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EFFICIENCY OF HYBRID CELL FORMATION FROM HETEROKARYONS FUSED BY HVJ

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SUMMARY Hybrid cell progeny were obtained in high yield when two kinds of parent cells were fused by UV-inactivated HVJ. The frequency of their formation was about 1×10^{-3}, or 1000 times more than on mixed culture of the two parent cell lines.

With these two parent lines three different hybrid clones were isolated differing in morphology, chromosomal number and generation time.

INTRODUCTION

Barski (1961) discovered that hybrid cells appeared when two different kinds of cells were cultivated together. This was valuable for genetic studies on somatic cells, but the efficiency of hybrid cell formation was low and so the separation of hybrids from the parent cells in culture was difficult. However, Littlefield (1963, 1964, 1965, 1966) developed an effective technique for hybridization. As the two parent cells, he isolated a thymidine kinase-less mutant and a guanilic acid-inosinic acid pyrophosphorylase-less mutant from L cells. On cultivation of the two mutants together in medium containing aminopterin (HAT medium), the parent cells degenerated and only the progeny of the hybrid cells could grow. Using this technique, studies on hybrids of many kinds of somatic cells became possible, and Ephrussi and co-workers extensively studied hybrids formed by this selection method (1965, 1966).

On the other hand, Okada and co-workers developed a technique for fusion of somatic cells in suspension by HVJ (1956, 1962). Heterokaryons readily appeared when a mixture of two different types of cells was treated with HVJ (Okada, 1961; Harris, 1965; Okada and Murayama, 1965). Yerganian and Nell (1966) first reported the appearance of hybrid cells from heterokaryons caused by HVJ.

Hence, it seemed natural to combine, Okada's fusion method and Littlefield's selection method for formation of hybrid progeny. This report is on the frequency of hybrid formation and as the most suitable combination of parent cells, two mutants derived from a single clone were used. The characters of the newly formed hybrids were studied.

MATERIALS AND METHODS

1. *Cells*

L cells of the wild type were kindly supplied by

Mr. Ono, of this Institute. They had been grown in Eagle's minimum essential medium (MEM) plus 10% calf serum. Two kinds of mutant of the L cells were selected in our laboratory. One was a mutant deficient in guanylic acid-inosinic acid pyrophosphorylase and resistant to 10γ/ml of 8-azaguanine (L_{AG}). The other was a mutant deficient in thymidine kinase and resistant to 100 γ/ml of 5-bromodeoxyuridine (L_{BUDR}). L_{AG} was selected as described by Littlefield (1963). For isolation of L_{BUDR}, clones resistant to 30 γ/ml of BUDR were first selected by Littlefield's method (1965). However, these clones were not completely deficient in thymidine kinase and could grow in the selection medium (HAT medium); cells containing thymidine kinase were grown in this medium. Next Puck's method (1967) was applied; cells integrating BUDR into their DNA were killed by exposure to visible light. Cells which had been grown in medium containing 30 γ/ml of BUDR were exposed to visible light and then grown in medium containing 100 γ/ml of BUDR, and the colonies forming on incubation were harvested.

L_{AG} and L_{BUDR} were cultured in Eagle's MEM and 10% calf serum supplemented by 10 γ/ml of 8-azaguanine (AG medium) for L_{AG} cells or with 100 γ/ml of BUDR (BUDR medium) for L_{BDUR} cells. They were each cloned 3 times and then used as parental cells in hybridization experiments. The two mutants did not grow in medium containing aminopterin (HAT medium), as shown in Table 2, and no revertants appeared during selection of the hybrids.

Ehrlich hyper diploid ascites tumor cells (ETC) were withdrawn from the peritoneal cavity of adult ddO mice inoculated 7 days previously. After washing 3 to 4 times with PBS(−), cells were resuspended in BSS-Ca medium and used as another parental cell line in hybridization experiments.

2. *Virus*

The HVJ-Z strain was propagated in the allantoic sacs of 10 day-old chick embryos. Infected allantoic fluid was harvested after 72 hr incubation at 35.5 C, and dialyzed against saline at 4C. The dialyzed fluid was stocked at −20 C. For fusion of cells, the stock was exposed to ultraviolet light for 5 minutes at room temperature: This UV dosage was twice that required to inactivate the infectivity of HVJ in the stock, under our conditions.

3. *Fusion of cells*

Monolayers of the two mutants were cultured in their respective growth media and then starved in HAT medium for about 24 hr. The appearance of hybrid progeny could be recognized faster after starvation. The monolayers were trypsinized, and the cells were washed and resuspended in BSS (NaCl 1.14×10^{-1}M; KCl 5.4×10^{-2}M; Na_2HPO_4 3.4×10^{-4}M; KH_2PO_4 4.4×10^{-4}M; buffered with 10^{-2}M of tris-HCl to pH 7.6) supplemented with 1 mM of $CaCl_2$ (Okada and Murayama, 1965, 1966). The concentration of cells was adjusted to 1 to 2×10^6 cells/ml. Equal numbers of two parental cells were mixed in a test tube (total 1 ml) at 4 C, and 0.5 ml of UV-HVJ was added. The tube was kept for 10 min. at 4 C, and then incubated for 20 to 30 min. at 37 C with shaking to complete the cell fusion reaction (Okada and Tadokoro, 1962). After this treatment, the cells (including multinucleated cells) were washed with low speed centrifugation. Cells were resupended in HAT medium, and plated after suitable adjustment of the cell number.

4. *Selection of hybrid progeny*

For isolation of hybrid cells, virus-treated cells were cultured in HAT medium: Eagle's MEM and 10% calf serum supplemented by 4×10^{-7}M aminipterin, 1×10^{-4}M hypoxanthine, 3×10^{-6}M glycine and 1.6^{-5}M thymidine (Littlefield, 1966). When endogeneous biosynthesis of purine and thymidylic acid is blocked by aminopterin, L_{AG} and L_{BUDR} can not survive, because they can not utilize hypoxanthine and thynidine, respectively, in HAT medium.

5. *Yield of hybrid progeny* To determine the frequency of formation of hybrid progeny, suitable numbers of cells appearing after treatment with HVJ (consisting of both single mutant cells, homokaryons and heterokaryons) were inoculated on to Falcon plastic plates (6 cm diameter). The plates were incubated at 37 C under 5% CO_2-air and the HAT medium was changed every 2 to 3 days. After 14 days, cultures were washed with PBS(−), fixed with methanol and stained by Giemsa solution and colonies were counted.

ETC cells are wild type, but they did not grow in vitro under our conditions, and hence hybrids between L_{AG} and ETC could also be selected in HAT medium. This system of selection is called a half selection system (Davidson and Ephrussi, 1965).

The frequency of formation of hybrid progeny was calculated as follows:

$$\text{Frequency}=\frac{\text{Number of colonies per plate}}{\text{Number of input parent cells per plate}^*}$$

* This number of cells is not the number of cells plated but the original number of cells before fusion by HVJ, which was calculated from the efficiency of cell fusion.

RESULTS

1. *Frequency of hybrid cell formation*

Results of two separate experiments are shown in Tables 1 and 2; in Table 1, L_{AG} and L_{BUDR} were used as parent cells, and in Table 2, L_{AG}-L_{BUDR} and L_{AG}-ETC were tested as parent combinations. In both experiments, cells were cultured in HAT medium after fusion by UV-HVJ. There were no survivors in cultures of cells of either parent line alone with or without UV-HVJ treatment. The rate of reversion of these L-mutants to the wild type or the rate of mutation of ETC to cells capable to grow in vitro was less than 10^{-5}, and no reactivation occurred on fusion of cells of one of these parents. Only when a mixture of the two kinds of parents was used, did new, growing cells appear in HAT medium.

TABLE 1. *Frequency of formation of hybrid progeny*

Input cell No. per plate	Hybrid colony No. per plate (average)	Frequency
10^5	333	$1.2/10^3$
10^4	14, 17, 15, 14 (15)	$0.56/10^3$
10^3	1, 3, 0, 0 (1)	$0.37/10^3$
10^2	0, 0, 0, 0 (0)	0

The parental cell number was 1.4×10^6 cells/ml for L_{AG} and 1.6×10^6/ml for L_{BUDR}.

TABLE 2. *Frequency of formation of hybrid progeny*

Parental cell	Input cell No. per plate[a]	Average colony No. per plate	Frequency
ETC	10^5	0	0
	10^4	0	0
L_{AG}	10^5	0	0
	10^4	0	0
L_{BUDR}	10^5	0	0
	10^4	0	0
L_{AG}-L_{BUDR}	10^4	40	$1.2/10^3$
	10^3	3	$0.9/10^3$
L_{AG}-ETC	10^5	floating cells	ND
	10^4	floating cells	ND

The parental cell number was 1.8×10^6/ml for L_{AG}, 1.7×10^6/ml for L_{BUDR} and 1×10^6/ml for ETC. One ml of cells and 0.5 ml of UV-HVJ were mixed for fusion of cells of one parent, and 0.5 ml each of the two parental cells and 0.5 ml of UV-HVJ were mixed for fusion of cells of both parents.

[a] Cell number after fusion.

Hybrids from the L_{AG}-L_{BUDR} combination grew forming colonies in HAT medium, and their appearance was clearly identified. In the experiment shown in Table 1, 333 colonies appeared when 10^5 parent cells which had been treated with the virus were plated, 15 colonies from 10^4 cells per plate and one from 10^3 cells as the mean value. From the results, "the efficiency of hybrid formation" was calculated from the formula described in Materials and Methods as $1.2/10^3$, $0.56/10^3$ and $0.37/10^3$, respectively: the frequency was based on the original number of cells before fusion and the cell number decreased to 1/2.7 on cell fusion treatment. In the experiment shown in Table 2, the number of cells in the parent cell mixture decreased to 1/3.4 on cell fusion and in culture 40 colonies appeared from 10^4 cells and 3 colonies from 10^3 cells. The frequencies were calculated as $1.2/10^3$ and $0.9/10^3$, respectively. The frequency in Table 2 was higher than that in Table 1. This was due to the difference in condition of these cell samples.

These two results show that the highest frequency was obtained with the highest concentration of seed cells, the value being $1.2/10^3$. Littlefield (1966) reported a frequency of 10^{-6} on cultivation by Barsky's method of L_{AG} with L_{BUDR}, which be isolated himself. Thus the artificial fusion of two parent cells by UV-HVJ is very effective for hybrid formation and the frequency is 1 000 fold that in the mixed culture method.

Ehrlich ascites tumor cells (ETC) have been passaged serially in adult ddO mice for over

10 years in our laboratory. When transfered to in vitro cultures, ETC grew initially as a suspension and then rapidly degenerated. Therefore, hybrids of L_{AG} and ETC can be detected in cultures in HAT medium. After treatment of the cell mixture with UV-HVJ, the fusion index (Okada and Tadokoro, 1962) was 8.3. The cells were plated and on cultivation, hybrids grew vigorously and tended to float on the culture. As shown in Table 2, when 10^5 cells and 10^4 cells were inoculated per plate, the plates had become full in floating cells after 14 days. The frequency of hybrid formation could not be estimated accurrately as the number of colonies per input cells. However, hybrid progeny were definitely produced at a very high frequency after fusion by the virus.

2. *Hybrids of L_{AG} and L_{BUDR}*

Eight colonies were randomly selected from those appearing on the plate of 10^5 cells shown in Table 1, to confirm their hybrid characters. The capacities of these eight clones to grow in HAT, AG or BUDR medium were examined. Cells were inoculated into falcon plates at a concentration of 10^3 cells per plate. The medium was changed every week and after incubation for 20 days at 37 C, the number of colonies was estimated. The result is shown in Table 3. All clones formed colonies in HAT medium but their growth was completely inhibited in AG 10γ/ml medium or in BUDR 100 γ/ml medium and this can only be true of cells containing both thymidine kinase and guanilic acid and inosinic acid pyrophosphorylase. This result and the evidence that revertants of the parents did not appear, shown in Table 2, indicate that the clones are hybrids.

It is expected that 1) phenotypical expression of the hybrids should be different from that of the parents, 2) hybrids with different expression should appear at a high frequency. We selected three of the eight clones differing in the growth rate: clones No. 3, 5 and 6. The patterns of their colonies were clearly different, as shown in Fig. 1: clone 3 formed very large colonies while those of clone 5 were compact and small. The colonies of clone 6 were thin and diffuse, so that the number of colonies could not be determined exactly although in Table 3 it is shown as the highest.

These three clones were purified by cloning them more than three times, and then their morphological, chromosomal and growth characters were compared with those of the parents in monolayers, and in suspensions after trypsinization. The cells of the pro-

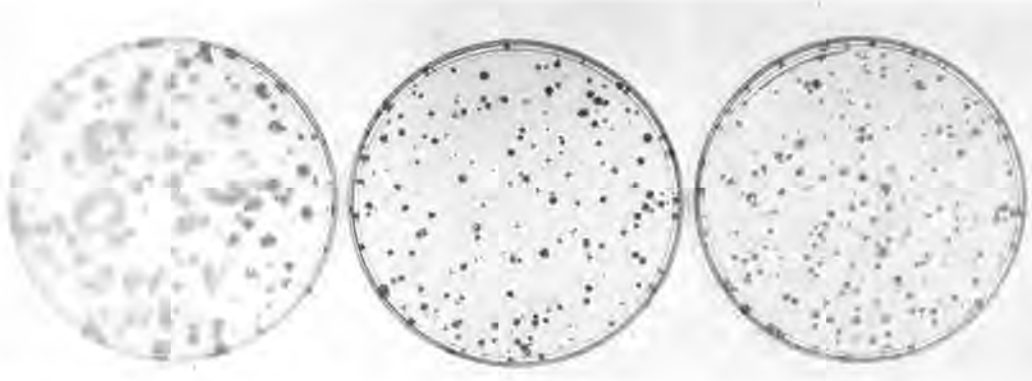


FIGURE 1. *Colonies of hybrid clones formed in HAT medium. Left: clone-3, Center: clone-5, right: clone-6*

TABLE 3. *Colony formation in HAT, AG and BUDR media.*

Clone	AG medium	BUDR medium	HAT medium
1	0	0	162
3	0	0	178
4	0	0	37
5	0	0	160
6	0	0	204
7	0	0	14
8	0	0	218

genies of the three hybrid clones were larger than the parent cells both in monolayer cultures and in suspensions formed after trypsinization of the monolayers (Fig. 2). The morphological character of clone 3 in monolayer culture was slightly different from that of clone 5. The cells of clone 3 were more fibroblastic than those of clone 5 and they had protrusions from their edges. Clone 6 was clearly different from clone 3 and 5. The cells appeared less refractible and flat, especially when unfixed cells were observed under a phase contrast microscopy. These morphological characters were stable and have been kept during serial passage of these cells.

Giant cells appeared more frequently in monolayer cultures of the hybrid cells more than in cultures of the parents. To see whether giant cell formation was mediated by HVJ, these hybrids in monolayers were tested by the hemadsorption method using chicken red blood cells. No cells adosorbed red blood cells. This shows that no reactivation of UV-HVJ occurred in the hybrid cells and giant cell formation was not due to fusion of cells by the reactivated virus.

The results of chromosomal analysis are shown in Table 4. The modal number of chromosomes was 106 in clone 3 and 103 in clone 6. These numbers may be compared with the total of the two parent chromosomes; the modal value of L_{AG} and L_{BUDR} being 57 and 50, respectively. In clone 5, the modal value was 78 and this value was clearly less

TABLE 4. *Mean chromosome numbers of parental cells and hybrid cells.*

Cell	Chromosome number	Range
L_{AG}	57	50- 63
L_{BUDR}	50	46- 54
ETC	45	41- 47
L_{AG}-L_{BUDR}-3	106	96-115
L_{AG}-L_{BUDR}-5	78	71- 85
L_{AG}-L_{BUDR}-6	103	101-105
L_{AG}-ETC	83	72- 86

Cells were treated with 0.2 γ/ml of Verban for 2 hr.

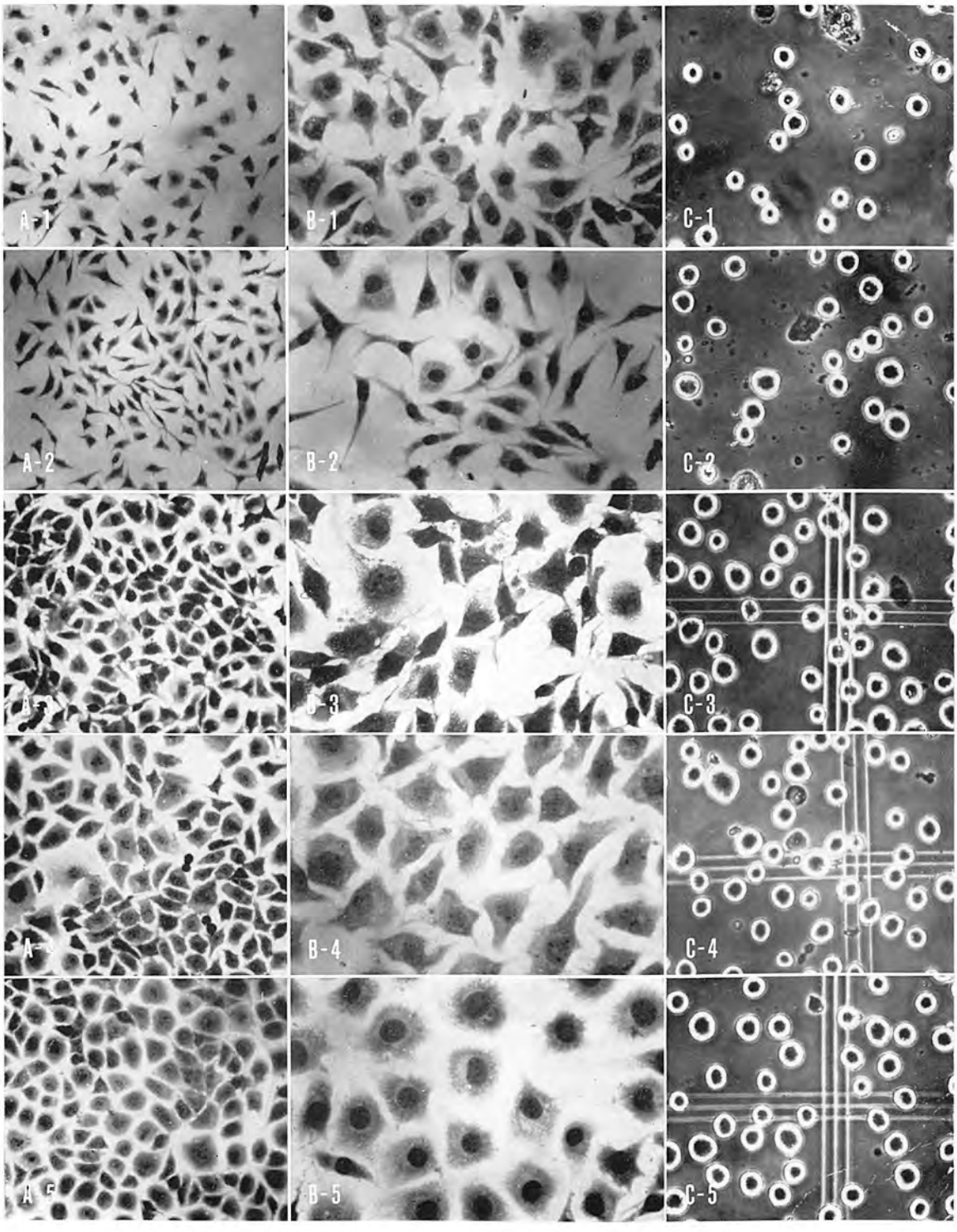
than of clones 3 and 6. As shown in Table 4, no parent cell containing over 70 chromosomes or under 40 chromosomes was detected. Therefore, clone 5 is not likely to be a revertant of a parent but a hybrid of the two L-mutants, a kind of segregation of the chromosomes occurring during growth and the finally stable chromosomes remaining as clone 5. Loss of chromosomes has been reported in some hybrids (Weiss and Ephrussi, 1966; Weiss and Green, 1967; Marin and Littlefield, 1968).

The growth rates of the two parents and the three hybrid clones were estimated and shown in Table 5 as generation times. The generation times of L_{AG} and L_{BUDR} were 18 hr and 24 hr, respectively. The growth rates of the hybrid clones were less than those of either parent and the generation time was estimated as 25 hr in clone 3, 39.5 hr in clone 5 and 43 hr in clone 6.

TABLE 5. *Generation time of parental cells and hybrid cells.*

Cell	Generation time (hr)
L_{AG}	24
L_{BUDR}	24
L_{AG}-L_{BUDR}-3	25
L_{AG}-L_{BUDR}-5	39.5
L_{AG}-L_{BUDR}-6	43

FIGURE 2. *Morphological feature of the parental cells and the hybrids cells.*
A: photographs of stained preparations at a low magnification.
B: photographs of stained preparations at a high magnification.
C: photographs of cell suspension under a phase-contrast microscopy.
1: L_{AG} cells, 2: L_{BUDR} cells, 3: hybrid clone-3, 4: hybrid clone-5, 5: hybrid clone-6.


A-1
B-1
C-1
A-2
B-2
C-3
B-3
C-3
B-4
C-4
B-5
C-5

DISCUSSION

Littlefield (1966) reported that the frequency of formation of hybrids of AG- and BUDR-resistant L cells was 10^{-6} on mixed culture of the parent cells. The present results show that the frequency was much greater when the two parent cell lines were fused artificially by UV-HVJ. Coon and Weiss (1969) reached the same conclusion on fusion of the two L-mutants of Littlefield or other parents by UV-HVJ. The frequency we obtained seems to be slightly higher than theirs.

When a mixture of two parent cell lines is fused by HVJ, the resulting mixture contains unfused parent cells, homokaryons and heterokaryons. The heterokaryons alone may form hybrid cells. The heterokaryon population consists of di-, tri-, tetra, and higher polykaryocytes. Yamanaka and Okada (1968) showed that dikaryons produced by UV-HVJ divided into daughter cells in culture as efficiently as the original cells before HVJ treatment. However, on increasing the number or nuclei per cell the efficiency decreased sharply. Chromosomal analysis in the present experment indicated that most of the hybrids seem to be formed from di-heterokaryocytes and this seems reasonable. We estimated the frequency of hybrid cell formation on the basis of the total number of parent cells used before fusion. We could not control cell fusion to produce di-heterokaryocytes alone or select di-heterokaryocytes from a randomly fused sample. If these were possible, the efficiency would be even greater.

From only 8 hybrid clones, three were selected and found to have different characters. Thus it seems that hybrids with different characters appear at high frequency among hybrids of the same parents. The difference in the characters of these hybrids could not be explained simiply from a difference in their numbers of chromosomes. Clones 3 and 5 were similar in morphology but clearly different from 6. The number of chromosomes in clones 3 and 6 was nearly the same of the sum of those in the two parents while the number in clone 5 was only 64% of this number. The growth rates of these three hybrids were less than those of the parents, the growth rate being fastest in clone 3 and slowest in clone 6. However, it is uncertain what factors determine the growth rate.

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