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Esterase Gene Expression in Chinese Hamster and Mouse Lymphoma Hybrids Isolated Under Nonselective Pressure

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Hybrids between a fibroblastic Chinese hamster cell line (CH23) and a mouse lymphoma cell line (P388F36) were produced and isolated by a simple new method without using selective media and avoiding contact with the parental cells. The chromosomal situation in the two hybrid types (PCM and PCS) isolated suggested that growth on glass surface (PCM) or in suspension (PCS) depended on the number of hamster and mouse chromosomes which existed in the hybrids. Chromosomal stability in hybrids grown as monolayers (PCM) was reached at a stage in which two to four mouse chromosomes coexisted with no fewer than 19 hamster chromosomes. In a study of gene linkage utilizing clones of this hybrid population, five out of nine genes regulating the synthesis of different esterases in the mouse cells used were found to be unlinked.

KEY WORDS: hamster–mouse hybrid selection; gene expression; esterases.

INTRODUCTION

The methods for hybrid cell selection involve mainly drug-resistant (Littlefield, 1964, 1966; Sobel *et al.*, 1971) or nutritional mutant (Kao *et al.*, 1969; Morrow *et al.*, 1973) cell lines either alone or the first in combination with cells requiring a specific temperature for growth (Goldstein and Lin, 1972). In all these systems, each parental cell line is continuous and possesses a different sensitive marker against which, under special conditions, the hybrids

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will be selected. Semiselective methods have been reported utilizing either a high concentration of drug-resistant cells fused with a low concentration of normal cells followed by cloning shortly after fusion (Davidson and Ephrussi, 1965) or a continuous resistant cell line and a noncontinuous cell population (Harris *et al.*, 1969; Klein *et al.*, 1971). Advantageous media that have also been reported contain methylcellulose (Watkins and Chen, 1969) or methylphenyldiazene carboxylate (Siniscalco *et al.*, 1969), in which hybrids can preferably grow.

A simple method for the selection of hybrids between normal monolayer and suspension cell lines and some aspects of the application of these hybrids for gene linkage analysis (Ruddle, 1970, 1971, 1972; Koprowski and Knowles, 1973) are presented here.

EXPERIMENTAL PROCEDURES

Cells and Cell Fusion

Chinese hamster fibroblasts (Tjio and Puck, 1958) were cloned in this laboratory, and the clone used (CH23) was characterized by the presence of 23 chromosomes (range 21–26, polyploidy 8.0%; Fig. 1a) and a generation time of 12–13 hr. P388F mouse lymphoma cells (Fischer and Sartorelli, 1964) were also cloned and the clone (Pe88F36) was characterized by the presence of 36 chromsomes (range 34–46, polyploidy 27.04%; Fig. 1b) and a generation time of 21–22 hr. Both cell lines were adapted to grow in Ham's F12 medium (Ham, 1965) plus 10% fetal bovine serum, penicillin, streptomycin, and amphotericin B. All cells used were free of contamination, as shown by thymidine-H^3 labeling and autoradiography (Nardone *et al.*, 1965; Fogh *et al.*, 1971). Throughout the hybrid isolation procedure, conditioned Ham's F12 medium supplemented with an additional 5% fetal bovine serum was used. This medium was the cell-free medium in which CH23 and P388F36 cells had been grown for 3 days; the cells had been removed by three repeated 10-min centrifugations at 2000 rpm under sterile conditions. Conditioned medium (CM) supplemented with serum was found to greatly promote the growth of viable single cells into colonies. It was used in order to facilitate the growth of very dilute cell populations. Cloning was accomplished by culturing dilute cell suspensions in plastic petri dishes containing tiny pieces of broken glass coverslips and transferring pieces carrying single cells to CM.

Cell fusion was carried out according to the method of Harris and Watkins (1965). UV-inactivated virus was used at 10^3 and 10^4 HAU/ml to fuse 3×10^6 CH23 and 9×10^6 P388F36 cells in 2 ml of Hanks' balanced salt solution.

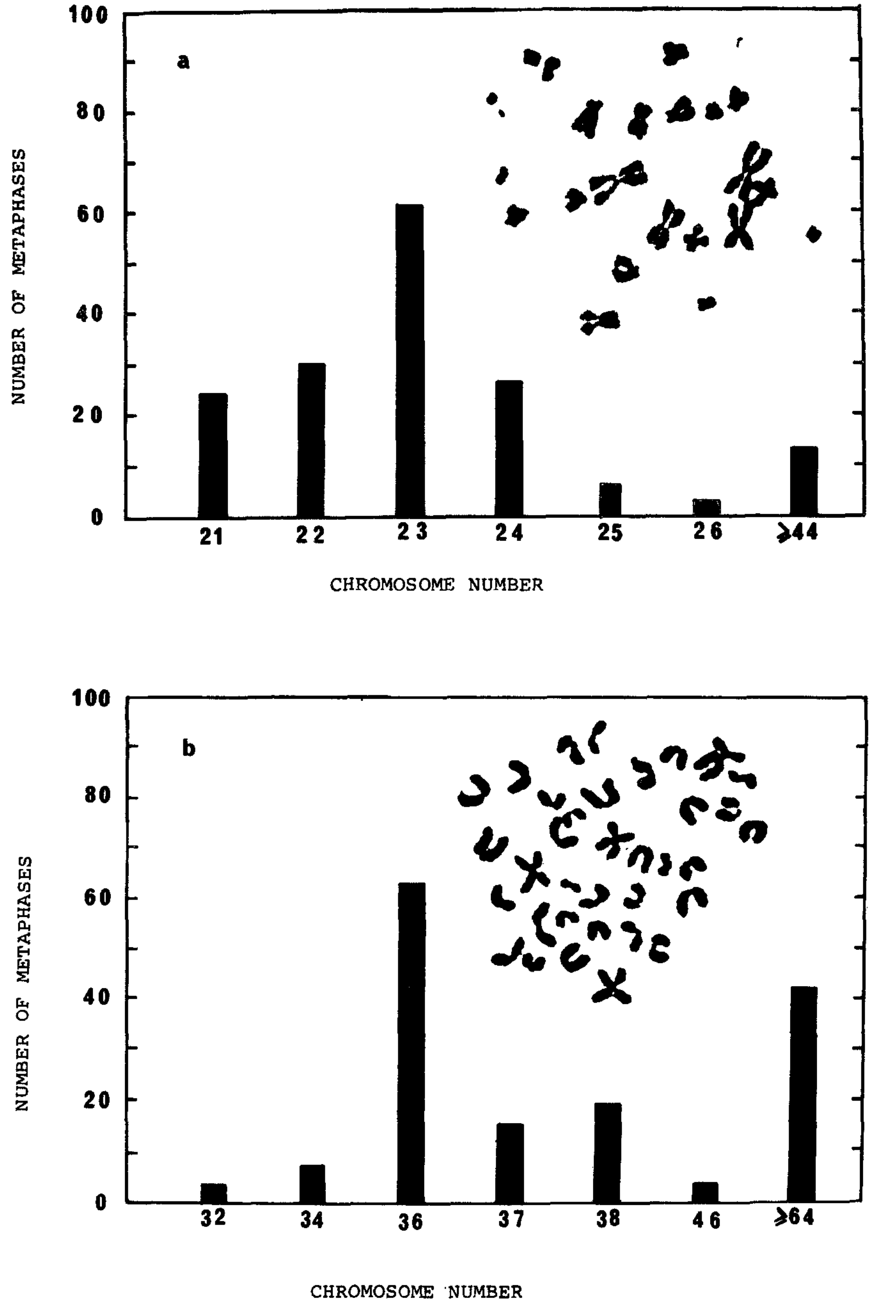


Fig. 1. Distribution of chromosome number and predominant karyotype of (a) CH23 and (b) P388F36 cell lines. Total metaphases examined: 162 and 159 for (a) and (b), respectively.

Isolation of the Hybrids

Simultaneous isolation of hybrids grown as monolayers (PCM) and hybrids grown in suspension (PCS) was accomplished as follows: After the fusion process, the cell suspension was transferred to a 16-oz tissue culture bottle (A) with fresh medium and incubated for 4 h. This was considered a sufficient length of time to permit most of the CH23 and multinucleate cells to adhere to the glass surface. The supernatant medium containing cells remaining in suspension (mainly P388F36) was then transferred to a new bottle (B). Bottle A was washed three times with medium, new medium was added, and bottle A was incubated for a further 3 hr, after which the supernatant medium was transferred to another bottle (C). The same procedure was repeated at least six more times, and so a series of bottles B–I were produced from bottle A. Immediately after their preparation, each of these bottles was incubated for 3 hr to permit CH23 metaphase cells to attach to the glass (Terasiman and Tolmach, 1963). The supernatant media were then again transferred to a series of new bottles (B_1–I_1 from B–I, respectively), and the new bottles were incubated for a further 3 hr to permit any more CH23 metaphase or even interphase cells still existing in suspension to adhere to the glass. Transfer of the supernatants of bottles B_1–I_1 to new bottles B_2–I_2, respectively, was carried out again after the 3 hr, and bottles B_2–I_2 were incubated for several days until colonies of monolayer cells could be seen in several of them. Cells from each of these colonies were transferred to new bottles by means of a rubber policeman, and after growth for about 7 days each bottle was screened for hybrids by preparing conventional metaphase spreadings. After the 7-day incubation, the supernatants of bottles C_2–I_2 were transferred to new bottles (C_3–I_3, respectively, and incubated for 3 h to permit the metaphase hybrid cells which were in suspension to attach to the glass of bottles C_3–I_3. Supernatants were again transferred to new bottles C_4–I_4 and screened for hybrids using the content of those bottles which contained growing suspension cell populations.

Electrophoretic Determination of Esterases

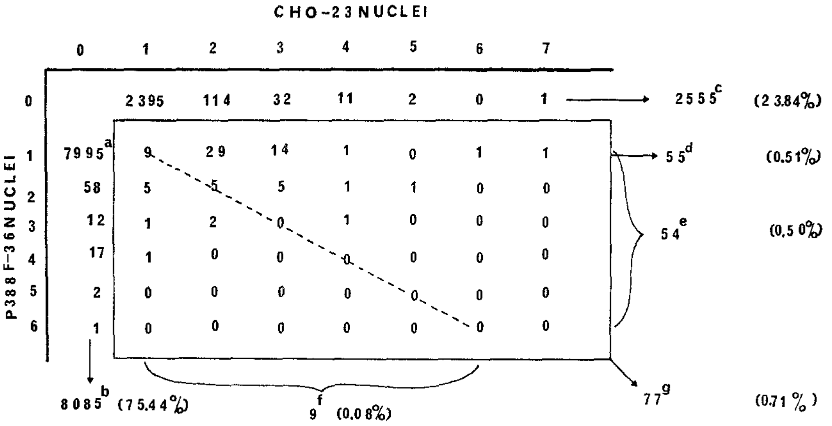
Cells were homogenized as previously described (Ayad and Delinassios, 1973) in double-distilled water, and the homogenate was centrifuged at 3500 rpm for 10 min at 0 C. Two to five drops of supernatant were used as sample for the electrophoretic processes.

Polyacrylamide gel disc electrophoresis was carried out according to the method of Ornstein and Davies (1964), and bands showing esterase activity were stained at *p*H 7.1 after 45 min in a fresh solution containing 0.1% fast red TR (Sigma) and 1 mM α-naphthylacetate (Sigma, predissolved in 50% acetone), in 0.05 M tris-HCl buffer.

RESULTS AND DISCUSSION

The yield of fused cells was 3.35% and 3.46% for the two virus doses 10^3 and 10^4 HAU/ml, respectively. Heterokaryons were present in 0.64% and 0.71%, respectively (Fig. 2), determined according to Harris and Watkins (1965).

The method for the isolation of hybrids, described in the Experimental Procedures section, can be explained as follows: After the fusion process, the multinucleate cells are attached to the glass and fusion of their nuclei gives rise to giant mononucleate cells (Fig. 3). Mitosis in these cells is completely abnormal, and the daughter cells produced, either diploid or polyploid, carry different numbers of mixed hamster and mouse chromosomes. During metaphase, these cells detach from the glass and the likelihood of growing in suspension or attached to the glass depends on the properties of the two newly combined hamster and mouse genomes. It is obvious that when polyploid cells are produced, with mixed mouse and hamster chromosomes, several successive abnormal mitoses might follow, resulting in a very large number of chromsome combinations in the daughter cells. In the case of polyploid hybrids carrying at least one complete mouse chromosome set



	0	1	2	3	4	5	6	7
0		2395	114	32	11	2	0	1
1	7995[a]	9	29	14	1	0	1	1
2	58	5	5	5	1	1	0	0
3	12	1	2	0	1	0	0	0
4	17	1	0	0	0	0	0	0
5	2	0	0	0	0	0	0	0
6	1	0	0	0	0	0	0	0

Fig. 2. Nucleogram of fusion between CH23 and P388F36 cells. Numbers immediately above the closed frame represent the number of CH23 cells containing one to seven nuclei per cell. Numbers to the left of the closed frame represent the number of P388F36 cells containing one to six nuclei. Heterokaryons (i.e., cells containing labeled and unlabeled nuclei) are included in the closed frame. Total cell counts: 10,717. (a) This number was calculated from the total number of CH23 nuclei, the P388F36 nuclei in homokaryons and heterokaryons, and the ratio 1:3 between CH23 and P388F36 cells during the fusion. (b) Total number of P388F36 single and multinucleate cells counted. (c) Total number of CH23 single and multinucleate cells counted. (d) Number of heterokaryons containing only one P388F36 nucleus. (e) Number of heterokaryons containing more CH23 than P388-F36 nuclei. (f) Number of heterokaryons containing more P388F36 than CH23 nuclei. (g) Total number of heterokaryons. Numbers in parentheses represent percentages.

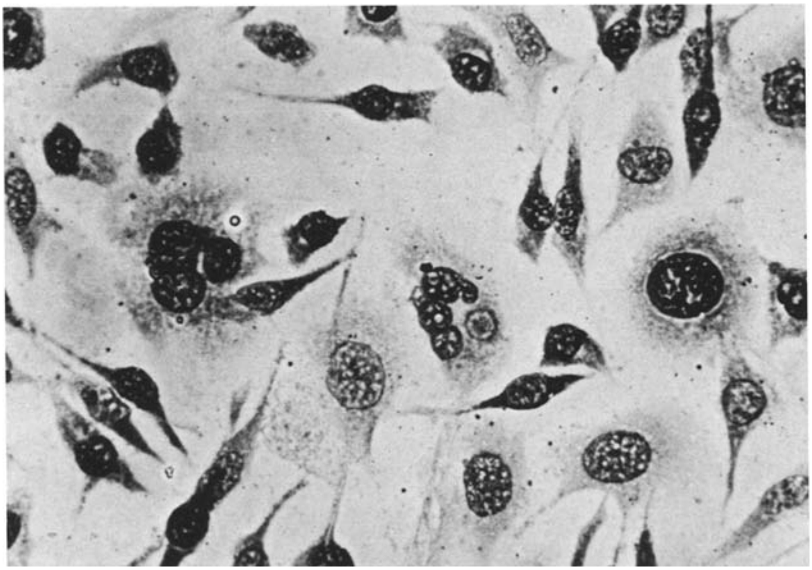


Fig. 3. Multinucleate cells, mononucleate cells, and cells with giant nuclei, adhered to the glass, 6 hr after the fusion process. Methanol fixed and safranin stained.

and only one complete or incomplete hamster chromsome set, growth in suspension might be favorable, although it would be almost impossible to see this type of cell because of very low frequency (Fig. 2) and short time of existence. Abnormal mitosis in this sort of hybrid could give two daughter cells: one containing all or a large proportion of hamster chromosomes with few mouse chromosomes, and one containing all or a large proportion of mouse chromosomes with few hamster chromosomes. The cells of the former type, if they survive, can attach to the glass and, if their genome permits, can divide and give hybrid cell populations such as those studied here. The aim of the successive transfer of the supernatant media from bottle to bottle is to eliminate the hamster cells so that when those polyploid hybrids which are growing in suspension are ready to divide, the new bottle to which they have been transferred will be free of attached CH23 cells.

The isolation of hybrids grown in suspension can be explained in a similar manner, but in this case the heterokaryons from which the hybrids will be produced must possess more hamster nuclei and one mouse nucleus. The aim is, similarly, to eliminate the suspension cells by successful washing out of the suspension cells from bottle A so as to provide a medium free of cells for the isolation of PCS hybrids not attached to the glass.

Hybrids between Chinese hamster and mouse cells have been isolated in several laboratories by using selective pressure (Scaletta *et al.*, 1967;

Koyama *et al.*, 1970; Handmaker, 1971; Pontecorvo, 1971; Marin and Pugliatti-Crippa, 1972; Marshall-Graves, 1972). The situation in these hybrids with respect to chromosome losses appears to be rather more complicated than in man × mouse (Weiss and Green, 1967; Ruddle, 1971), or man × Chinese hamster (Kao and Puck, 1970; Westerveld *et al.*, 1971) hybrids, which preferentially lose human chromosomes. Preferential loss of mouse (Scaletta *et al.*, 1967) or hamster chromsomes (Koyama *et al.*, 1970), or chromosomes of either parental type, carrying the enzymatic activity against which selection is favored (Marin and Pugliatti-Crippa, 1972) and also nonpreferential loss accompanied by great chromosomal variation (Handmaker, 1971; Marshall-Graves, 1972) comprise all the possible combinations of chromsomal situations in mouse × Chinese hamster hybrids, and suggest a further investigation of these hybrids. Pontecorvo (1971) has induced directional loss of the chromosomes of either parental hamster or mouse cell by X- or γ-irradiation or BUdR treatment before fusion.

During the course of the present hybrid isolation method, it is presumed that a predominance of hamster chromosomes is correlated with adherence to the glass and a predominance of mouse chromosomes with nonadherence. This hypothesis is proven below by examination of the hybrids produced. Our simple method provides an opportunity to study hybrid cells isolated from their parental cells immediately following their production, avoiding possible phenomena of cell-to-cell communication (Subak-Sharp *et al.*, 1966, 1969; Stoker, 1967; Pitts 1971, 1972; Bendich *et al.*, 1967) as well as the use of selective media. This system might also be useful for further research into the mechanism of chromosome loss in the hybrid cells, for which explanations have been proposed and discussed in works based only on hybrids produced under selective pressure (Matsuya *et al.*, 1968; Kao and Puck, 1970; Ruddle, 1970; Handmaker, 1971; Marshall-Graves, 1972). Separation and extensive chromsomal analysis of hybrids from fusion between other cell types should also be attempted before any conclusions can be made about the general applicability of this method.

Karyotypic analysis of a PCM hybrid clone during its 21st generation (Table I) revealed a completely heterogeneous population, showing (1) retention of varying numbers of mouse chromosomes from cell to cell (range 2–14), (2) loss of one to ten hamster chromsomes in 96.39% of the hybrids examined, (3) formation of abnormal hamster chromosomes (range 1–4) in 7.2% of the hybrids, and (4) formation of dot chromsomes (range 1–13) in 72.07% of the hybrids (Fig. 4).

During the 105th generation of the same hybrid population, a second karyotypic analysis (Table I) was carried out, revealing (1) further loss of mouse chromosomes and retention of numbers ranging only from two to five, (2) complete loss of mouse chromosomes in 12.6% and return to a

Table I. Karyotypic Analysis of PCM Hybrid Cell Population During 21st, 105th, and 255th Generations

	No. of cells in metaphase			Percentage		
	21st	105th	255th	21st	105th	255th
Hybrid metaphases examined	111	189	138	100	100	100
Hybrids containing dot chromosomes	80	74	29	72.07	39.15	21.01
Hybrids with fewer than 23 hamster chromosomes (diploid cells) plus hybrids containing fewer than 46 hamster chromosomes (tetraploid cells in relation to CH-23 cells)	107	137	101	96.39	72.48	73.18
Hybrids containing abnormal hamster chromosomes	8	1	1	7.20	0.52	0.72
Hybrids containing abnormal mouse chromosomes	0	0	0	0	0	0
Hybrids containing one mouse chromosome	0	0	0	0	0	0
Hybrids containing two mouse chromosomes	26	80	67	23.42	43.32	48.55
Hybrids containing three mouse chromosomes	37 } 81	77	38	33.33	40.74	27.53
Hybrids containing four mouse chromosomes	18	6	13	16.21	3.17	9.42
Hybrids containing five mouse chromosomes	5	2	0	4.50	1.05	0
Hybrids containing six mouse chromosomes	8	0	0	7.20	0	0
Hybrids containing more than six mouse chromosomes	17	0	0	15.31	0	0
Hybrids containing no mouse chromosomes	0	24	20	0	12.60	14.49
Total hybrids containing 20–30 chromosomes	90	158	114	81.08	83.59	82.60
Total hybrids containing 31–45 chromosomes	5	5	3	4.50	2.64	2.17
Total hybrids containing 46–88 chromosomes	16	26	21	14.41	13.75	15.21

karyotypic pattern similar to CH23 in 8.4% of the cell population, (3) almost complete disappearance of abnormal chromosomes, and (4) presence of dot chromosomes (range 1–5) in 39.15% of the metaphases examined (Figs. 5 and 6).

During the 25th generation, the hyhrid population under study exhibited a chromosomal situation, showing (1) some further loss of mouse chromosomes and retention of two to four of them, (2) complete loss of mouse

Fig. 4. Metaphase of a hybrid cell containing 77 hamster chromosomes, 14 mouse chromosomes, and 6 dot chromosomes, prepared during the 21st generation of the hybrid population.

chromsomes in 14.49%, which resembled the hamster parental cells, (3) presence of dot chromosomes (range 1–5) in 21.01% of the metaphases examined, and (4) presence of no fewer than 19 hamster chromsomes in every hybrid cell examined.

From the examination and comparison of the karyotypes present during the 21st, 105th, and 255th generations, it is concluded that the hybrid population actively establishes more stable chromosomal patterns, since the majority of the variable-hybrid karyotypes existing at the 21st generation disappeared by the 105th and 255th generations. It is thought that this and

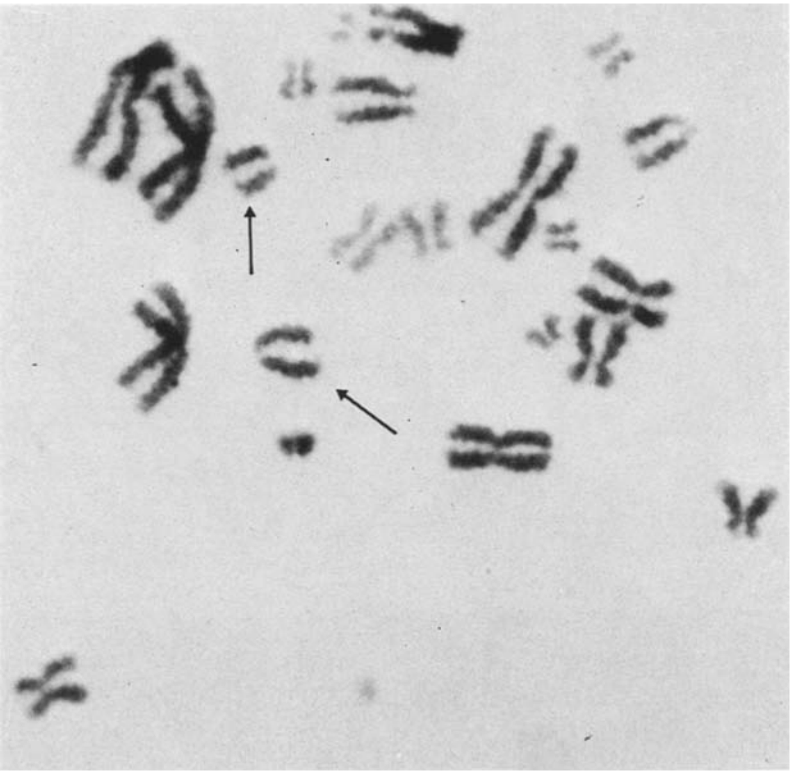


Fig. 5. Metaphase of a hybrid cell containing 21 hamster chromosomes and three mouse chromosomes (arrows), prepared during the 105th generation of the hybrid population.

also the phenomenon of gradual decrease of dot chromosomes in an increasing number of cells are indications of reconstruction processes of the chromosomal material caused by a kind of discordant cohabitation of two different genomes within a cell. The situation with regard to the PCS hybrids is different; they tend to preserve at least most of the mouse chromosomes, gradually losing the hamster ones (work in progress).

PCM hybrids did not show any change in morphology or generation time compared with the CH23 cells.

As the PCM hybrids were found to be highly heterogeneous with regard to their distribution of chromosomal constitution and as different mouse chromosomes were carried by different cells, it would be impossible to use such a hybrid population directly for genetic mapping (Rubble, 1970, 1971, 1972). It was thus decided to isolate single cells from this population

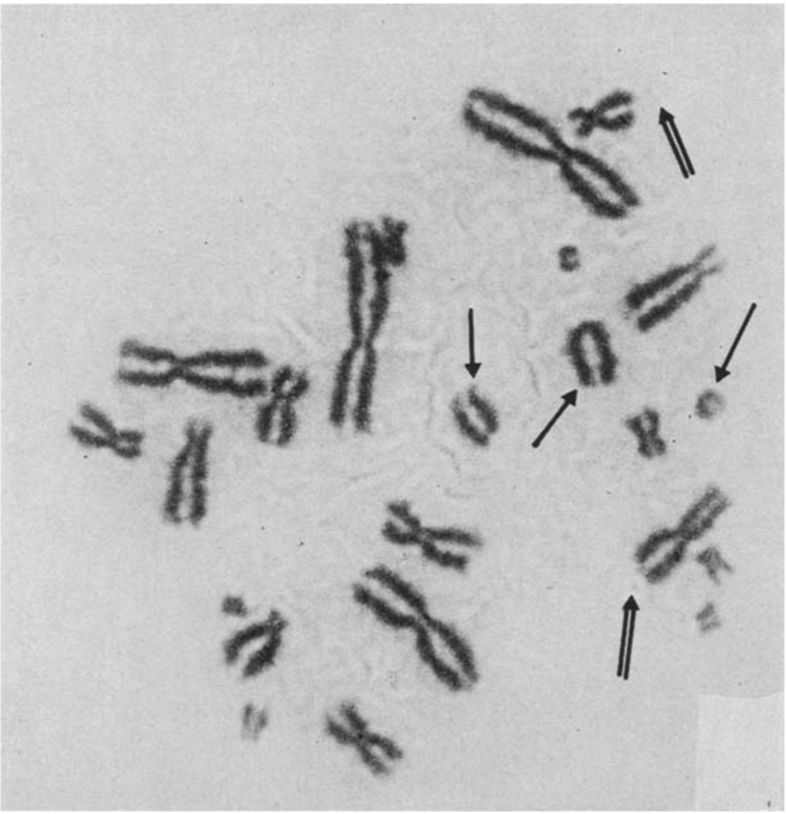


Fig. 6. Metaphase of a hybrid cell containing 20 hamster chromosomes, 3 mouse chromosomes (arrows), and 2 dot chromosomes (double arrows), prepared during the 105th generation of the hybrid population. Mouse chromosomes are different from those present in the metaphase of Fig. 4.

and let them divide until they formed confluent monolayers. These new cell populations were separately harvested, and their electrophoretic esterase activities on polyacrylamide gells were examined.

CH23 cell extracts showed three electrophoretically separated bands (Fig. 7a,b). The second and especially the third lower bands needed much longer time to stain than the first and in some cases either were not recovered at all or stained as one faint band (Fig. 7c). P388F36 cell extracts were found to be much richer in esterase activity, exhibiting about nine visible bands (Fig. 7k). Table II shows the results of esterase pattern examination of 11 hybrid clones. Figure 7 shows remarkable differences in the electrophoretic patterns of the esterases of the two parental and six hybrid clones. From

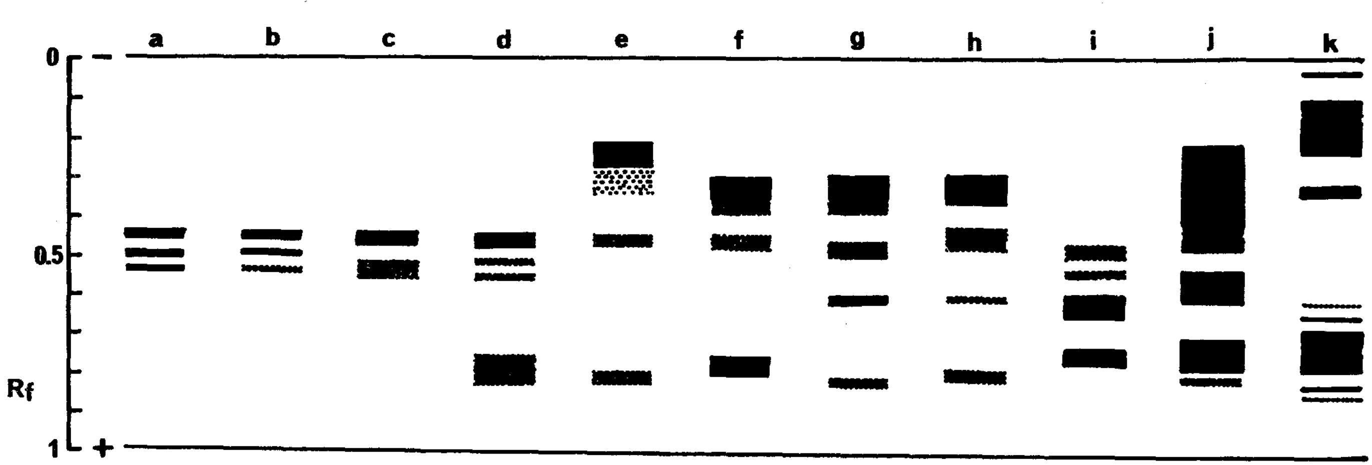


Fig. 7. Diagram of polyacrylamide gels showing the electrophoretic patterns and R_f values of esterases of parental and hybrid cells. (a, b) CH23 cell esterases, two different preparations, (c) CH23 esterases after freezing of the cell extract for 1 week, (d) PCM1, (e) PCM2, (f) PCM3, (g) PCM4, (h) PCM5, (i) PCM6 hybrid clone esterases, (j) mixture of CH23 and P388F36 cell extract esterases, (k) P388F36 cell esterases.

Table II. Esterase Pattern Examination in 11 Hybrid Clones[a]

Hybrid clone	CH23 esterase bands	P388F36 esterase bands	Extra unidentified bands
PCM1	+	+	–
PCM2	?	+	+
PCM3	?	+	+
PCM4	?	+	+
PCM5	?	+	+
PCM6	?	?	+
PCM7–PCM11	+	–	–

[a] Symbols: +, presence; –, absence; ?, unable to identify.

these results, it can be concluded that mouse genes regulating the synthesis of P388F36 cell esterases can be activated and expressed in the hamster–mouse hybrids studied. As different esterase bands attributed to mouse chromosomes are present in different hybrids, it might be inferred that several esterase genes are located on different mouse chromosomes. Gene–chromosome linkage could be studied in future by using the new chromosome identification techniques (Hsu, 1973).

From Fig. 7, by comparison with the esterase patterns of different hybrid clones, it is seen that at least five out of nine expressed esterase genes are located on different mouse chromosomes. Absence of a given band at its characteristic position is therefore directly connected with the absence of a particular mouse chromosome. Of course, problems of gene regulation, gene suppression, or, generally, gene interactions may complicate the issue, but obviously such difficulties cannot be resolved at present.

The tissue-specific esterases of the mouse have been found to give numerous isozyme bands when electrophoresed on starch gel (Ruddle and Harrington, 1967; Ruddle *et al.*, 1969; Ruddle and Nichols, 1971). The polyacrylamide gel phenotypes of the mouse lymphoma cell esterases of this study obviously do not correspond with those obtained by starch gel electrophoresis.

We believe that the present method of hybrid isolation provides a tool for study of genome interactions within a hybrid cell without using selective pressure and therefore avoiding any preferential retention of chromosomes carrying specific markers. Expression of the genetic material of the randomly maintained chromsomes has been shown.

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