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GENETIC RECOMBINATION IN MAMMALIAN
CELLS IN CULTURE

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FINAL REPORT

GENETIC RECOMBINATION IN MAMMALIAN CELLS IN CULTURE

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The purpose of this investigation was to study the mechanisms underlying the formation of auxotrophic mutants in the quasi-diploid Chinese hamster cell line, CHO-K1. Cytological examination of a sequential set of auxotrophic mutants showed chromosomal changes at each mutant step. Quasi-tetraploid, non-complementing mutants were synthesized by cell fusion. When these non-complementing mutants yielded nutritional independent progeny, cytogenetic analysis showed chromosomal rearrangement, and in the case of glycine requirement, changes in one of the chromosomes were present. A classical mitotic recombination process appears not to be involved in mutant formation in the Chinese hamster cell.

Mutation must be achieved by some other mechanism such as gross chromosomal rearrangement and gene inactivation.

Karyotype Changes Accompanying Nutritional Independence:
The Change From Glycine Requirement to Independence in Hybrid Cells of
the Chinese Hamster Cell, CHO-K1

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(20)

Abstract

A glycine requiring hybrid, synthesized from two independent glycine requiring non-complementary mutants, was examined for its ability to produce glycine independent progeny spontaneously. The hybrid produced glycine independent progeny at a rate two orders of magnitude above the spontaneous mutation rate of either parent culture. Karyotype analysis of the progeny indicated a high incidence of rearrangement in one chromosome accompanying the change to glycine independence. These observations are compatible with a recombination process.

Introduction

The mechanism by which auxotrophic mutants are formed in the quasi-diploid cell line of Chinese hamster, CHO-K1 is not clear. There have been some speculations (1, 2, 3) and it was recently shown that mutation, both forward and backward, is accompanied by karyotype change (4). These results suggest that a recombination mechanism, or a breakage-gene inactivation mechanism is involved. To further investigate genetic change we have synthesized some hybrid CHO cells auxotrophic for glycine and have inquired as to karyotype changes when these hybrids generate glycine independent progeny. The results of these investigations are presented here.

Materials and Methods

Culture Conditions

Routine growth of cells in F12 medium containing 8% fetal calf serum (F12FC8) and preparation and use of nutrient deficient F12 supplemented with the macromolecular portion of fetal calf serum (FCM), and other manipulations involved in culturing the cells have been described previously (5).

In Table 1 are listed the strains of CHO-K1 utilized in this study. A spontaneous proline prototroph was derived from 155.25 by plating these cells at densities of 2×10^5 cells (100 mm plate containing F12 without proline plus 10% FCM). The revertants were recovered at frequencies of approximately 2×10^{-7} .

Karyotype Analysis by G. Banding

The techniques of Deaven and Peterson (6), and Worton et al. (2) were generally followed. After a 45 minute exposure to 0.05 M colcemid, the cells from a nearly confluent 60 mm plate were collected by trypsinizing and centrifuging for 5 minutes at 600 rpm. The cells were treated with hypotonic KCl (0.075M) and subjected to two fixation cycles of methanol acetic acid (3:1).

The chromosomes of the swollen fixed cells were spread by dropping two drops of the cell suspension in fixative on cold, wet slides. These preparations were air dried and stored for at least one week prior to being banded and stained.

The spread chromosomes were trypsinized and stained with Giemsa at room temperature as follows. The slides were immersed in 0.025 mg/ml trypsin (Difco 1:250) in D.P. buffer (0.23 M NaCl, 0.0024 MKH₂PO₄, 0.014 M Na₂HPO₄ at pH 7.0) for 45-60 seconds, rinsed in two consecutive rinses of D.P. buffer, and then stained with Giemsa for approximately 1 minute 45 seconds. The Giemsa was prepared by mixing, prior to use, 4 ml of Gurr's r66 Improved Giemsa in 100 ml of Gurr's pH 6.8 buffer (Biomedical Specialties, Santa Monica, CA). After staining, the slides were rinsed three times in distilled water and air dried.

Results

From 51.11, pro⁻glyAl⁻glyA2⁺ser⁺, and 155.25 pro⁺glyAl⁺glyA2⁻ser⁻, hybrids were synthesized. Hybridization was by the polyethylene glycol method (7). Selection of the hybrids was achieved by using the proline and serine mutations. Thus:

	<u>pro⁻</u>	<u>glyAl⁻glyA2⁺</u>	<u>ser⁺</u>
P1	<u>pro⁻</u>	<u>glyAl⁻glyA2⁺</u>	<u>ser⁺</u>
	<u>pro⁺</u>	<u>glyAl⁺glyA2⁻</u>	<u>ser⁻</u>
P2	<u>pro⁺</u>	<u>glyAl⁺glyA2⁻</u>	<u>ser⁻</u>

The parental cell lines are pictured as if they are diploid for the mutations considered. Selection for the hybrids was on the basis of proline and serine independence. A number of hybrids were isolated and examined for glycine requirement. As expected from previous results, all required glycine for growth.

In accordance with this observation, and with the knowledge that the glyA mutations involved were independent events, the glyA genotype of the hybrids is pictured as two non-identical, non-complementing mutations in the same cistron.

The next objective was to see if such glycine requiring hybrids could produce glycine independent derivatives. Presumably this would be accomplished as follows:

$$\underline{\text{glyA1}^{-}\text{glyA2}^{+}}$$

$$\begin{array}{cc} \hline - & + \\ \hline \end{array}$$

X exchange to yield a $\underline{++}$, which is functional for glycine synthesis.

$$\begin{array}{cc} \hline + & - \\ \hline \end{array}$$

$$\underline{\text{glyA1}^{+}\text{glyA2}^{-}}$$

The glyA $\underline{++}$ hybrids can be selected in glycine-free medium which will permit only glycine independent clones to grow.

The hybrids, were karyotyped as soon after formation as possible. These hybrids were of two kinds: those that yielded glycine independent clones subsequently and those that did not. The subsequent analyses were limited to those that did produce glycine independents, but it is realized that study of those that did not is important since they may yield additional information on limitations to the mechanism that permits becoming prototrophic for glycine. They will be the subject of future studies.

The glycine requiring hybrids that yielded glycine independent subclones were found to be characterized by at least two properties: (1) they rarely had chromosome counts that represented the sum of the two parental chromosomal complements, and in general they usually had several chromosomes less than that figure; (2) each was characterized by having changed chromosomes in its

karyotype.

Although many parental chromosomes can be recognized in the hybrids, they also contained chromosomes that could not be identified with the present methods of resolution with either parent. These new "marker" chromosomes usually were two to five in number. Even with the ability to detect "missing" normal chromosomes it was not possible to identify these marker chromosomes with the chromosomes of the parent. Perhaps more refined cytogenetic analysis will resolve this problem.

Thus, the fusion process itself produced karyotypic changes. This was not expected, although Worton reports (2) similar observations. The basis for the generation of new chromosomes during hybridization remains obscure, but may indicate something about the mechanism of hybridization or of events taking place during it. It may be necessary to fuse cells at specific stages in the life cycle since it has been shown (10) that fusion of cells greatly out of phase results in chromosome fragmentation.

The formation of new chromosomes during hybridization presents new problems for the analysis but many parental chromosomes are readily recognizable. The correlation of karyotypic changes with the formation of glycine independent clones can still be undertaken with well characterized hybrids.

Glycine independent clones are produced spontaneously at the approximate frequency of $1-10/10^5$.

According to Kao et al. (11) the reversion frequency from gly^- to gly^+ in the parental 51.11 strain is less than 10^{-8} . Three experiments in this laboratory indicate the frequency to be less than 10^{-6} . The 155.25 pro^+ parent also reverts to gly^+ at a low frequency, less than 10^{-6} in seven experiments in this laboratory. Thus, the production of glycine independent clones in the hybrids is two-three orders of magnitude higher than the spontaneous reversion rate in either parent.

The glycine independent clones were analyzed cytogenetically and karyotypes prepared for comparison with the glycine requiring parents.

A representative karyotype of one hybrid, 2x-6, from which three independent glycine independent clones were derived is shown in Figure 1, which shows the readily identifiable chromosomes plus "new" chromosomes not immediately identifiable with any parental chromosomes. A total of six karyotypes was prepared of 2x6. The chromosome numbers in these karyotypes varied from 36 to 40. A summary of the karyotypes is presented in Table II. In making this summary it is assumed that the initial hybrid contained four chromosome one's, four chromosome two's, two chromosome x's, etc., except for chromosome's 2a and eleven for which there would be only two per hybrid. The karyotypes are characterized as having either identifiable chromosome losses or readily recognizable changes in an identifiable chromosome. In this way background is provided against which to detect changes accompanying the change to glycine independence. Two changes characterize the six karyotypes of the parental 2x6; each indicates a loss of one chromosome five, and one chromosome eight from the sum of the complements of the two parents 51.11 and 155.25 pro⁺. These characteristics also serve to relate the glycine independent derivatives to the parental hybrid: of nineteen karyotypes prepared of three independent glycine independent derivatives of 2x6, eighteen showed the loss of at least one chromosome five and one chromosome eight.

Analysis of the karyotypes of three independent glycine independent derivatives of 2x6 was made and is also shown in Table II. There appears to be three qualitative changes accompanying the changes to glycine independence: changes in chromosome one, chromosome two and chromosome ten.

The changes can be thought of in a quantitative manner: with regard to chromosome one, in the case of the 2x6 parent there were six karyotypes (each with four chromosomes one) with twenty-four possible chances for changes or losses in chromosome one, whereas only one loss and no structural change was in fact observed. In the case of chromosome one for the nineteen karyotypes of the glycine independent derivatives, there were seventy-six opportunities for

changes or loss of chromosome one and nineteen were found. The ratio 19/76 is greater than 1/24 by a considerable amount.

For chromosome two, the comparison is similar: 3 out of 24 chances for change or loss in the 2x6 parent karyotypes as compared with 23 out of 76 in the karyotypes of the glycine independent derivatives. All but four of those changes were chromosomal losses.

In the case of chromosome ten the difference appears not to be so great: 1/24 for the 2x6 parent as compared with 11/76 in the glycine independent derivatives. All these changes were chromosome losses.

For the remaining chromosomes the differences between parent and glycine independent derivative appear not to be significant and there were no structural changes detected.

Most of the data in Table II represents chromosome "losses", that is, a chromosome could not be identified as being present, even though the total number of chromosomes present is nearly the sum of the two parents' contribution. This is not the case with the chromosomes one. With hybrid 2x6, of the nineteen changes in chromosome one accompanying change to glycine prototrophy, six were losses, whereas 13 were noted as recognizable structural changes in one of the chromosomes one. These changes are summarized in Table III.

Unfortunately, the parental hybrid 2x6 has been lost because of contaminated medium and analysis of additional glycine independents cannot be made in this hybrid. We have additional independent glycine requiring hybrids and the karyotypic data on one of them, 3x3, indicates that of 28 opportunities for change in chromosome one, there were eleven changes noted in the glycine independents as compared to only one in twelve in the glycine requiring parent.

A summary of the chromosomal change observed when hybrid 3x3 becomes glycine independent (three independent gly⁺ changes) is given in Table IV. Some examples of these changes are shown in Figure 2.

A careful study of the remaining identifiable chromosomes in the karyotypes showed only 4 structural changes. The sum of all of these chromosomes in the karyotypes of the glycine independent chromosomes examined was 95. Thus, 4/395 is an indication of chromosome stability from the standpoint of recognizable structural change.

Discussion

The results presented a study of a non-complementing hybrid cell line which undergoes some genetic event to yield cells which no longer require glycine is supportive of the thesis that recombination does occur in Chinese hamster cells in culture.

The changes occur at a frequency at least two orders of magnitude higher than the spontaneous reversion rate of either parental nutritional mutation. This is accompanied by a cytogenetic change, whose precise nature is not yet known, but involves a single member of one of the chromosomes one in six of six changes to glycine independence.

Because of the unexpected chromosomal changes occurring during hybrid formation, the study has been made more complicated since it raised the background against which nutritional change with accompanying cytogenetic change, must be measured.

Several things can be employed to reduce this background: (1) early examination of non-complementing hybrids and selection of those that have the fewest changes for additional study; (2) variation in the hybrid formation procedure so that less altered (in the chromosomal sense) hybrids will be produced and (3) use of more refined cytogenetic procedures now available.

With the glycine independent hybrids that are available, additional study is possible. The identity of the chromosome containing the information for serine hydroxymethylase can be sought in a further hybridization step, similar to a back-cross. In these experiments, a glycine independent hybrid, believed

recombinant for some chromosome, can be hybridized with a diploid glyA strain and placed in a glycine-free medium to select for the chromosome that contains the information for glycine synthesis. This can be achieved (F.-T. Kao, personal communication) by irradiating the glycine independent recombinant with 5000 rads of gamma radiation which decreases the number of chromosomes from the irradiated parent that will survive in the second-step hybrid. In this way selection for the gly^+ recombinant chromosome can be made. Although the background of chromosome change generated by the hybridization process plus the additional possible change produced by the gamma radiation exposure to the glycine independent parent may make for additional difficulties, careful analysis of these second-step hybrids may reveal cases where a structurally modified chromosome one noted after the change to glycine independence is the only chromosome to be found in the glycine independent hybrids produced in the secondary hybridization. We have shown elsewhere that recognizable karyotypic change always accompanies nutritional requirements.

As to the mechanism underlying the change to glycine independence, we can only speculate. It appears not to be a mitotic recombination in the classical sense, i.e. at a four strand stage. However, other mechanisms are possible. Cox and Masson (12) have found that radiation induced mutations in mammalian cells to resistance to 6-thioguanine are usually associated with gross chromosomal change. Esposito (13) has found in yeast that mitotic recombinations occur principally at the two-strand stage. We will need to reexamine the situation in Chinese hamster cells taking into account this observation.

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Table I
Description of Cell Cultures Utilized

Culture	Parent	Phenotype	Mutagenic Agent	Reference
CHO-K1		pro ⁻ ser ⁺ gly ⁺	spontaneous	8
51.11	CHO-K1	pro ⁻ gly ⁻ (glyA)ser ⁺	E.M.S.	9
155.25 pro ⁺	155.25	pro ⁺ gly ⁻ (glyA)ser ⁻	spontaneous	this study

Table II

Frequency of Changes or Losses of Chromosomes in Glycine Requiring
Hybrids as Compared with Glycine Independent Progeny Derived from Them.

Chromosome	1	2	3	4	5	6	7	8	9	10	11
A. Glycine Requiring Parent Hybrid	1/24	3/24	2/12	3/24	9/24	4/24	4/24	9/24	9/24	1/24	3/12
B. Glycine Indepen- dent Progeny	19/76	23/76	7/38	13/76	33/76	8/76	15/76	37/76	29/76	11/76	7/38
C. B/A	6.0	2.5	1.1	1.4	1.1	0.65	1.2	1.3	1.0	3.5	0.72

The frequency of changes or losses in chromosomes 1, 2, 10 is higher in the glycine independent progeny than in the parental glycine requiring hybrid. In the case of chromosome one in the glycine independent hybrid progeny, most of the changes were structural changes rather than losses.

Table III

Changes in Chromosome One Accompanying the Change to
Glycine Independence in Hybrid 2x6

Glycine Independent	Number of Karyotypes	No. of Karyotypes with	
		Structural Changes in Chromosome One	Probable Nature of Change
2x613	9	8	p ⁻
2x614	6	3	q ⁻
2x615	4	2	q ⁻

Table IV

Changes in Chromosome One Accompanying the Change to
Glycine Independence in Hybrid 3x3

Glycine Independent	Number of Karyotypes	No. of Karyotypes with	
		Structural Changes in Chromosome One	Probable Nature of Change
3x311	3	3	p ⁻
3x312	3	3	p ⁻
3x313	7	7	q ⁺

Figure Legends

Figure 1. Karyotype of the hybrid 2x6, the result of the fusion of line 51.11 with line 155.25 pro⁺ by selecting for proline and serine independence. Note the marker chromosomes generated during the fusion process.

Figure 2. Examples of the change occurring in one of the four chromosomes one accompanying the change to glycine independence. These changes occurred independently. From left to right: glycine independents 2x-613; 2x-614; 2x-615; 3x-315; and 3x-313. The first three are from glycine requiring hybrid 2x6, the last two from glycine requiring hybrid 3x3.

FIGURE 1. Morse, Boehm, Morse and Dahl

TOP

FIGURE 2. Morse, Boehm, Morse and Dahl

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