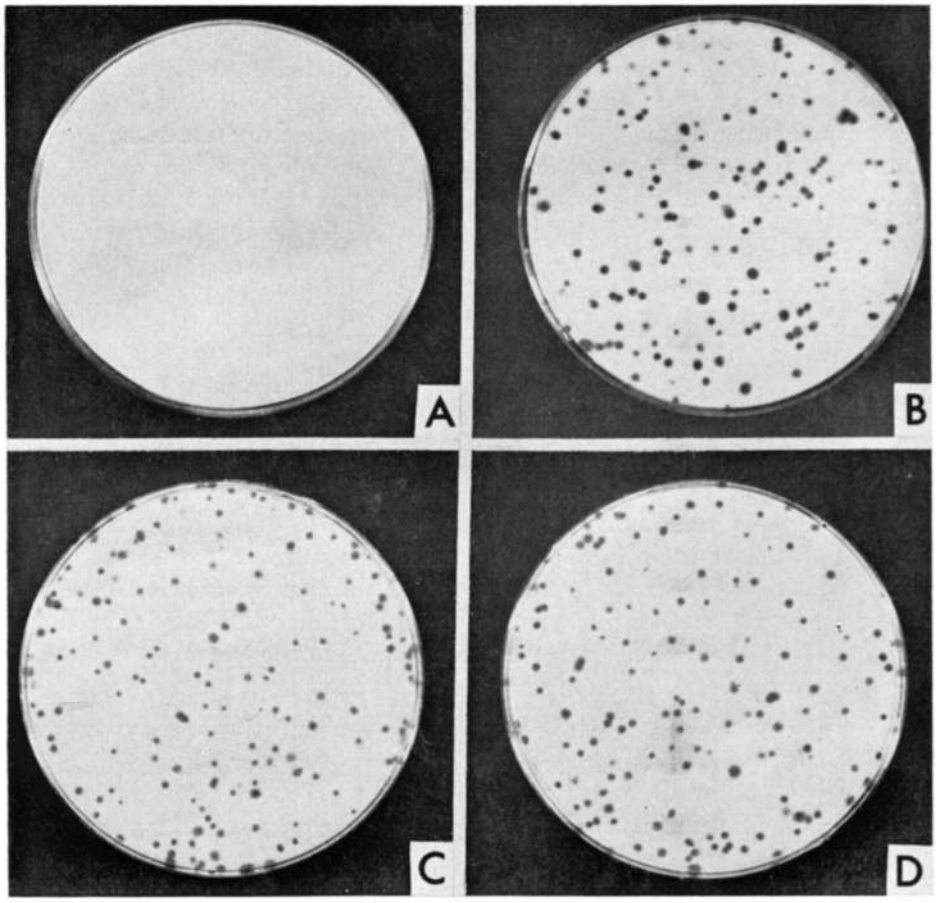

FIGURE 1.—Difference in growth responses to proline of CHO Pro$^-$ and CHL Pro$^+$ cells. Two hundred cells were plated in each case, and all plates were incubated seven days. A. CHO Pro$^-$ cells; no proline; B. CHO Pro$^-$ cells; 3×10^{-4} M proline; C. CHL Pro$^+$ cells; no proline; D. CHL Pro$^+$ cells; 3×10^{-4} M proline.

(HAM and PUCK 1962). All cell strains were routinely farmed in complete F12 supplemented with 2% of pretested fetal calf serum.

The Chinese hamster ovary cell used is one of a series of sub-clones of a culture which was first isolated in 1957 (PUCK, CIECIURA and ROBINSON 1958). These subclones have been variously designated as the Chinese hamster ovary culture, CHBOCIDI, CHO, CHD-3 or CHD-3A (PUCK) in previous publications (PUCK, CIECIURA and ROBINSON 1958; HAM 1962, 1963, 1965; PUCK, SANDERS and PETERSON 1964). The Chinese hamster lung cell CHL, was isolated here approximately two years later from another animal, an embryo presumably male.

RESULTS

Quantitation of the proline response: In Figure 1 is shown a typical set of platings demonstrating the dependence of the Chinese hamster ovary cell culture (CHO, Pro^-) on proline, in contrast to the independence of the Chinese hamster lung (CHL, Pro^+) culture. The data of Table 1 establish the following facts: (a) The maximum plating efficiency of CHO cells under optimum conditions is about 78%. (b) The plating efficiency for normal colony formation (defined as colonies with at least 50 cells (PUCK and MARCUS 1956)) is uniformly zero for all proline concentrations from zero up to 1×10^{-5} M. (c) For proline concentrations between 3×10^{-5} M up to about 1×10^{-2} M, the plating efficiency is constant and maximal. (d) For higher proline concentrations, the plating efficiency falls, approaching zero for concentrations beyond 0.10M. Similar experiments with the CHL cell demonstrated that the same maximum plating efficiency is obtained either in the absence of proline, or in its presence up to concentrations as high as 0.01M.

TABLE 1

Dependence of single cell growth of CHO Pro^- cells on proline concentration

Proline concentration (M)	Plating efficiency (%)	Colony size
0	0	0
1×10^{-7}	0	0
5×10^{-7}	0	0
1×10^{-6}	0	0
5×10^{-6}	0	0
1×10^{-5}	0	0*
2×10^{-5}	75	small
3×10^{-5}	79	normal
5×10^{-5}	74	normal
1×10^{-4}	75	normal
5×10^{-4}	78	normal
1×10^{-3}	77	normal
5×10^{-3}	77	normal
1×10^{-2}	74	normal
5×10^{-2}	70	small
1×10^{-1}	51	small
5×10^{-1}	0	0

* However, "micro" colonies containing less than 50 cells appear in a number corresponding to a plating efficiency of 25%.

Measurement of the growth rate of these two cell types was carried out by the life-cycle method described by PUCK and STEFFEN (1963) in which a collection function was established by counting accumulation of mitotic figures in the presence of colcemide. The generation time for the CHL cell is 10 to 14 hours regardless of the presence or absence of proline. The generation time for the CHO Pro$^-$ cell is infinite in the absence of proline, and decreases with addition of proline, ultimately reaching the same value as for the CHL cells at proline concentrations of 3×10^{-5}M or greater.

If the proline marker is to be useful for mutagenesis experiments of the simplest form, it is necessary to be able to plate Pro$^-$ cells in the absence of proline, and to have them remain viable for a reasonable period (i.e., at least a full generation period) so that mutations can be expressed. To determine the survival of CHO Pro$^-$ cells under these conditions, such cells were plated in the absence of proline, and incubated at 37°C. After various time intervals, proline was added to the plates to a concentration of 3×10^{-4}M. The plating efficiency as a function of the period of proline starvation is shown in Figure 2. The data indicate an initial shoulder followed by an exponential fall in reproductive ability in the

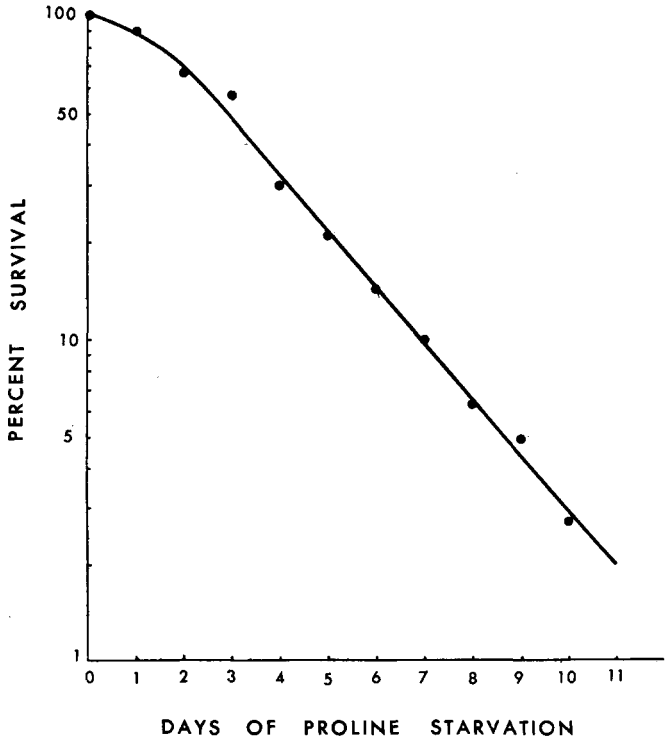

FIGURE 2.—Survival of colony-forming ability of CHO Pro$^-$ cells as a function of time of proline starvation. Cells were plated in complete medium minus proline, and 3×10^{-4} M proline was added after various periods of incubation. The absolute percent survival of these cells when proline was supplied from the very beginning is about 80%.

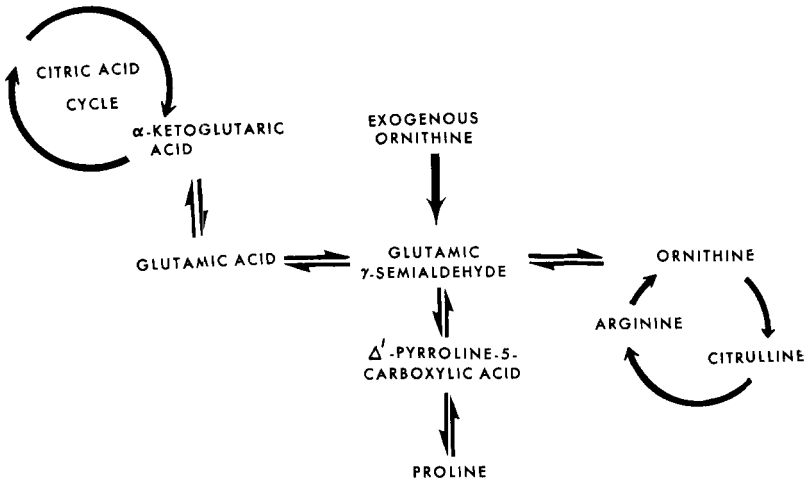

FIGURE 3.—Metabolic pathways involved in proline biosynthesis. While the general scheme has been demonstrated to be similar in *E. coli*, Neurospora, and mammalian systems, the reversibility of all the individual steps has not been demonstrated in all of these organisms. Also the acetylated pathways present in some microorganisms are not indicated.

absence of proline. After this initial shoulder, the number of viable cells decays with a half-life of 1.3 days. Thus, even after four generation-times, approximately 80% of the cells are still viable.

Biochemistry of the block in proline synthesis: We assume the validity of the relations between proline, glutamate, ornithine and arginine shown in Figure 3 which have been demonstrated in various organisms (STETTEN 1955; VOGEL 1953; VOGEL and BONNER 1954). A companion pathway involving the acetyl derivatives of ornithine, glutamate, and glutamic semialdehyde has been demonstrated in some microorganisms but not in mammals (MEISTER 1965). Nutritional experiments were carried out to locate the point of block in the proline-deficient mutant:

(a) Addition of ornithine to a medium lacking proline, produced definite growth of the CHO Pro$^-$ cells, but at a rate slower than maximal so that smaller colonies were produced by the standard plating procedure, as shown in Figure 4 in which the result of an addition of 0.01 M ornithine is presented. If 0.01 M ornithine is added to the basal medium supplemented with 1×10^{-5} M proline, an amount insufficient by itself to produce any visible growth, the colony size approaches that characteristic of optimal growth rate (Figure 4). It may be concluded that the pathway from ornithine to proline is intact in the deficient mutant, but does not supply sufficient proline to permit achievement of maximal growth rate.

(b) Addition of arginine in any concentration up to 0.01 M to a proline-free medium produced no visible growth. However, this arginine addition in the presence of 10^{-5} M proline did produce visible growth stimulation, though to a smaller extent than that achieved by ornithine under these circumstances. Again

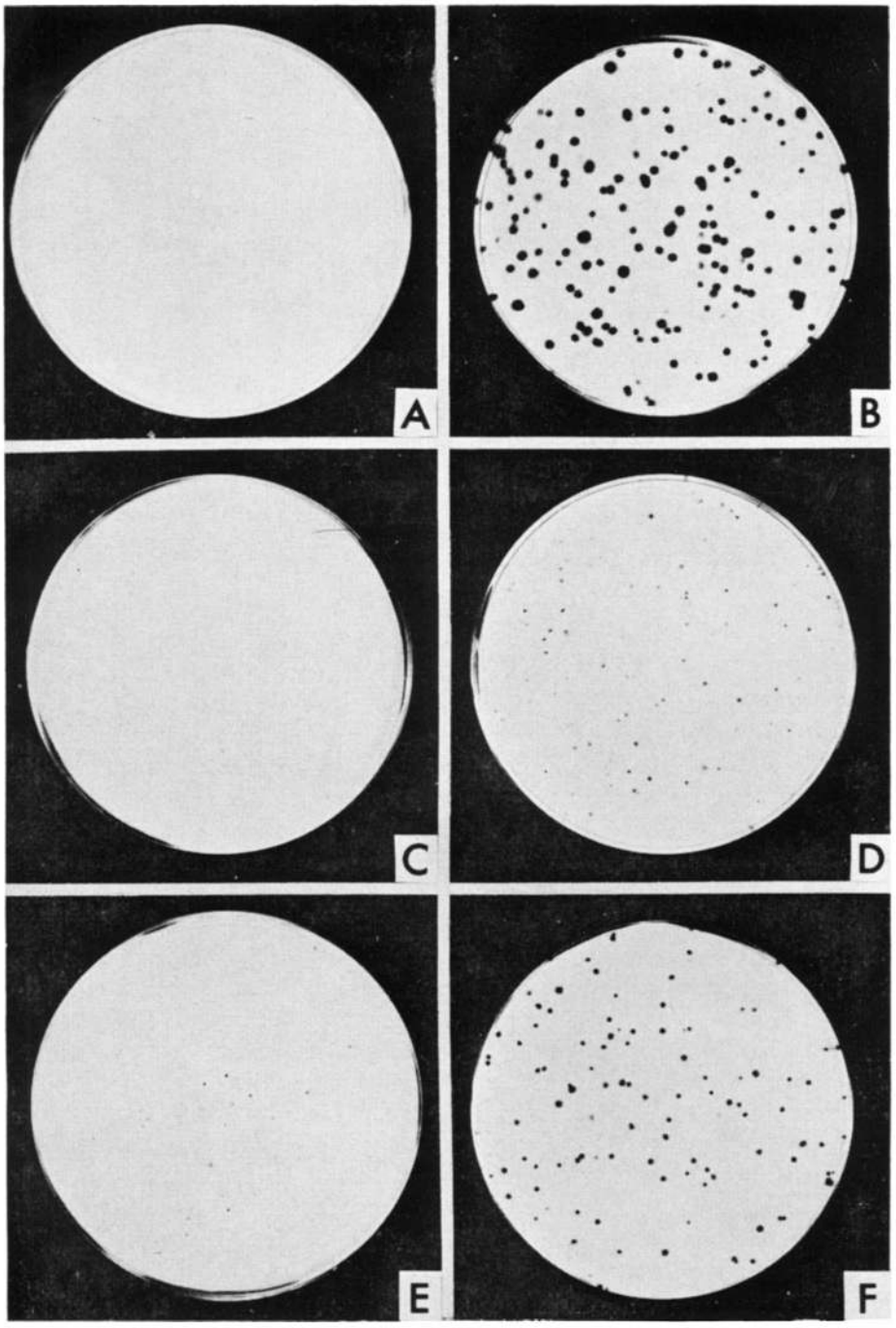

FIGURE 4.—Colony formation by 200 CHO Pro$^-$ cells in the standard proline-deficient medium supplemented with the following amino acids: A. 10^{-5} M proline; B. 3×10^{-5} M proline; C. 10^{-2} M arginine; D. 10^{-2} M arginine + 10^{-5} M proline; E. 10^{-2} M ornithine; F. 10^{-2} M ornithine + 10^{-5} M proline.

the conclusion appears tenable, subject to the validity of the assumptions noted, that the pathway from arginine to proline is open but permits less proline synthesis than when ornithine is used. Similar findings were obtained in Neurospora where it was demonstrated that endogenous ornithine dervied from arginine is less readily converted to proline than exogenous ornithine (VOGEL and BONNER 1954).

(c) As expected, addition of arginine to medium containing 0.01 M ornithine did not increase the plating efficiency or the growth rate. If either arginine or ornithine was added in concentrations exceeding a total of 0.01 M, alone or together, growth was suppressed.

The growth response produced by ornithine and arginine indicates that the steps of the pathway from ornithine to proline, via glutamic γ-semialdehyde are all intact. Therefore it can be concluded, subject to the reservations noted, that the block in the proline deficient mutant occurs in the conversion of glutamic acid (which is normally supplied in the medium) to the semialdehyde.

(d) As demanded by this formulation, Δ^1-pyrroline-5-carboxylic acid (which in solution spontaneously forms an equilibrium mixture with glutamic γ-semialdehyde) functioned as well as proline in promoting growth of the deficient mutant. This compound, kindly supplied by DR. H. STRECKER, produced maximal plating efficiency and growth rate at all concentrations between 5×10^{-5}M and 5×10^{-3}M.

(e) Omission of either or all of the following components of the medium did not affect the plating efficiency response of this cell strain to the presence or absence of proline, although the growth rate was usually somewhat reduced: glycine, alanine, thymidine, hypoxanthine, inositol, aspartic acid, vitamin B12, lipoic acid, glutamic acid, and 1,4-diamino butane.

Chromosomal analysis of the deficient mutant: This cell culture has a modal chromosome number of 21, which is fairly constant (Table 2). The karotype demonstrates a number of changes from the normal Chinese hamster cell. Analysis is now in progress in an attempt to identify the missing parts of the chromosomal complement. It seems a reasonable working hypothesis that the proline requirement is a result of loss of part of the chromosomal complement and mutation of the critical gene on the homologous chromosome.

TABLE 2

Distribution of chromosome numbers in CHO Pro⁻ cells

(A) Analysis of chromosome number distribution among the essentially diploid cells. (Counts were made on 90 well spread mitoses)

Chromosome number:	18	19	20	21	22	23	24
Percent of cells:	0	0	5.6	85.6	8.8	0	0

(B) Ploidy analysis:

	Diploid range	Tetraploid range
Number of cells:	272	15
Percent:	94.8	5.2

Rate of spontaneous reversion to proline independence: If the mutant form is the result of a gene mutation on a monosomic chromosome region, reversion should be demonstrable. Experiments were performed in which 10^5 Pro$^-$ cells were plated in a series of 60-mm plastic petri dishes containing 5 ml of the standard proline-deficient medium. The plates were incubated for seven days and colonies were picked and grown up into large cultures. Sixty-six such new cultures were established. To test whether these are indeed genetically different clones, they were replated in proline-free medium. All but two of the cultures were clearly true mutants, exhibiting maximal plating efficiency either in the presence or absence of proline just like the CHL Pro$^+$ cell. If a single mutated gene without complex regulatory phenomena is responsible for this reversion, one would expect the Pro$^+$ cells so produced to be able to make less proline than those of the CHL Pro$^+$ which contains 22 apparently normal chromosomes plus an additional unidentified member. Presumably, therefore, CHL Pro$^+$ is at least diploid with respect to the genetic determinants involved. Hence, one might expect the CHO Pro$^+$ revertants to display evidence of a smaller proline biosynthetic capacity than the CHL Pro$^+$ cells. In accordance with this expectation it was found that unlike the CHL Pro$^+$ cell, the CHO-Pro$^+$ revertants do not grow as rapidly in the absence of proline as they do in its presence. However, when added proline is furnished, these reverted CHO Pro$^+$ clones all grow as rapidly as any of the Chinese hamster cell lines in the laboratory. Preliminary chromosomal analysis of several revertants revealed these to be grossly similar to the parental CHO Pro$^-$ clone.

A total of 24 Pro$^+$ revertants was found in a series of nine cultures of Pro$^-$ cells in which 1.12×10^7 cells were tested. The average frequency of reversion estimated from these data by the median method of approximation was $(0.9 \pm 0.7) \times 10^{-6}$ (LEA and COULSON 1949).

Properties of the revertants: The revertants picked and tested are not all identical since while they all form colonies in the absence of proline, and display a stimulation of growth rate on addition of proline, small but demonstrable differences in growth rate under identical conditions exist among some of the selected clones.

In earlier studies, it was shown that an irradiated, nonreproducing feeder cell layer can supply needed nutrients to single cells of a nutritionally deficient culture plated on top of it (PUCK, MARCUS and CIECIURA 1956; FISHER and PUCK 1956). Experiments were carried out to determine whether the Pro$^-$ cell would show the expected differences when grown on feeder layers of irradiated cells of the different types here described, in the absence of proline. In a first series, Petri dishes were seeded with 2×10^5 CHL Pro$^+$ cells and then irradiated with 3000 rads of 230 kv X rays in the standard proline-free medium. Immediately afterward an inoculum of 200 CHO Pro$^-$ cells was plated in the same dishes. Two types of control plates were prepared, in which the irradiated feeder cells, or the unirradiated Pro$^-$ cells, were separately omitted. Invariably, colonies were obtained in the number closely equal to that of the Pro$^-$ cell inoculum, only when these cells and irradiated feeders were placed in the same

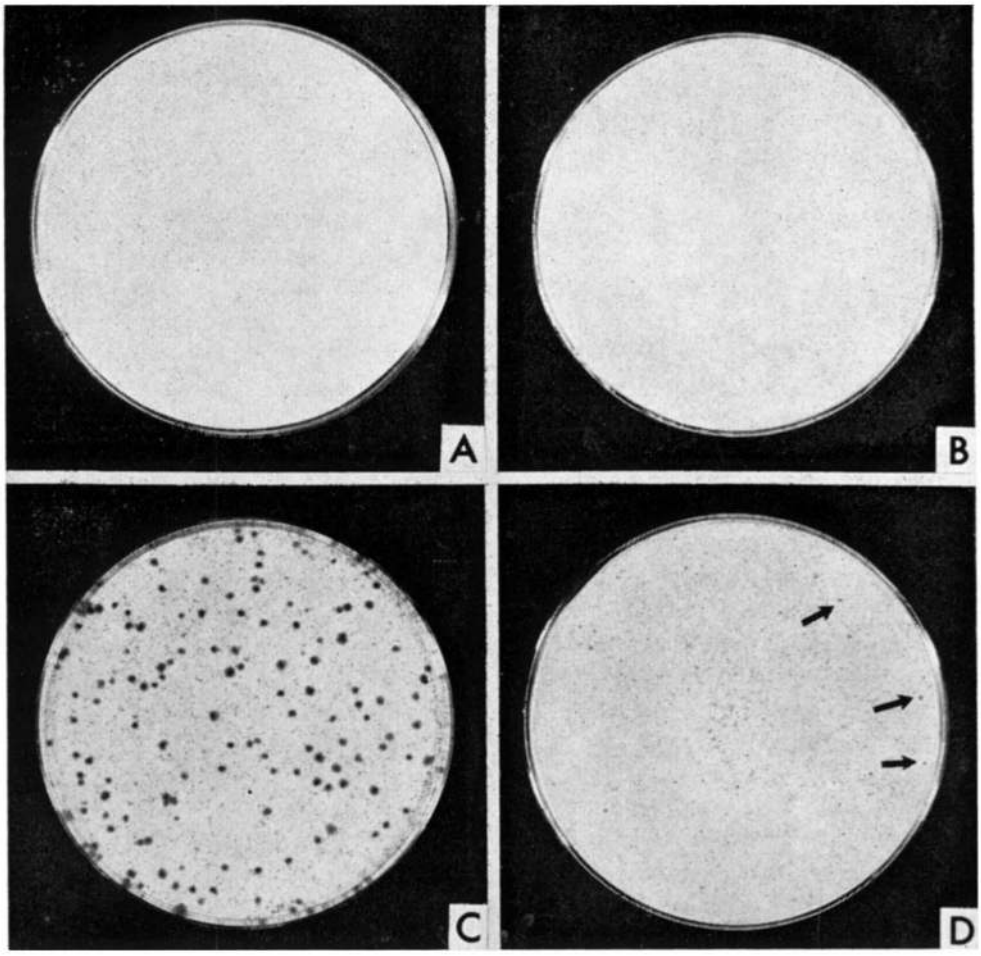

FIGURE 5.—Demonstration of the use of the feeder layer technique to differentiate between the various cell strains here described. All plates contain the proline-free medium. A. Control: 200 CHO Pro$^-$ cells alone. B. Control: Feeder layer of irradiated CHL Pro+ alone. C. 200 cells of CHO Pro$^-$ plus feeder layer of irradiated CHL Pro+. D. 200 cells of CHO Pro$^-$ plus feeder layer of irradiated CHO Pro+ revertant. The colonies are definite but smaller and usually fewer in number than in C.

plate (Figure 5), while both types of control plates uniformly displayed no colonies. In a second series, where irradiated CHO Pro$^-$ cells were used as a feeder layer, no growth was obtained from an inoculum of unirradiated CHO Pro$^-$ cells. Finally, when single cells of CHO Pro$^-$ were plated on top of an irradiated feeder layer of the CHO Pro+ revertant, intermediate behavior was obtained: some colony formation occurred, but with plating efficiency and colony size always less than that produced by a feeder layer of the CHL Pro+ cell. Thus, the feeder layer technique permits easy differentiation between different types of Pro+ cells described here (Figure 5).

Direct confirmation of the intermediate level of proline synthesis by the revertant clones has been obtained in current experiments measuring the amount of radioactive proline produced by cells fed with C^{14}-L-glutamic acid. At present writing, all of the four CHO Pro^+ revertants tested have demonstrated about half the rate of proline synthesis of the CHL Pro^+, while no synthesis was detected in the CHO Pro^-. (Table 3). Details of these experiments will be published later.

DISCUSSION

These experiments demonstrate a well behaved revertible mutation involving a simple biochemical growth factor, with a spontaneous frequency of revertants similar to that of single gene mutations in bacteria. Hence, genetic experiments with good resolving power should be possible with this marker. Experiments measuring mutation rates produced by various physical and chemical agents in this system are in progress.

Three kinds of Chinese hamster cell with clearly differing proline responses are described (Table 3). CHO Pro^- cells can synthesize little or no proline; the

TABLE 3

Aspects of growth behavior and biochemical properties of the three types of Chinese hamster cells here studied

	CHL-Pro^+	CHO-Pro^-	CHO-Pro^+ (Revertant cultures)
Plating efficiency in absence of proline	maximal	0	maximal
Growth rate in absence of proline	fast	0	slow
Minimum proline requirement for maximal growth	0	3×10^{-5} M	1×10^{-5} M
Growth stimulation by arginine or ornithine	0	+	0
Amount of radioactive proline synthesized from C^{14}-glutamic acid by a massive culture, relative to that produced by CHL-Pro^+ cells	100%	0	51 to 63% in four representative cultures
Ability to act as a feeder layer for CHO-Pro^- cells in a proline-free medium	maximal	0	intermediate

(Maximal plating efficiency is about 80%; fast growth rate represents a generation time of 12 to 14 hours; slow growth rate represents a generation time of 20 to 22 hours.)

Pro^+ revertants can make intermediate amounts, and the CHL Pro^+ can make maximal amounts. These data, together with the chromosome constitutions of the various cells involved, are consistent with the hypothesis that the CHO Pro^- has no active form of the gene required for proline synthesis; the various CHO Pro^+ revertants have one; and the CHL Pro^+ has two (or possibly even three). A similar relationship between gene multiplicity and cellular enzyme activity has been found in mammalian tissue culture cells for the galactose transferase system (PUCK 1966).

These experiments suggest that the chromosomal location of at least some mammalian genes could be determined by obtaining stable cultures of mammalian cells with specific monosomies, and testing them for the haploid condition of various genes. Such screening tests may include testing the ability of single cells to grow but at rates less than that of the wild type unless supplemented with specific metabolites not required by the wild type; testing the ability of such mutants to act as feeder layers for standard cell cultures with known nutritional deficiencies; and direct measurement of relative enzyme activities of different components of known, unbranched reaction chains by methods described previously (ROBINSON 1963). Genes on such monosomic chromosomes should be more readily mutated by various physical and chemical mutagens.

For such studies it becomes necessary to obtain clonal stocks of cells with particular chromosomal monosomies. Nondisjunctional events which occur spontaneously both in the mammalian body and in culture can serve as the basis for such stocks. In the present case, the cell as isolated from the animal had a modal chromosome number of 22, but on recloning, a culture with 21 chromosomes was isolated. Hence, a virtue resulted from the otherwise obnoxious tendency of mammalian cells to undergo nondisjunction. For cultures in which this process is rare, it may be possible to increase its frequency (for example by use of low temperatures as described by RAO and ENGELBERG 1966).

SUMMARY

A previously described proline-requiring mutant of a Chinese hamster cell, which produces maximal plating efficiency when proline is present and none in its absence, has been studied further. Biochemical experiments place the probable point of block in the proline deficient cell at the conversion of glutamic acid to glutamic γ-semialdehyde.—The deficient mutant has only 21 chromosomes. It is postulated that one gene resided on the lost part of the chromosome complement, and a mutation occurred in the sister gene.—Spontaneous revertants to proline independence occur with a frequency of about 1×10^{-6}. The revertants are distinguishable from other proline-independent Chinese hamster cells with no missing chromosomes, by their slower growth rate in the absence of proline, their inability to function efficiently as feeders for the proline deficient cells, and by their reduced rate of conversion of glutamic acid to proline. The revertant behavior is compatible with that expected from the haploid state of the gene under study.

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