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Stable Haploid Cultured Cell Lines from frog Embryos*

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Abstract. Two haploid cell lines have been established from androgenetic embryos of the frog, *Rana pipiens;* one line has been maintained in culture for 150 generations, the other for 200 generations. Karyotypes of the two lines agree well with the standard for the species although some chromosomes show small differences in length. The cells multiply in the same defined basal medium used for culture of other anuran cell lines; this medium consists of the usual amino acids, vitamins, and serum macromolecules plus an exogenous purine source. Both the haploids resemble "permanent" cell lines in their prolonged multiplication in culture. The two lines differ in their mode of growth, one being epithelial-like, the other forming an overlapping meshwork of fibroblast-like cells. Both have the low plating efficiency characteristic of "unaltered" cells. These two lines are exceptional in their ability to compete successfully with the diploid variants which arise by endomitosis or cell fusion and which usually overgrow the haploid population. The more vigorous line, RPH 68.2A, should provide the long-desired haploid material for genetic studies in cell culture.

The use of cell cultures for genetic studies has been hampered by the difficulty of detecting recessive mutants in diploid cells. Since the experimental system which would yield the highest frequency of detectable mutations is a uniform population of proliferating haploid cells, we undertook some years ago to establish haploid cultured cell lines from embryos of the frog, *Rana pipiens*. Haploid amphibian embryos can be produced by experimental manipulation of the fertilization process[1] and the free-living embryos develop to an extent that permits initiation of serially cultured cell lines.[2] A further advantage of amphibian cells for genetic work is the possibility of using the nuclear transfer technique[3] to determine the nature of a presumed mutation and its expression in developing embryos.

By the tenth subculture generation, most haploid frog cell lines are overgrown by diploid variants which are presumed to arise either by endomitosis or cell fusion.[2] Two instances have now been observed in which nearly pure lines of haploid cells were maintained for more than 50 subcultures, with the occurrence of at least 150 mitotic generations.

In this paper we describe the origin and some pertinent properties of these two lines, and summarize the karyotype studies made to determine the extent to which the chromosomes constitute a true haploid set. We believe that one of these lines (RPH 68.2A) is the first haploid cell line of animal origin suitable for

genetic experimentation. It has been in use for genetic work in our laboratory for more than a year.

Materials and Methods. Culture techniques were those developed in our laboratory for frog embryo cell lines.[4] Androgenetic haploid embryos from *Rana pipiens* (J. M. Hazen Co., Alburg, Vt.) were produced by removing the maternal nucleus of the fertilized ovum with a glass needle.[1] At late tail bud stage, the embryos were bathed in Methiolate solution, washed, and exposed to EDTA in calcium- and magnesium-free Niu-Twitty solution[5] to loosen the epidermis. Carcasses freed of epidermis and yolky material were placed in small plastic Petri dishes, minced, and covered with growth medium.[6] The cultures were incubated at 25°C.

For chromosome preparations, cultures were exposed to colchicine for 6 hr or longer to allow metaphases to accumulate. After the cells were removed from the flask surface by trypsinization and pretreated with hypotonic KCl solution,[7] they were fixed in 3:1 methanol:acetic acid, spread on glass slides, and dried by being ignited. Measurements of chromosome length were made from photomicrographs of Giemsa-stained preparations: the length of chromosome arms was determined by application of thread along the center line of each chromatid.[8] Multiplication rates of cultures were estimated from counts of the number of cells in a series of sample areas (windows) of the growth surface.[4]

For measurements of cell volume, and of nucleic acid and protein, cells were removed from the growth surface by trypsinization. The amounts of DNA, RNA, and protein per cell were determined according to standard procedures using the diphenylamine, orcinol, and Lowry reactions, respectively.

Results and Discussion. Derivation of the haploid lines: The two lines to be described are the only haploid lines which have been established to date; they derive from several of the more than 150 cultures prepared since the present medium was adopted. Line RPH 67.205 was initiated from tissue of about 12 embryos in August 1967; line RPH 68.2A from about 9 embryos in January 1968. Sperm from a single male frog was used to fertilize each set of eggs.

In a consideration of the possible reasons for the stable haploid nature of these lines, several observations may be pertinent. First, in both cases the series of primary cultures produced from the same sperm and clutch of eggs showed exceptionally vigorous growth from the first day. Second, line RPH 67.205, which was consistently monitored for its content of haploid cells, remained haploid from its initiation as shown in Table 1. In this series, two brother cultures were 48 and 66 per cent haploid, respectively, by subculture 6, although many such lines are diploid by subculture 10. Finally, both stable haploid lines were obtained from experiments in which cultures of the same parentage gave rise to diploid

TABLE 1. *Percentage frequency of haploid metaphases in various subculture generations of line RPH 67.205.*

Subculture	Haploids	Subculture	Haploids
6	90	26	97
7	85	30	91
15	(87)	31	84
19	100	36	98
25	84	52	84
26	92		

The frequency of haploid metaphases was estimated from chromosome counts under oil immersion; the first 50 metaphases encountered in scanning the slide were counted. All plates with 10 to 14 chromosomes were included as haploids.

The datum in parentheses is an estimate based on counts of nucleolar number; i.e., 87% of the interphase nuclei exhibited a single nucleolus.

lines. These observations suggest that only rarely does a male frog possess a genotype relatively free of defective loci and that, as a result of segregation, exceptional sperm may occur which possess all of the genes required for vigorous haploid growth in culture.

Cloning from mixed populations has so far been unsuccessful as a means of establishing new haploid lines, although haploid cloned lines have been established from RPH 68.2A. Diploid colonies are also obtained from this line, suggesting that a lethal diploid condition does not explain the stable haploid state. The plating efficiency of both the haploid and diploid lines is 10–15 per cent, as would be predicted on the basis of data for nontransformed mammalian lines.

Nutritional requirements: The mean doubling-time of line RPH 68.2A is about 40 hours at 25°C in the standard growth medium (diluted Leibovitz L-15[9] supplemented with 10% fetal calf serum). A comparable rate of growth is characteristic of the diploid cell lines. If, in place of whole serum, the synthetic medium is supplemented with serum macromolecules purified by filtration through Sephadex G-25,[10] the cells fail to multiply (Fig. 1). However, the addition of any one of a number of purine compounds to this minimal medium will allow the cells to grow, although more slowly than when whole serum is used. A purine is also required by the several other frog cell lines which have been tested.[11]

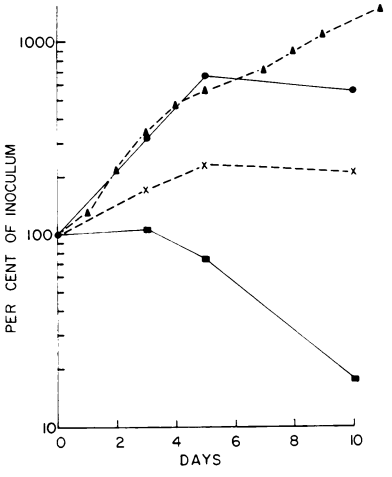

FIG. 1.—Growth of RPH 68.2A cells in L-15 medium with the following supplements added: ■—■, a macromolecular portion of fetal calf serum purified by Sephadex G-25 filtration; X---X, the same supplement plus 0.2 mM hypoxanthine; ●—●, 10% fetal calf serum; ▲--▲, 10% fetal calf serum. The first three growth curves are for cells in plastic flasks; the last growth curve is for cells in a roller bottle. At zero time the cell density for each curve was about 1.5×10^4 cells/ml or 3×10^3 cells/cm^2.

Line RPH 68.2A multiplies readily in roller bottles, in the same medium used for stationary cultures (Fig. 1). However, when placed in a calcium- and magnesium-free modification of Leibovitz medium and maintained as suspensions, the cells fail to increase in number although they remain morphologically intact for up to three days.

Karyotype: A typical metaphase of RPH 68.2A is shown in Figure 2. It has the modal chromosome number of 13, the haploid number. When tabulating the proportion of haploids in a culture, metaphases with 11 or 12 chromosomes are included as haploids; these constitute 10–15 per cent of the total count and may be due to loss of chromosomes during slide preparation. In line RPH

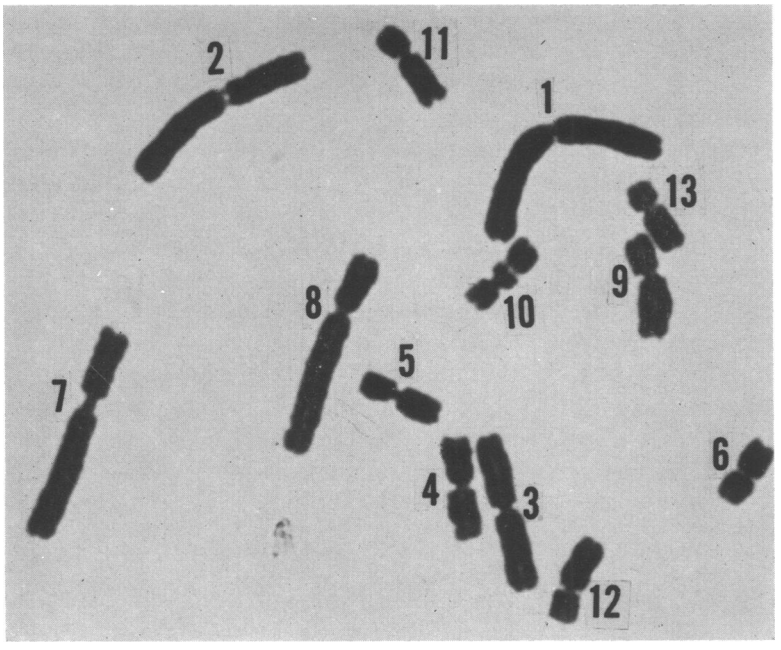

FIG. 2.—Metaphase plate from line RPH 68.2A at subculture 32. The 13 chromosomes are numbered according to the system of DiBerardino. Chromosome 10 has the prominent secondary constriction believed to be the nucleolar organizer. ×2000.

68.2A the percentage of metaphases which are haploid is 95 or more and in RPH 67.205, it is 85 per cent. The remainder of both the populations is diploid.

To determine whether the chromosomes of these lines constitute a true haploid set, they were compared to a standard species karyotype, using measurements made by DiBerardino on 48 metaphases from various developmental stages of *Rana pipiens* embryos.[12] (Original data were kindly made available for our use by Dr. DiBerardino.) In Figure 3, measurements of relative length of 25 haploid metaphases from each line are compared with similar data from the embryo tissues. The chromosome length and the position of the centromeres are in approximate agreement with the standard. The significance of the small differences in length were calculated for a number of chromosomes using Student's *t* test. The results are shown in Table 2. Differences from the standard karyotype (significant at the 1% level) were found for several chromosomes in each line and between the two haploid lines. The chromosomes which differed from the standard karyotype were not the same in the two lines. Differences found were both positive and negative and ranged from 4 to 11 per cent of a chromosome length. In the absence of any detailed analyses of chromosome length in various *Rana pipiens* populations, the genetic significance of the observed differences is difficult to assess. It is possible that duplication of small crucial genetic regions is responsible for the survival of the haploid lines.

The continued euploidy of the haploid lines is in marked contrast to the aneuploidy of mammalian cell lines which have been cultured for comparable num-

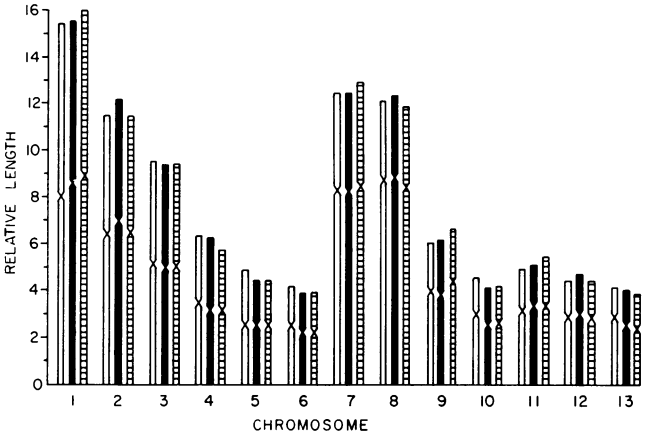

FIG. 3.—Idiogram comparing chromosome lengths and centromere positions from metaphases of the haploid lines with measurements of embryo metaphases. Ordinate: relative chromosome length as per cent of the total chromosome length per metaphase. Abscissa: chromosome number according to the system of DiBerardino.[12] The centromere position is shown by the notched region of each bar. Open bars: embryo chromosomes; solid bars: line RPH 68.2A; hatched bars: line RPH 67.205.

bers of generations. The minimal genetic constitution of haploids may favor stability; any aneuploid would be substantially unbalanced.

Nucleic acid and protein content: The haploid nature of the cells of line RPH 68.2A is supported by a comparison of its DNA content per cell to that of a diploid line. Nucleic acid and protein content per cell were determined in cells prepared from a roller bottle culture of the haploid line and from a similar culture

TABLE 2. *Comparison of relative chromosome length in haploid cultured lines and in embryonic cells of Rana pipiens.*

	Chromosome no.	Relative Length (%)*			
		Haploid	Embryo	Difference	P
RPH 68.2A	2	12.22	11.51	+0.71	<0.1%
	5	4.38	4.83	−0.45	<0.1%
	6	3.87	4.26	−0.39	<0.1%
	10	4.06	4.51	−0.45	<0.1%
	12	4.71	4.39	+0.32	<1.0%
RPH 67.205	4	5.70	6.19	−0.49	<1.0%
	9	6.58	5.98	+0.60	<0.1%
	11	5.46	4.88	+0.58	<0.1%
		RPH 67.205	RPH 68.2A		
RPH 68.2A and RPH 67.205	2	11.47	12.22	−0.75	<0.1%
	7	12.91	12.44	+0.50	<1.0%
	8	11.83	12.34	−0.51	<1.0%
	9	6.58	6.12	+0.46	<1.0%
	12	4.42	4.71	−0.29	<1.0%

* The relative length of a chromosome is the length of the chromosome divided by the total length of all the chromosomes in that metaphase times 100. Each datum is the mean value from measurement of 25 metaphases for each haploid or 48 metaphases for the embryo cells. Chromosomes in which no significant difference was observed are omitted from the table.

of RPH 67.132, a near diploid of haploid origin.[13] (We are indebted to Robert P. Perry, and Dawn E. Kelley for providing these data.) The DNA content per haploid cell (4.66 pg) is almost exactly half that of the diploids (9.17 pg). The ratio of protein to RNA is similar in the two cell lines, but the ratios of protein to DNA and of total RNA to DNA are about one-fourth higher in the haploids than in the diploids. The basis of this difference in cellular composition requires further investigation.

Cell volume: The volume of a haploid cell in culture is half that of a diploid cultured cell; there is a similar size difference between the cells of haploid and diploid embryos, as has been known for some time. Table 3 shows the distribution of cell volumes found in cell suspensions prepared from the two haploid lines, from a normal diploid cell line (RPH 67.132) and from a cloned line (RPH 67.134c4) in which the chromosome number is near triploid. It may be seen that the modal cell volumes are approximately in the ratio of the chromosome numbers. It should be noted that all four of these lines were initiated from haploid cultures, providing direct proof of the dependence of cell volume on chromosome number.

TABLE 3. *Comparative cell volume of cultured frog lines.*

Cell line	Subculture	Modal cell volume*	Chromosome constitution
RPH 68.2A	44	20	95% haploids
	47	20	
RPH 67.205	44	26	85% haploids
	49	31	
RPH 67.132	75	39	86% diploids, 14% tetraploids
	83	44	
RPH 67.134c4	64	70	heteroploid, modal number 40
	69	80	

* Distribution of cell volumes was plotted using the Coulter Counter (model B), with increments of 2.5 threshold units per window. The data are in threshold units.

Cellular morphology: The mode of growth of cultured cells is often correlated with the nature of their gross chromosome constitution. However, not only do the two haploid lines reported here differ substantially from one another, but line RPH 68.2A is unique among our frog cell lines in that it more nearly resembles neoplastic cell lines than normal ones. In sparsely seeded cultures, cells of this line are commonly elongated and fibroblastlike and, less frequently, attenuated and spindle shaped. As the cells become more crowded, there appears to be little contact inhibition of movement[14] since extensive overlapping is observed. Multicellular cords and aggregates are formed, so that dense cultures assume the stellate netlike appearance shown in Figure 4*a*. (In roller bottle cultures, rounded compact aggregates of adhering cells form and eventually reach several millimeters in cross section.) The nuclei show no prominent heterochromatic blocks, as is characteristic of amphibian cells, and a single nucleolus is found in more than 95 per cent of the nuclei. Attenuated cytoplasmic processes connect widely separated cells.

Line RPH 67.205 is more typical of epithelial-like frog cell lines.[13, 15] The cells tend to be extremely flattened and form limited overlaps (Fig. 4*b*). Post-

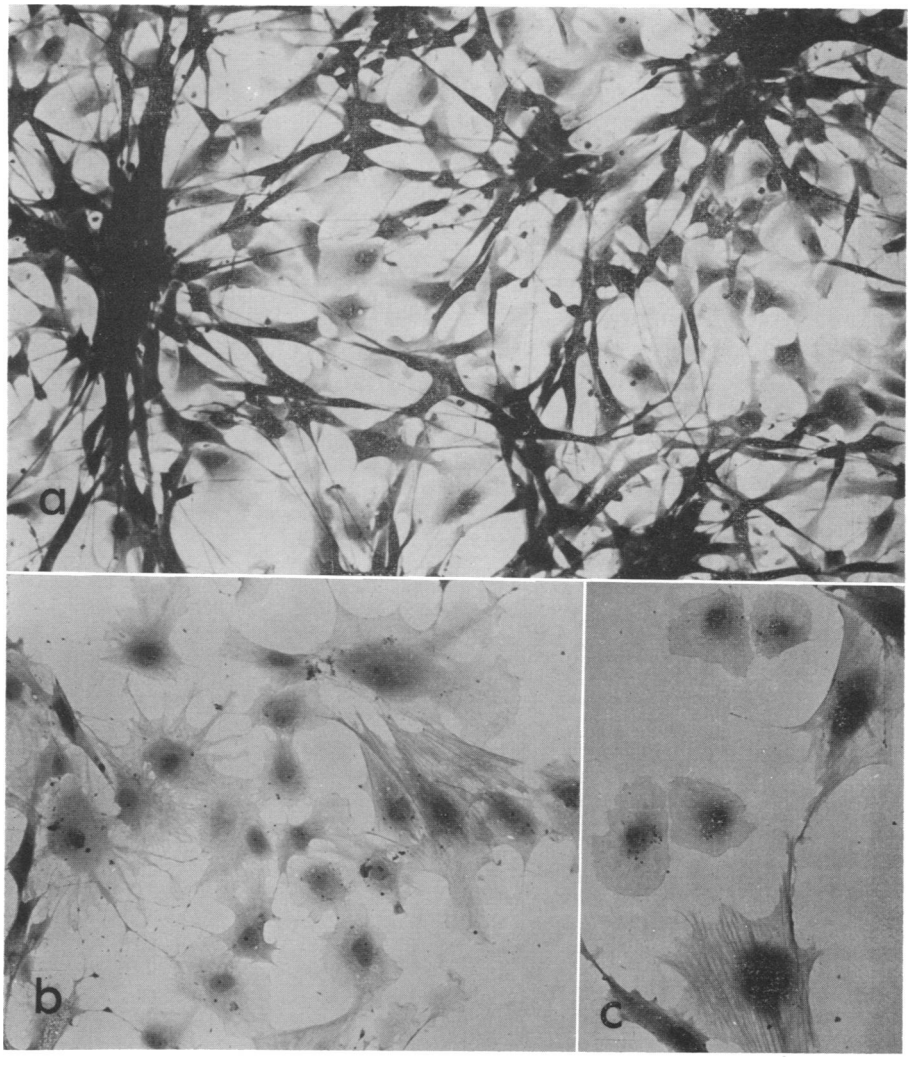

FIG. 4.—Haploid cultured frog cells, fixed in glutaraldehyde and stained with iron hematoxylin. (*a*) Line RPH 68.2A, showing netlike, multicentric growth. The cells overlap freely and form cords and aggregates. Attenuated cytoplasmic processes connect widely separated cells. ×200. (*b*) Line RPH 67.205, showing flattened polygonal cells with limited tendency to form cytoplasmic overlaps. ×160. (*c*) Line RPH 67.205, from a sparse culture. Two postmitotic pairs of apparently haploid cells may be recognized by the small size of the nuclei and the presence of single nucleoli.

mitotic cell pairs remain in contact along an extended common border (Fig. 4*c*) and form epithelial-like plaques of polygonal cells. Enlarged and presumably diploid cells with two nucleoli are more common in this line. Very large, often multinucleated, cells with prominent cytoplasmic striations are also found (Fig. 4*c*).

Conclusion. The cell line RPH 68.2A is the result of attempts begun almost a decade ago to develop a haploid amphibian cell line for genetic studies. In addi-

tion to the unique property of a stable haploid chromosome complement (now over 200 generations), these cells have other advantages. They can be grown in both stationary and roller bottle cultures; clonal colonies can be routinely recovered; the cells can be frozen for storage; the chromosome constitution is simple enough to facilitate cytogenetic work; a basal medium has been defined, and the cells can be used as donors in nuclear transfer studies of mutants.

In this laboratory we are currently isolating and analyzing mutants with resistance to certain drugs (puromycin, BUdR) and attempting to recover auxotrophic mutants. Studies of variants by cytochemical techniques and by nuclear transfer are also in progress. The possibility that the cells are tumorigenic is being tested by injecting them into anterior eye chambers of adult frogs and into the tail tips of tadpoles.

The stimulus to begin this work came from Dr. Jack Schultz, who pointed out to us the genetic possibilities of amphibian haploid cells and encouraged our persistence. The authors respectfully dedicate this paper to him.

Susan Schatz Reed provided valued assistance in the management of the cultures and in performing the measurements of the chromosomes.

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