Biochemical and Cytological Evidence for Triple Hybrid Cell Line Formed from Fusion of Three Different Cells

Abstract. *Hybrids between two parental lines of somatic cells have been observed in various genomic multiplicities by ourselves and others. Possible explanations for the higher multiplicities include the fusion of cells in a 1:1 ratio with one genomic set undergoing an asynchronous replication either before or after fusion or the fusion of two or more cells. We now provide evidence for multiple genomic hybrids arising from the fusion of more than two cells in a mixture of three different cell lines. This proof is based on unique chromosomal and biochemical markers characteristic of the three parental cell lines. The distinctive phenotypes of all three lines are expressed in a clonally derived hybrid. Thus, we conclude that long-term, proliferating somatic cell hybrids can arise from the fusion of three or more cells.*

Somatic cell hybrids have been reported with multiplicities greater than 1:1 (one human to one mouse genome) (*1*). The multiple hybrids were detected on the basis of karyotypic analysis. It would be interesting to determine whether hybrids in the ratio of two mouse to one human genome resulted from the fusion of a tetraploid cell with a diploid cell, from the fusion of a single mouse cell with a single human diploid cell with the subsequent asynchronous replication of the mouse cell only, or from the fusion of three different cells. To provide definitive evidence for the latter, we performed a hybridization experiment using three cytologically and biochemically different cell lines.

We chose RAG, A9, and WI-38 cell lines for the hybridization study for the following reasons. The tissue culture cell line RAG, derived from a BALB/c mouse renal adenoma, is resistant to 8-azaguanine (*2*). This resistance is due to a deficiency in hypoxanthine-guanine phosphoribosyl-transferase (HG-PRT), an enzyme involved in purine biosynthesis. Cells of this line have a modal number of 74 chromosomes with two marker chromosomes distinct from A9 and human chromosomes. The tissue culture cell line A9, derived from the C3H mouse strain, is also deficient in HG-PRT (*3*). It has a modal number of 54 chromosomes and possesses a unique acrocentric marker chromosome. The normal diploid cell line WI-38, which is derived from embryonic lung, has 46 chromosomes (*4*). It possesses some chromosomes whose morphologies are different from those of A9 and RAG chromosomes. All three cell lines have different electrophoretic mobility patterns for the enzymes phosphoglucomutase (PGM) (E.C. 2.7.5.1) (*5*), glucosephosphate isomerase (GPI) (E.C. 5.3.1.9, phosphoglucose isomerase) (*6*), and glucose-6-phosphate dehydrogenase (G6PD) (E.C. 1.1.1.49) (*7*). These enzyme phenotypes can be distinguished from each other in the hybrid cells.

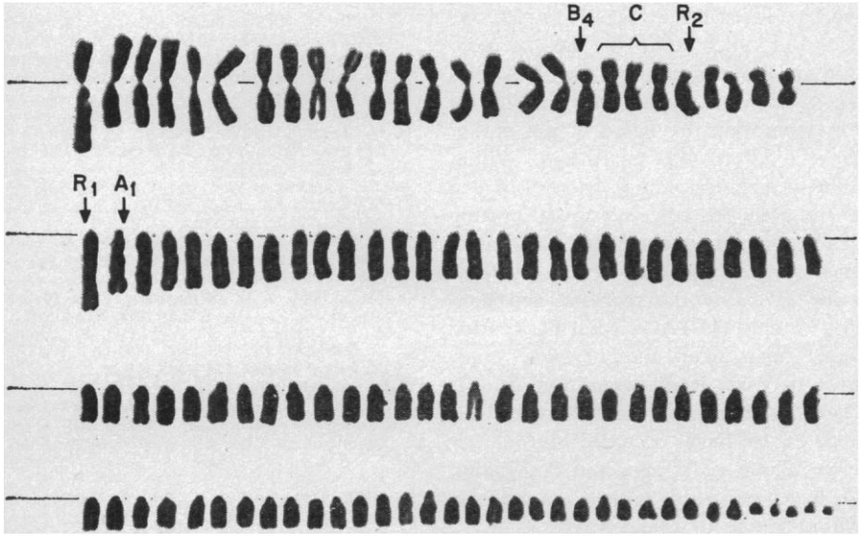

Fig. 1. Karyotype of triple hybrid cell (RAW-A4) showing the two distinct marker chromosomes; R_1 (RAG) marker is a long acrocentric chromosome; A_1 (A9) marker is a long acrocentric chromosome with a prominent secondary constriction in the middle of the long arm; B_4 and C are presumed human chromosomes; R_2 (RAG) marker is a submetacentric chromosome. Chromosomes were prepared as follows: Colcemid in a final concentration of 1 μg/ml was added to the cultures 4 to 6 hours prior to harvesting the cells. The cells were treated with trypsin in a hypotonic medium of 0.075M KCl for 6 minutes, followed by fixation in two changes of a mixture of methanol and acetic acid (3 : 1) for 15 minutes and 10 minutes. Heat-dried slide preparations were made and stained in 1.5 percent aceto-orcein. See also (*10*).

The differences in the enzyme phenotypes of the mouse cell lines is a consequence of their derivation from inbred mouse strains of different genotypes. The HG-PRT deficiency of the mouse lines and the presence of HG-PRT in WI-38 permits the selection of hybrids of mouse and human cells by the application of Littlefield's (*3*) selective system originally devised by Szybalski and Szybalska (*8*). However, there is no reason to expect the preferential selection of hybrids of A9, RAG, and WI-38 over combinations of A9 and WI-38 or RAG and WI-38.

Two million cells from each of the three cell lines were plated out in DMVE (Dulbecco-Vogt modified Eagle's medium) in a small Falcon flask (25 cm^2). The cells were incubated overnight at 37°C. The next day the cultures were drained and chilled to 4°C. Sendai virus inactivated with β-propiolactone (1000 hemagglutinating units per milliliter) was added with enough DVME medium to allow the viral suspension to flow freely over the surface of the cells. The flask was again chilled to 4°C for 30 minutes with gentle rotation. The flask was incubated for 30 to 50 minutes at 37°C after chilling to effect virus-mediated cell fusion. After 50 minutes the cells were disaggregated with a Viokase solution of trypsin and dispensed into three large Falcon flasks (75 cm^2) with HAT medium. A HAT selection medium containing hypoxanthine, aminopterin, and thymidine was used to select for hybrid cells. Aminopterin prevents de novo synthesis of both hypoxanthine and thymidine. The RAG and A9 cell lines, being deficient in HG-PRT, cannot utilize the exogenously supplied thymidine and hypoxanthine in the HAT medium, and, therefore, cannot grow. Being a normal cell line, WI-38 can use the thymidine and hypoxanthine, but it grows slowly and is contact

inhibited. Hybrid cells with WI-38 as one of the input cell lines will grow in HAT medium as a result of intergenic complementation.

At the end of 3 weeks, colonies of presumed hybrid cells were evident. The cells were disaggregated with trypsin, diluted, and plated at 100 and 50 cells per tissue culture dish. After 3 weeks, clones were visible and were then picked by means of the clonal isolation technique of Puck (*9*). Chromosome preparations and enzyme extracts were made on all the clones previously described (*10*).

In the single clone (RAW-A4) to be described here, karyotypic ideograms from 12 cells revealed chromosome numbers ranging from 118 to 133 with a modal number of 124 chromosomes. The modal number of chromosomes of the hybrid cell line was less than the total number of chromosomes of the three input lines which would have been 174 chromosomes. The lower number of chromosomes is due in part to the random loss of human chromosomes, a characteristic feature of hybrids of human and mouse cells noted previously by Weiss and Green (*11*). The long acrocentric chromosome characteristic of the RAG cell line was present in 11 of 12 cells (Fig. 1). The distinguishable A9 marker chromosome was also observed in 11 of 12 cells. There was a chromosome that resembled a B group human chromosome detectable in 6 of 12 cells. The use of the HAT selection system depends on the presence of the gene for HG-PRT which is X-linked. Thus, a part of the human X, or the whole human X chromosome should be retained in the hybrid cells for their growth in HAT.

The mobility patterns of GPI, PGM, and G6PD on starch gel are different for the three input cell lines. The gene for G6PD, which behaves as a dimeric enzyme upon electrophoresis, is X-linked in man and, most probably, in the mouse (*7*). No electrophoretic variants for this enzyme have been detected in *Mus musculus*. However, the expression of G6PD phenotype on starch gel is different for mouse and man. The mouse RAG and A9 forms of the enzyme have the same migration pattern, migration being faster than it is for the human form of the enzyme (Fig. 2a). Heteropolymeric enzyme formed from the subunits of the RAG or A9 (or both) and human G6PD can be seen migrating to a position intermediate between the more rapidly migrating mouse forms and the slower human form. This result proves that the hybrid is one of mouse and human cells, but does not indicate the presence of both mouse parental genomes.

Genetically controlled phenotypic differences between BALB and C3H mice have been described for phosphoglucomutase (PGM) by Shows *et al.* (*5*). The mouse PGM phenotype consists of several distinct molecular forms which can be divided into at least two zones (Fig. 2b)—PGM-1 and PGM-2. The former, which migrates toward the anode, is composed of five distinct bands. Electrophoretic variants exist between RAG and A9 in zone PGM-1. The PGM-1 from RAG appears as five bands which rapidly migrate toward the anode, whereas PGM-1 from A9 appears as five bands which migrate more slowly toward the anode. The hybrid cell line RAW-A4 expresses both the RAG and A9 PGM-1 phenotypes. No heteropolymeric isozymes have been observed in heterozygotes. The more slowly migrating zone, PGM-2, is composed of three isozymes. Neither RAG nor A9 can be distinguished from each other in the PGM-2 region nor from human PGM-1 and human PGM-2 which presumably migrate in this zone. The PGM electrophoretic phenotype of RAW-A4 suggests that both RAG and A9 contributed genetic material to the hybrid cell. Taken together with the evidence from the analysis of G6PD phenotypes, it seems quite likely that RAW-A4 is a hybrid of RAG, A9, and WI-38.

Additional and more conclusive evidence was obtained from the enzyme patterns for GPI, which is a dimer (*6*). A rapidly migrating form of the enzyme was noted in the inbred strain C3H from which the A9 line is derived. A more slowly migrating form of GPI was noted in the inbred strain BALB/c from which the RAG cell line is derived. In the zymogram (Fig. 2c) the three parental forms (C3H, BALB, and human) of the enzyme can be seen with the human form migrating most rapidly. Since GPI is a dimer, the three heteropolymeric forms of the enzyme—human/A9, human/RAG, and A9/RAG—were anticipated. All three homopolymers and all three heteropolymers were actually identifiable (Fig. 2c). The various enzyme assays together with the cytological informa-

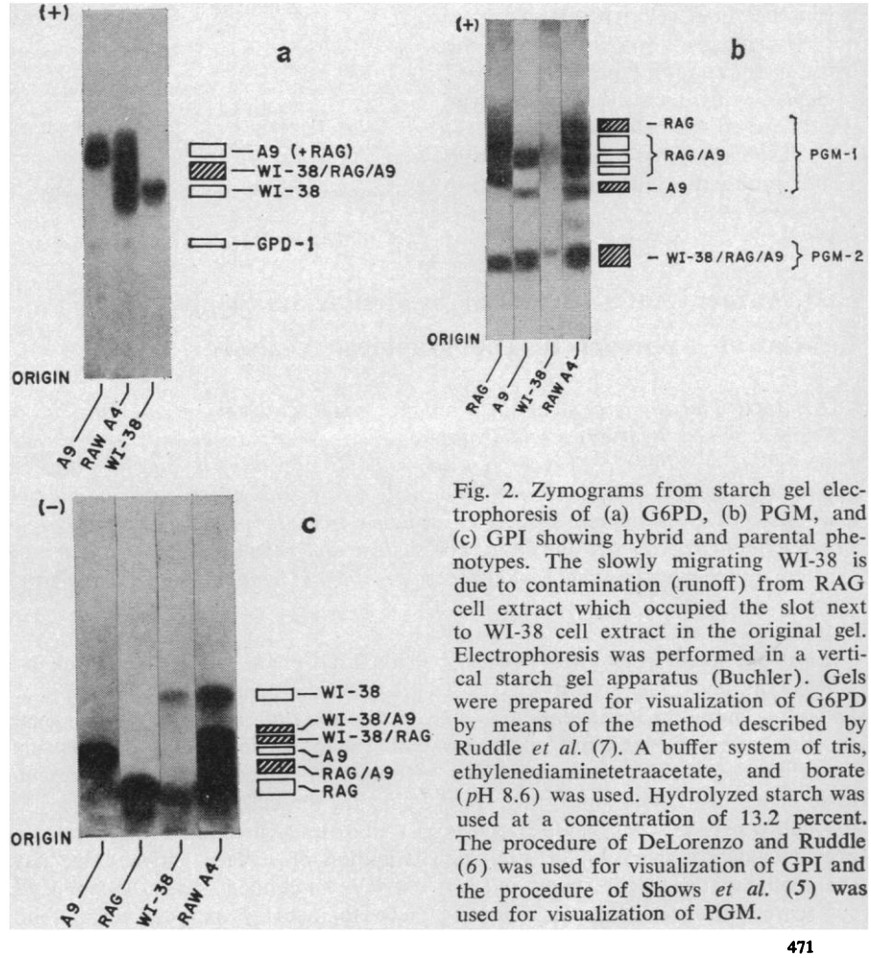

Fig. 2. Zymograms from starch gel electrophoresis of (a) G6PD, (b) PGM, and (c) GPI showing hybrid and parental phenotypes. The slowly migrating WI-38 is due to contamination (runoff) from RAG cell extract which occupied the slot next to WI-38 cell extract in the original gel. Electrophoresis was performed in a vertical starch gel apparatus (Buchler). Gels were prepared for visualization of G6PD by means of the method described by Ruddle *et al.* (*7*). A buffer system of tris, ethylenediaminetetraacetate, and borate (*p*H 8.6) was used. Hydrolyzed starch was used at a concentration of 13.2 percent. The procedure of DeLorenzo and Ruddle (*6*) was used for visualization of GPI and the procedure of Shows *et al.* (*5*) was used for visualization of PGM.

tion proves conclusively that the hybrid was formed from three different parental cells.

A simplistic view of somatic cell hybridization indicates that each hybrid is the result of a fusion between two cells—one human cell and one mouse cell. Although generally multiplicities of one mouse cell and one human cell do occur, other multiplicities are possible and do occur. Nabholz *et al.* (*1*) and Matsuya *et al.* (*1*) have reported multiplicities of greater than 1 : 1 in hybrids of human and mouse cells as well as in hybrids of mouse cells. Similar observations in our own laboratory show that cells from a fusion between RAG and WI-38 occur in multiplicities of 1 : 1 as well as 2 : 1.

Three possible explanations for the formation of hybrids with the greater multiplicities have been previously stated. In the case of the triple hybrid reported here, we have shown that three different cells are capable of fusing and forming a viable, long-lived hybrid. In one other instance, a bona fide triple hybrid has been reported (*12*). Knowles *et al.* reported the rescue of infectious SV40 after fusion between three different cells transformed by SV40. In this instance, the fusion between the three cell types was carried out sequentially. Suspensions of the two transformed cell types were exposed to Sendai virus; then the third cell type was added to the cell mixture. Also, it is not established that their triple hybrid would proliferate indefinitely. It is unclear how many generations this triple hybrid underwent after rescue of the virus.

Additional questions remaining to be answered are: Do higher multiplicities arise also by fusion of tetraploid and diploid cells or by fusion of single human and mouse cells with subsequent asynchronous replication of the mouse cell; in the case of triple hybrids, do the cells hybridize simultaneously, or as a series of events separated in time; and at what frequency do viable hybrids of higher multiplicities arise?

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Cell Surface Antigens: Serial Sectioning of Single Cells as an Approach to Topographical Analysis

Abstract. *The topographical distribution of H-2 antigens on the surfaces of C57BL/6 mouse thymocytes and lymph-node lymphocytes was investigated by a new technique. Single cells were reacted with visually labeled antibody directed against H-2 antigen. Each cell was processed for electron microscopy individually and was serially sectioned. Models constructed from serial electron micrographs provided detailed views of the entire cell surface and showed that H-2 antigen occurs on thymocytes in small isolated regions and on lymphocytes in large interconnected regions.*

Electron microscopy with visually labeled antibody has shown that H-2 and other mouse alloantigens, as well as virus-specified antigens, occur on discrete areas of the cell surface rather than being distributed uniformly over the entire cell (*1, 2*). These antigens provide valuable markers for studying the topographical organization of cell surface constituents. However, conventional use of the visual labeling method restricts the investigator to defining the distribution of antigen on the perimeter of single sections, and permits only limited inferences as to its representation on the cell as a whole.

In order to determine the precise distribution of surface antigens, we have serially sectioned single lymphocytes and thymocytes exposed to antibody labeled with visual markers, enabling us to construct models of the complete surfaces of these cells. We chose to work with single cells maintained in droplets to avoid the repeated cycles of centrifugation and resuspension required for the preparation of labeled cell suspensions, which could cause cellular damage and loss of labeled antibody.

For this initial study, H-2 antigens on the cell surface were labeled by the indirect method of Hämmerling *et al.* (*3*); southern bean mosaic virus (SBMV) (*4*) was used as the visual marker. Cells were reacted first with the appropriate H-2 antibody (γG), then with the hybrid antibody (rabbit anti-mouse γG/rabbit anti-SBMV), and finally with SBMV.

To observe the distribution of H-2 antigen on mouse lymphoid cells, we prepared models of the surfaces of four thymocytes and four lymph-node lymphocytes from C57BL/6 mice. More than 20 antigens are specified by the *H-2* locus, and mouse strains express characteristic constellations of antigens (designated H-2^a, H-2^b, and so forth) (*5*). The C57BL/6 strain is H-2^b. The cytotoxic antiserum used was C57BL/6/H-2^k anti-C57BL/6 (H-2^b) prepared in an H-2 congenic mouse strain to ensure limitation of serological reactions to H-2 (the cytotoxic titer against H-2^b lymph node cells was 1/640). This was diluted 1 : 10; the hybrid antibody and SBMV were diluted to 0.5 mg of protein per milliliter. The following negative controls were used: (i) an H-2^b antiserum to H-2^a, which should not react with C57BL/6 cells, was substituted for positive antiserum; (ii) normal mouse serum was substituted for antiserum; (iii) all mouse serum was omitted; (iv) the C57BL/6 test cells were replaced by cells from the strain in which the H-2 antiserum was prepared, C57BL/6/H-2^k. For each of these four controls, one thymocyte and one lymphocyte were serially sectioned.

During the reactions, each cell was processed separately in droplets (volume, 0.3 μl) under a layer of silicone oil (dimethylpolysiloxane, Dow Corning 200 fluid, viscosity 100 cs) in the bottom of a plastic petri dish (60 by 15 mm). Medium 199 was used for suspending cells and for diluting reagents. After each step of the reaction sequence the cells were washed with medium 199 containing 2 percent γG-free fetal calf serum (Grand Island Biological); this protein supplement