

CONTROLLED PRODUCTION OF PROLIFERATING SOMATIC CELL HYBRIDS

ROBERT J. KLEBE, TCHAW-REN CHEN, and FRANK H. RUDDLE

From the Department of Biology, Kline Biology Tower, Yale University,
New Haven, Connecticut 06520

ABSTRACT

The techniques described permit the controlled production of large numbers of proliferating somatic cell hybrids in a relatively short period of time. Sendai virus is used to promote cell hybridization. β -propiolactone is employed as the inactivating agent of Sendai virus since it produces complete loss of viral infectivity while preserving viral fusion capacity. Cells are fused in monolayer, instead of in suspension, since fixing cells in two dimensions permits one to control cell contacts during the fusion event through the expedient of varying multiplicities of the parental cells and the total cell density. Under the conditions described, a several hundred fold increase in the number of hybrid clones obtained is seen as compared to the controls.

INTRODUCTION

Somatic cell hybridization of mammalian cells involves the fusion of parental cells to produce a single proliferating hybrid cell with the genetic complement of both parental cells (15). Okada and co-workers (17-19) have demonstrated that live Sendai virus can be used to fuse suspensions of mononucleated tissue culture cells to yield non-proliferating single cells which contain large numbers of nuclei. The feasibility of using UV-inactivated Sendai virus in promoting the production of growing somatic cell hybrids was first described by Yerganian and Nell (25). Heretofore, somatic cell hybridization relied on chemical selection for infrequent spontaneous fusion events (15). The techniques to be described permit the controlled quantitative production of large numbers of growing hybrid clones with the use of convenient numbers of cells and an uncomplicated methodology.

MATERIALS AND METHODS

Preparation of Inactivated Sendai Virus

Sendai virus (Para-influenza I) was grown and harvested according to Okada, Murayama, and

Yamada (19). In brief the method is as follows: 0.2 ml of a 10^{-6} dilution of infected allantoic fluid [1000 hemagglutinating units of virus (HAU)/ml] was inoculated into the allantoic cavity of 9-day embryonated eggs. After 72 hr, the eggs were placed in the cold room at 7°C for 12 hr to induce clotting in order to prevent bleeding during the harvesting procedure. Yields of 1000 HAU/ml or more were routinely obtained as determined by the Salk pattern method (3), employing 0.5 ml of 0.5% chicken red blood cells with phosphate-buffered saline (PBS) as diluent and 0.5 ml of two-fold dilutions of infected allantoic fluid. Titrations were carried out in 96-U-WS Dispo-Trays (Linbro Chemical Co., New Haven, Conn.). The infected allantoic fluid was clarified at 2000 *g* for 20 min and the virus was pelleted at 16,000 *g* for 1 hr. The pellet was resuspended in one-tenth the starting volume with PBS + 1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). The addition of bovine serum albumin at this step prevents a two-fold decrease in virus titer during the inactivation step. This material was clarified again and immediately inactivated with β -propiolactone (β -prone, Fellows Testagar, Detroit, Mich.) as described by Neff and Enders (16). β -prone was diluted to 10% with triple glass-distilled water and then the 10%

aqueous solution was diluted to 2% with a saline bicarbonate solution (1.68 gm of NaHCO_3 + 0.5 ml of 0.4% phenol red + 100 ml of isotonic NaCl). A virus suspension containing 10,000 HAU/ml was brought to a final concentration of 0.05% β -prone with the above 2% β -prone solution and incubated with shaking at 4°C for 10 min, at 37°C for 2 hr to inactivate the virus, and at 4°C overnight to insure the complete hydrolysis of the remaining β -prone. The inactivated virus was diluted to 2000 HAU/ml with culture medium (Dulbecco-Vogt (24) modified Eagle's medium + 10% agamma calf serum + 100 units/ml penicillin + 100 $\mu\text{g}/\text{ml}$ streptomycin [GIBCO, Grand Island, N. Y.]) and frozen at -70°C in ampoules.

Adaptation of Cells to Tissue Culture and Isolation of Mutants

We would like to acknowledge the gracious assistance of Dr. Albert Claude in providing us with a transplantable renal adenocarcinoma derived from the BALB/cd strain of mice in which renal adenocarcinoma occurs at a high incidence in adults (9). The tumor cells were adapted to in vitro culture by alternate animal-to-tissue culture passage (2). Tumor tissue was fragmented mechanically into small clumps and single cells, and was planted in F-12 medium + 10% agamma calf serum + 100 units/ml penicillin + 100 $\mu\text{g}/\text{ml}$ streptomycin (GIBCO). After 1 wk in culture, the surviving cells were inoculated into the appropriate host and the process was repeated again. After two animal-to-tissue culture passages, a population of proliferating renal adenocarcinoma cells was obtained which, however, was contaminated by normal fibroblasts of stromal origin. The renal adenocarcinoma cells were freed of the contaminating fibroblasts by growing the mixed population in suspension in untreated plastic bacteriological petri plates. Under these conditions, the contact-inhibited normal cells perish while the non-contact-inhibited renal adenocarcinoma cells proliferate. Since the renal adenocarcinoma forms diffuse colonies when seeded at low density in monolayer, the cell population was serially cloned three times in 0.125% semisolid agarose (5). The cloned population of renal adenocarcinoma cells is termed Renal. All subsequent experiments were performed with Dulbecco-Vogt medium.

A nonreverting 8-azaguanine resistant mutant was obtained by exposing 10^7 cells to 5 $\mu\text{g}/\text{ml}$ of 8-azaguanine (AG) (Mann Research Laboratories, New York, N.Y.) and then by exposing the survivors to 20 $\mu\text{g}/\text{ml}$ of the drug. The resistant mass population was then cloned and each clone derived was tested for revertants in the selection medium HAT (15). One clone, termed RAG, was identified which has proven to be free of revertants despite prolonged

growth in culture medium lacking the drug. This cell line has been submitted to the American Type Culture Collection.

Biochemical Selection of Somatic Cell Hybrids

The biochemical selection technique of Littlefield (15) was employed. The selection medium consists of Dulbecco-Vogt medium + 10% agamma calf serum + 100 units/ml penicillin + 100 $\mu\text{g}/\text{ml}$ streptomycin, supplemented with 1.6×10^{-6} M thymidine, 10^{-4} M hypoxanthine, and 4×10^{-7} M aminopterin (15). In brief, the basis of the selection procedure is the following. Aminopterin inhibits dihydrofolate reductase which converts dihydrofolate to tetrahydrofolate (12). In the absence of the cofactor tetrahydrofolate, the *de novo* synthesis of purines is blocked (12) and, in addition, the methylation of dUMP to dTMP by thymidylate synthetase (EC 2.1.1.2) (12) and the conversion of serine to glycine by serine transhydroxymethylase (EC 2.1.2.1) (12) are inhibited. Cells treated with aminopterin proliferate if exogenous hypoxanthine, thymidine, and glycine (supplied in Dulbecco-Vogt medium [24]) are added (10). The conversion of hypoxanthine into purines is catalyzed by hypoxanthine-guanine phosphoribosyl transferase (HG-PRT) (EC 2.4.2.8). Exogenous hypoxanthine is not utilized by 8-azaguanine-resistant mutants (22), since HG-PRT is reduced in activity or lacking (22). Cells resistant to 5'-bromo-deoxyuridine (BUdR) do not utilize exogenous thymidine (8) due to the lack of the enzyme thymidine kinase (EC 2.7.1.21) (8). Thus, in Dulbecco-Vogt HAT medium, the parental 8-azaguanine-resistant and BUdR-resistant cells die while somatic cell hybrids between these mutants proliferate (15) due to intergenic complementation.

Monolayer Fusion Procedure

In the experiments to be described a nonreverting 8-azaguanine-resistant renal adenocarcinoma, RAG, derived from the BALB/cd strain of mice (described above), and a nonreverting BUdR-resistant L-cell LM(TK⁻), (13) derived from a C3H mouse were studied; however, similar results have been obtained with several other pairs of cell lines. The fusion indices (F.I.) (18) of the RAG and LM(TK⁻) cell lines are similar, being 0.6 and 0.4, respectively, at 1000 HAU of inactivated virus under Okada's conditions (18). In all cases, stationary phase populations were used as starting material since it has been reported that cells in interphase may undergo chromosome pulverization as a result of fusion with a cell in mitosis (23). At the time of the addition of virus, cells were in mid-lag phase as deduced from their growth curves.

Following trypsinization and resuspension in fresh medium, a predominantly single cell suspension of the two cell lines was mixed in culture tubes at multiplicities and densities appropriate to the experiment prior to being planted in Falcon T-30 flasks (Falcon Plastics, Los Angeles, Calif.) (surface area = 25 cm²). The culture flasks were incubated for 16 hr at 37°C to allow the cells to attach and spread with a minimum of cell division. Once the cells had spread, the medium was removed and the flask was placed at 7°C for 10 min. 1 ml of chilled virus suspension was then added and the cells were left at 7°C for another 20 min to permit viral adsorption. At the end of the adsorption period, 4 ml of warm culture medium was added, and the cultures were immediately returned to the 37°C incubator for 2 hr. At the end of this time, the medium was removed and the cell sheet was washed twice

with culture medium before the selection medium, HAT, was added. In this medium, only fusion products between parental cells can grow, whereas the parental cells die. After 10 days in HAT medium, the culture flasks were washed with isotonic saline, air dried, and stained with Wright stain for colony counting.

Biochemical and Cytogenetic Methods Employed in Characterizing the Somatic Cell Hybrids Produced with Inactivated Sendai Virus

The starch gel assay for glucose-6-phosphate isomerase (EC 5.3.1.9) has been described by DeLorenzo and Ruddle (7).

Chromosome preparations were made according

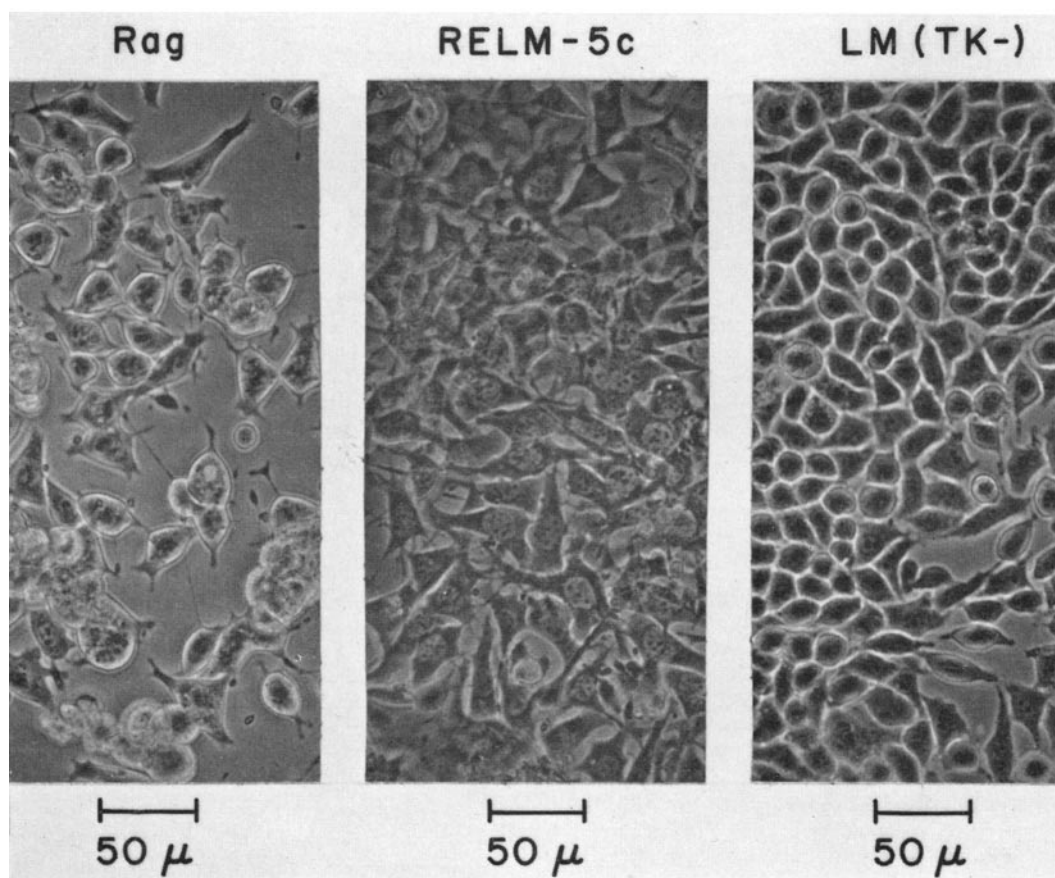


FIGURE 1 Phase-contrast photomicrographs of the parental and hybrid cell lines. Rag has an ameboid to epithelioid morphology with prominent cytoplasmic processes; LM(TK⁻) displays a characteristic cobble-stone growth pattern; and RELM appears to possess morphological properties which are the intermediate of the parental cell lines. Note the large number of prominent nucleoli in the hybrid cells.

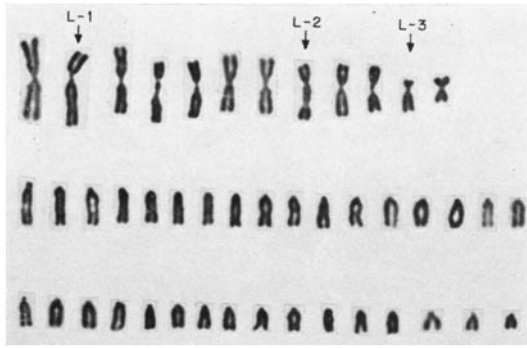


FIGURE 2 LM(TK⁻) karyogram. Arrows indicate three marker chromosomes (L-1, L-2, and L-3) which are readily distinguishable in the hybrid metaphase plate.

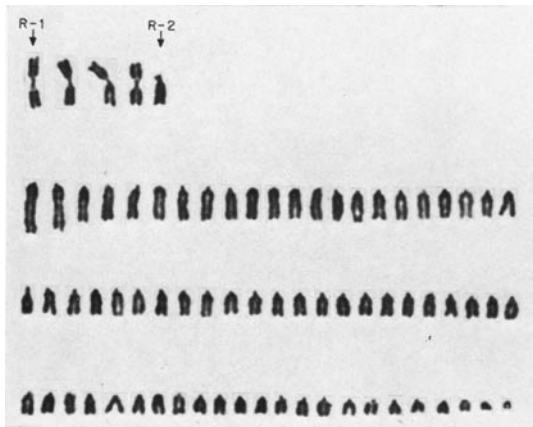


FIGURE 3 RAG karyogram. Arrows indicate two marker chromosomes (R-1 and R-2).

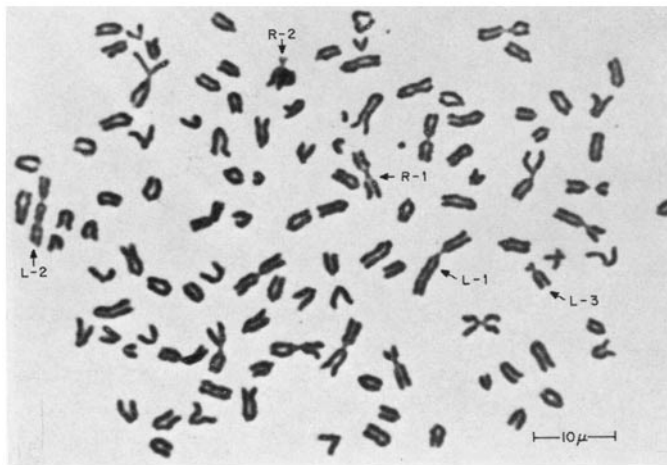


FIGURE 4 Metaphase plate of a hybrid cell [LM(TK⁻) × RAG.] Arrows demonstrate marker chromosomes derived from both parental cell lines.

to the method described by Chen (4). Semiconfluent cultures were treated with a final concentration of 1 $\mu\text{g}/\text{ml}$ of colcemid (CIBA Products Company, Fair Lawn, N.J.) for 4 to 5 hr, collected with hypotonic trypsin-versene solution (2 g/liter NaCl, 0.2 g/liter KCl, 0.2 g/liter KH_2PO_4 , 1.15 g/liter Na_2HPO_4 , 1 g/liter glucose, 0.10 g/liter trypsin 1:250 (Difco Laboratories, Detroit, Mich.), and 1.0 g/liter disodium versene), swollen in hypotonic KCl (0.075 M) for 6 min, pelleted, and fixed twice for 15 min with 1:3 acetic acid-methanol. Slides were prepared by air drying and were stained with 1.5% aceto-orcein.

RESULTS

Hybrid Characteristics

After 3–4 days in HAT medium, the parental cells had rounded up and begun to detach from the substratum; and within 7 days, colonies of cells appeared that were larger than either of the parents and morphologically dissimilar (Fig. 1). Several of these colonies were cloned and characterized as hybrids on two grounds. First, marker chromosomes derived from each of the parents were present in each independently derived clone (Figs. 2–4) and, secondly, the expression of the parental and heteropolymeric forms of the glucose-6-phosphate isomerase isozymes serves to indicate the presence in the hybrid cells of allelic genes derived from the BALB/cd- and C3H-derived parental cell lines (Fig. 5). The hybrids of the renal adenocarcinoma and the LM(TK⁻) cell lines have been designated RELM followed by a number indicating their clonal origin.

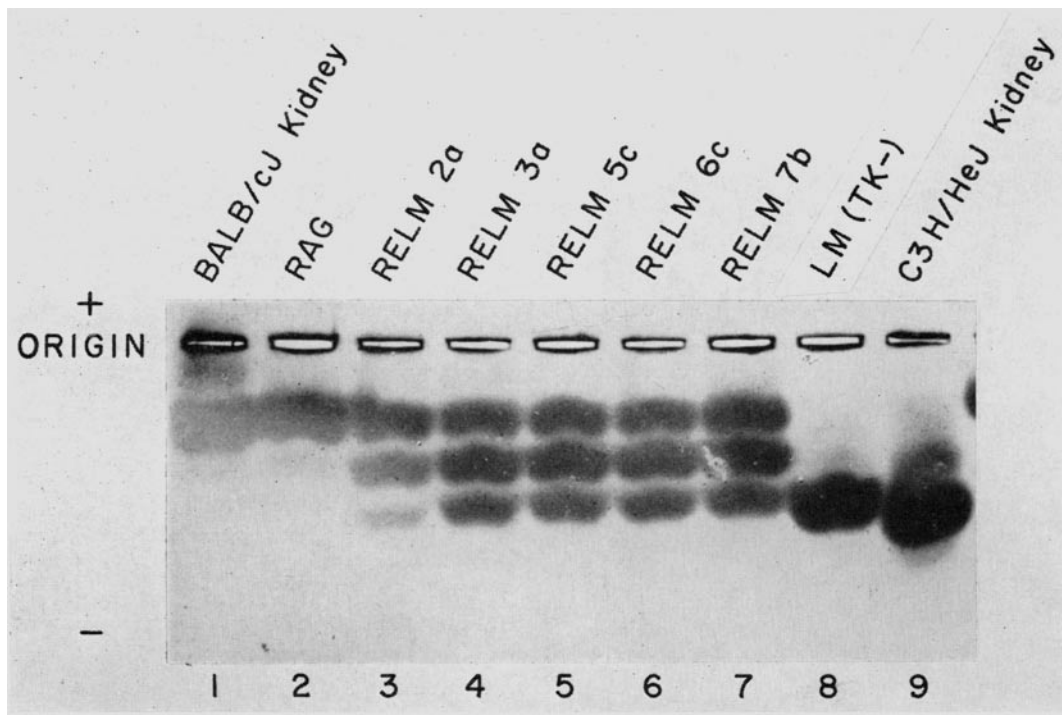


FIGURE 5 Glucose phosphate isomerase isozyme patterns. Channel 1, BALB/cJ kidney homogenate; Channel 2, BALB/c derived renal adenocarcinoma cell culture; Channels 3-7, hybrids; Channel 8, C3H derived cell line; Channel 9, C3H kidney homogenate. Note that the cell cultures retain isozyme patterns distinctive of the mouse strain of origin and also note that the independently derived hybrid clones express the isozyme patterns of both cell lines and a band migrating at an intermediate rate between the parental bands, indicating the formation of a heteropolymeric molecule with subunits coded by both parental genomes.

Quantitation of Cell Hybridization

The parental cell lines were mixed in a 1:1 multiplicity and then planted at 5×10^5 , 10^6 , and 2×10^6 total cells/25 cm² surface area culture flask and treated with inactivated virus as described previously. The yields of proliferating hybrid clones is dependent on the titer of virus employed and increases as the cell density increases (Fig. 6). The number of hybrid clones produced increases as the ratio of virus particles to cells increases up to a plateau at 2000 HAU, as shown in Fig. 7. Above 2000 HAU, a slight decline in the number of virus-promoted hybrid clones is encountered; and this effect is most pronounced at the lowest cell density. The decrease in hybrids at low cell density and high virus titer may be due to the inability of cells to repair more than a certain number of virus-induced cell surface lesions in a given period of time. It should be noted that

the ability of Sendai virus to fuse cells falls off above 1000-5000 HAU (18). When the parental cell lines are mixed in different multiplicities, the optimal yield of hybrid clones occurs at or near a 1:1 ratio of the parents (Fig. 8).

Quantitatively, no deterioration of the ability of β -prone inactivated virus to produce growing hybrids occurs over a period of 5 months when the virus is stored ampouled at -70°C (Table I). Ampoules of virus are frozen rapidly to -70°C and thawed rapidly to 37°C .

DISCUSSION

In order to obtain large numbers of growing somatic cell hybrid clones, the viral fusion method of Okada and co-workers (18), the viral inactivation procedure of Neff and Enders (16), and the monolayer fusion stratagem of Kohn (14) have been employed for the reasons cited below.

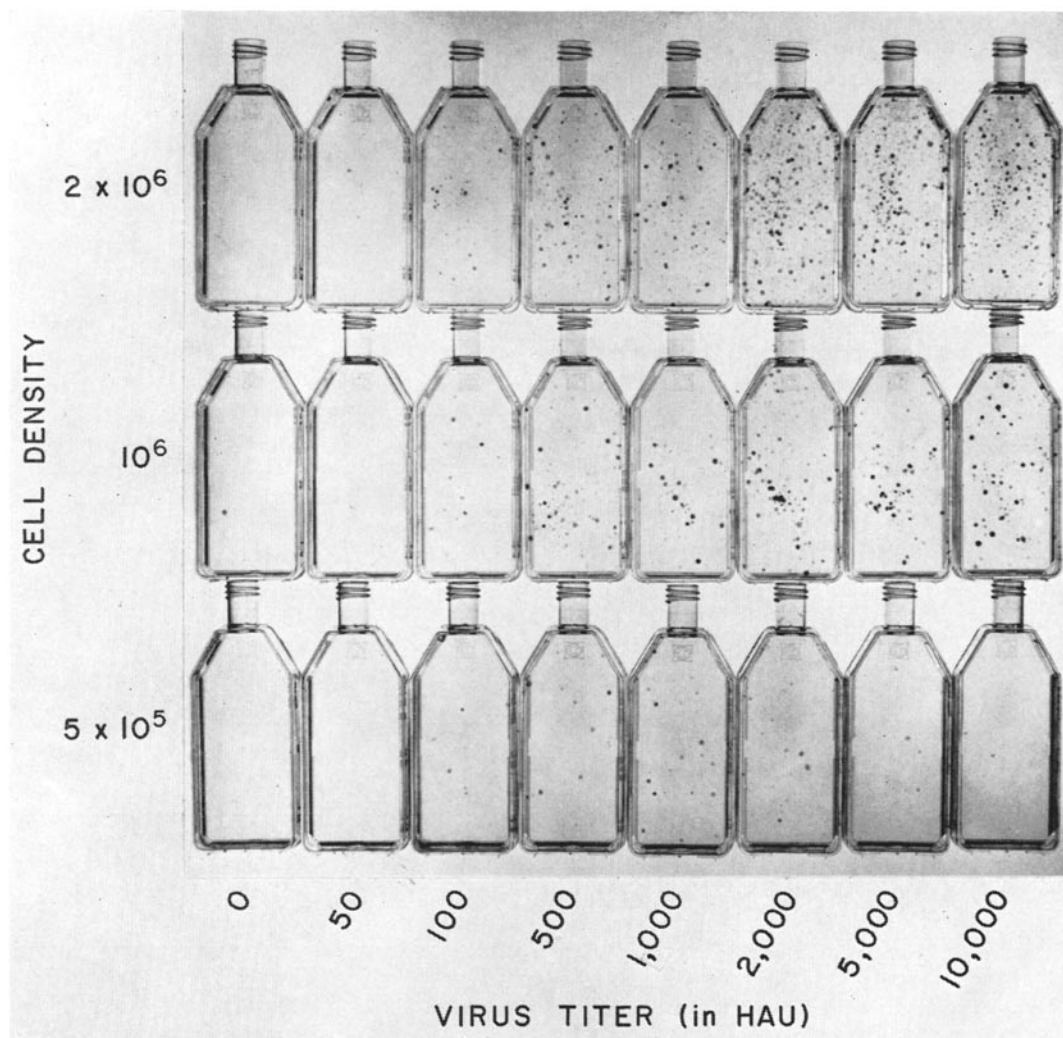


FIGURE 6 Hybrid clones produced at varying virus titer and cell densities. Note particularly the heterogeneity in clonal sizes.

First, Harris et al. (11) report that UV inactivation reduces viral infectivity by a factor of 10^6 , while it has been our experience and that of Neff and Enders (16) that β -prone completely inactivates Sendai virus. Since 1 HAU of Sendai virus represents 2.4×10^7 virus particles (17) and since the particle-to-infectivity ratio for Sendai virus is about 10:1 (17), one would be introducing approximately 2.4×10^2 live virus particles in a hybridization experiment employing only 100 HAU of UV-inactivated virus. Infection of cells at a low multiplicity of infection can lead to the permanent residence of para-influenza viruses in

their host in a carrier state without loss of cell viability or noticeable cytopathic effects (21), but with the chance of chromosomal abnormalities (23). Chemical inactivation of Sendai virus has the double advantage of technical simplicity and of producing complete viral inactivation. β -proprionolactone is an alkylating agent which reacts with the guanosine moiety in the viral RNA when the reaction is carried out in a slightly basic environment (20). Since the para-influenza group of myxoviruses does not exhibit multiplicity reactivation (1), hybridization experiments, which by necessity must employ a high multiplicity of virus

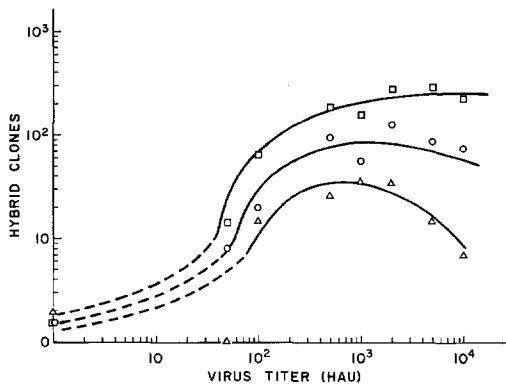


FIGURE 7 Relationship between virus titer employed and yield of hybrid clones. (\square), 2×10^6 total cells; (\circ), 10^6 total cells; (\triangle) 5×10^5 total cells.

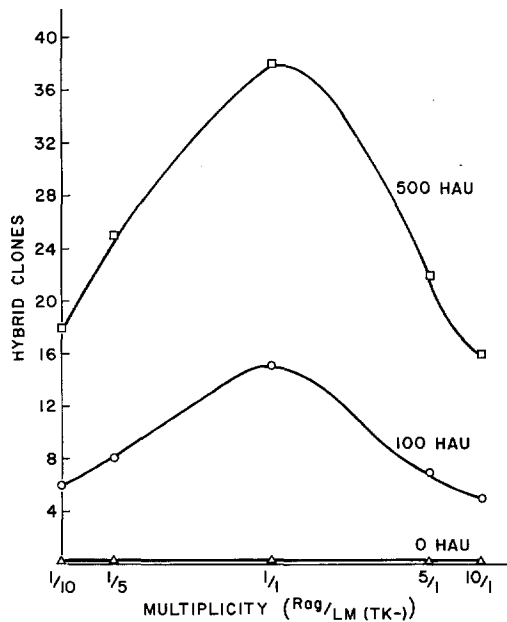


FIGURE 8 Relationship between input multiplicity of parental cells and yield of hybrid clones at constant cell density and virus titer. All points represent a total cell density of 10^6 cells. (\square), 500 HAU employed in fusion; (\circ), 100 HAU; (\triangle), 0 HAU.

particles to cells, do not run the risk of yielding live virus as a result of genetic crossing-over between virus genomes with lesions at different sites.

Secondly, hybridization of cells in monolayer allows one several controls over the extent of fusion and kinds of progeny derived which are not available when cells are fused in suspension. Fixing cells in two dimensions restricts fusion to cells

which are nearest neighbors. The nearest neighbor of a given cell can be predetermined statistically by varying the multiplicity of one parent cell line versus the other parent and can be manipulated still further by the cell density employed. At low cell density and at a multiplicity of one cell *A* to one cell *B*, the nearest neighbor of cell *A* would most probably be cell *B*.

In a similar study (6), cells were fused in suspension. Under these conditions, the extent of hybridization did not appreciably increase with increasing virus concentration. Possibly the formation of nonviable multiple fusion products during fusion in suspension has decreased the extent of hybridization with increased virus titer.

In employing the monolayer fusion method, the following recommendations may be useful. If the fusion index of the parental lines is similar and low at 1000 HAU, the parents should be mixed in a 1:1 multiplicity, planted at a high cell density,

TABLE I
Effect of Storage Time at -70°C on Ability of Inactivated Sendai Virus to Promote Cell Hybridization

	Freshly inactivated virus	Inactivated virus stored for 5 months
500 HAU	190	182
250 HAU	175	161
100 HAU	79	78
0 HAU	5	3

Parental cell lines were mixed at a 1:1 multiplicity and planted at a density of 2×10^6 total cells/25 cm² surface area flask.

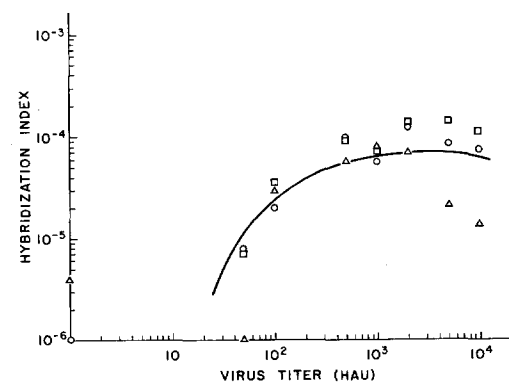


FIGURE 9 Relationship between virus titer and hybridization index. (\square), 2×10^6 total cells; (\circ), 10^6 total cells; (\triangle), 5×10^5 total cells.

and fused with 1000 HAU or less. If both parents have a high fusion index at 1000 HAU, the parents should be mixed at a 1:1 multiplicity, seeded at a low cell density, and fused with 1000 HAU or less. If parent *A* has a much higher fusion index than parent *B*, the multiplicity of *A*:*B* should be low, and the cells should be planted at a low density and treated with less than 1000 HAU to minimize $A \times A \times B$ fusions. After the period at 37°C, virus-treated cells can be safely trypsinized and replanted at low density in HAT medium in order to obtain large numbers of independent clones.

Okada (18) has used the term fusion index (F.I.) to describe the extent of cell fusion produced by Sendai virus (F.I. = [number of cells prior to fusion/number of cells after fusion] - 1). The term hybridization index (H.I. = the number of hybrid clones produced by a given concentration of virus/total cells employed at a 1:1 multiplicity) is meaningful in describing the extent of hybridization and is nearly constant at a given dose of virus at the cell densities employed here (Fig. 9). A hybridization index of 10^{-4} would indicate that

in a subpopulation consisting of 5×10^8 *A*-cells and 5×10^8 *B*-cells, one particular fusion between an *A*-cell and a *B*-cell gave rise to a colony of hybrid cells. Since the relationship between the fusion index and virus titer and between the hybridization index and virus titer are qualitatively similar, the fusion index of a cell line should serve as a useful gauge of its hybridization index.

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REFERENCES

1. BARRY, R. 1962. Failure of Newcastle disease virus to undergo multiplicity reactivation. *Nature (London)*. **193**:96.
2. BUONASSISI, V., G. SATO, and A. I. COHEN. 1962. Hormone-producing cultures of adrenal and pituitary tumor origin. *Proc. Nat. Acad. Sci. U.S.A.* **48**:1184.
3. CASALS, J. 1967. Immunological Techniques for Animal Viruses. K. Maramorosch and H. Kroprowski, editors. Academic Press Inc., New York. **3**:113.
4. CHEN, T. R. Fish chromosome preparation: Air-dried displays of cultured ovarian cells in two killifishes (*Fundulus*). *J. Fish. Res. Board Can.* In press.
5. CHU, M. Y., and G. A. FISCHER. 1968. The incorporation of ³H-cytosine arabinoside and its effect on murine leukemic cells (L₅₁₇₈Y). *Biochem. Pharmacol.* **17**:753.
6. COON, H. G., and M. C. WEISS. 1969. A quantitative comparison of formation of spontaneous and virus-produced viable hybrids. *Proc. Nat. Acad. Sci. U.S.A.* **62**:852.
7. DELORENZO, R. J., and F. H. RUDDLE. 1969. Genetic control of two electrophoretic variants of glucosephosphate isomerase in the mouse (*Mus musculus*). *Biochem. Genet.* **3**:151.
8. DUBBS, D. R., and S. KIT. 1965. The effect of temperature on induction of deoxythymidine kinase activity by herpes simplex mutants. *Virology*. **25**:256.
9. FELLUGA, B., A. CLAUDE, and E. MRENA. 1969. Electron microscope observations on virus particles associated with a transplantable renal adenocarcinoma in BALB/cf/Cd mice. *J. Nat. Cancer Inst.* **43**:319.
10. HAKALA, M. T., and E. TAYLOR. 1959. The ability of purine and thymine derivatives and of glycine to support the growth of mammalian cells in culture. *J. Biol. Chem.* **234**:126.
11. HARRIS, H., J. F. WATKINS, C. E. FORD, and G. I. SCHOEFL. 1967. Artificial heterokaryons of animal cells from different species. *J. Cell Sci.* **1**:1.
12. HITCHINGS, G. H., and J. J. BURCHALL. 1965. Inhibition of folate biosynthesis and function as a basis for chemotherapy. *Advan. Enzymol.* **27**:417.
13. KIT, S., D. R. DUBBS, L. J. PIEKARSKI, and T. C. HSU. 1963. Deletion of thymidine kinase activity from L cells resistant to bromodeoxyuridine. *Exp. Cell Res.* **31**:297.
14. KOHN, A. 1965. Polykaryocytosis induced by Newcastle disease virus in monolayers of animal cells. *Virology*. **26**:228.

15. LITTLEFIELD, J. W. 1964. Selection of hybrids from matings of fibroblasts *in vitro* and their presumed recombinants. *Science (Washington)*. **145**:709.
16. NEFF, J. M., and J. F. ENDERS. 1968. Poliovirus replication and cytopathogenicity in monolayer hamster cell cultures fused with beta propriolactone-inactivated Sendai virus. *Proc. Soc. Exp. Biol. Med.* **127**:260.
17. OKADA, Y., S. NISHIDA, and J. TADOKORO. 1961. Correlation between the hemagglutination titer and the virus particle number of HVJ. *Biken J.* **4**:209.
18. OKADA, Y., and J. TADOKORO. 1963. The distribution of cell fusion capacity among several cell strains or cells caused by HVJ. *Exp. Cell Res.* **32**:417.
19. OKADA, Y., F. MURAYAMA, and K. YAMADA. 1966. Requirement of energy for the cell fusion reaction of Ehrlich ascites tumor cells by HVJ. *Virology*. **28**:115.
20. ROBERTS, J. J., and G. P. WARWICK. 1963. The reaction of β -propriolactone with guanosine, deoxyguanylic acid and RNA. *Biochem. Pharmacol.* **12**:1441.
21. RODRIQUEZ, J. E., and W. HENLE. 1964. Studies on persistent infections of tissue cultures. V. The initial stages of infection of L(MCN) cells by Newcastle disease virus. *J. Exp. Med.* **119**:895.
22. SUBAK-SHARPE, H., R. R. BURK, and J. D. PITTS. 1969. Metabolic co-operation between biochemically marked mammalian cells in tissue culture. *J. Cell Sci.* **4**:353.
23. TAKAGI, N., T. AYA, H. KATO, and A. A. SANDBERG. 1969. Relation of virus-induced cell fusion and chromosome pulverization to mitotic events. *J. Nat. Cancer Inst.* **43**:335.
24. VOGT, M., and R. DULBECCO. 1960. Virus-cell interaction with a tumor-producing virus. *Proc. Nat. Acad. Sci. U.S.A.* **46**:365.
25. YERGANIAN, G., and M. B. NELL. 1966. Hybridization of dwarf hamster cells by UV-inactivated Sendai virus. *Proc. Nat. Acad. Sci. U.S.A.* **55**:1066.