*EVIDENCE FOR INTERGENIC COMPLEMENTATION IN HYBRID CELLS DERIVED FROM TWO HUMAN DIPLOID STRAINS EACH CARRYING AN X-LINKED MUTATION**

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Abstract.—Two male diploid fibroblast strains, each carrying deficiency mutations at different X-linked loci (glucose-6-phosphate dehydrogenase and hypoxanthine-guanine-phosphoribosyltransferase) have been successfully hybridized. The resulting mononucleated hybrid cells have been shown to synthesize both normal gene products, indicating that both X chromosomes are functionally active in the hybrid cells. We believe this is the first reported example of intergenic complementation in fused human diploid cells.

Introduction.—Although the possibility of applying the techniques of cell hybridization[1, 2] to human diploid cells for studies of somatic cell genetics has been discussed,[3] the difficulty of finding suitable cells has been a major deterrent. Ideally the cells to be used should carry several markers, each the primary gene product of a single chromosomal gene. Moreover, genic polymorphism should exist at each locus, the allelic products should be identifiable at the level of the single cultured cell, and the cells should be amenable to *in vitro* selection procedures.

Of the several thousand examples of simple Mendelian traits in man,[4] only a dozen or so can be identified at the cell level.[5] Three involve mutations of genes located on the X chromosome: glucose-6-phosphate dehydrogenase deficiency (g-6-pd),[6] hypoxanthine-guanine phosphoribosyltransferase deficiency (HGPRT),[7] and the Hurler-Hunter syndrome or gargoylism II.[8] Cells from patients with one of the first two deficiencies have been used in the experiments here described. Diploid fibroblasts derived from a male with the g-6-pd deficiency were fused with those from a male with the HGPRT deficiency in an attempt to obtain a hybrid tetraploid strain of cells which, in respect to their two X chromosomes, would be equivalent to cells doubly heterozygous in repulsion. Hybrid cells were obtained in which gene products of the X chromosomes of both parental strains could be identified.

Materials and Methods.—Skin biopsies were obtained from male patients known to be affected either by the Lesch-Nyhan syndrome[9] or by a severe form of congenital nonspherocytic anemia associated with g-6-pd deficiency and from females heterozygous at these loci. Bits of tissue approximately 1–2 mm^3 were placed in a 60 mm Petri dish with 1–1.5 ml of minimum growth medium[10] containing 5% calf, 5% fetal calf, and 5% human AB sera; they were then incubated in a 5% CO_2 atmosphere. A normal amount of medium (5–10 ml) was added after 1–2 days. After 5 to 10 days, when there had been extensive growth of a fibroblast layer, the culture was trypsinized and grown in stoppered bottles with the same growth medium, omitting human serum. All experiments were conducted before the strains had undergone 20 culture passages (30–40 cell divisions).

Cytochemical demonstration of g-6-pd and HGPRT activities: For the identification of cellular phenotypes, cells were plated at low density on slides placed in Petri dishes. Two to three days later the activity of g-6-pd was examined with a modification of the method described by Wajntal and DeMars.[11] The slide cultures were washed with Hanks solution and dried rapidly in an air stream. A large coverslip was then suspended about 1 mm above the surface of the slide, supported by four pillars of clay, and the intervening capillary space was filled with about 0.5 ml of a freshly prepared reaction mixture containing one part of 0.2 *M* phosphate buffer, pH 7.4; one part glucose-6-phosphate solution (60 mg/ml of disodium salt); one part triphosphorpyridine nucleotide solution (10 mg/ml of sodium salt, 99% purity); two parts of nitro-blue-tetrazolium (1 mg/ml, grade III); and one part of phenazine methosulfate (0.02 mg/ml) (all reagents from Sigma Chemical Co.). The slides were incubated at 37°C and 100% humidity for $^1/_2$ to 2 hr, depending upon the amount of formazan precipitation desired. The coverslips were then removed, the slides drained, fixed in formol vapors for 5 min, washed in Hanks solution, counterstained with 0.1% neutral red for 10 min, washed again, and dried. Since the formazan grains are soluble in organic solvents, reagents such as xylol and permount were avoided.

An autoradiographic procedure was used to estimate HGPRT activity.[12] Cells were grown for 24 hr in a medium containing 10 μc/ml ^{3}H-hypoxanthine (specific activity 3.15 c/mM, New England Nuclear), rinsed with normal saline, fixed in absolute methanol for 5 min, treated with ice cold 5% trichloracetic acid for 25 min, quickly washed with water, and dried. The slides were then coated with Kodak NTB-3 emulsion, exposed in the dark for 5 to 8 days, and developed.

Fusion technique: This was essentially as described by Harris *et al.*[13] and modified by Koprowski *et al.*[14, 15] One major difference was that the cells were mixed immediately after being freed from the glass and prior to centrifugation, in order to assure random intercellular contact. The cells were exposed for 30–60 sec to serum-, Mg-, and Ca- free minimal medium[10] containing 0.25% trypsin and 0.01% versene. The fluid was then withdrawn, the cells allowed to stand for 2–5 min, and then suspended in a large amount of serum-free medium and counted. Cells were then collected by centrifugation at 800 rpm (25 cm head diameter) for 30 min and resuspended in 1 ml of complete medium containing 2% calf serum. The fusions, either with or without Sendai virus, were carried out at concentrations of 10^7 cells/ml. β-Propiolactone-inactivated Sendai virus (4,000 HAU) was used. To estimate fusion efficiency, cells of one of the parental strains in each fusion series were prelabeled with ^{3}H-thymidine and the cultures examined autoradiographically after the fusion experiments.[14]

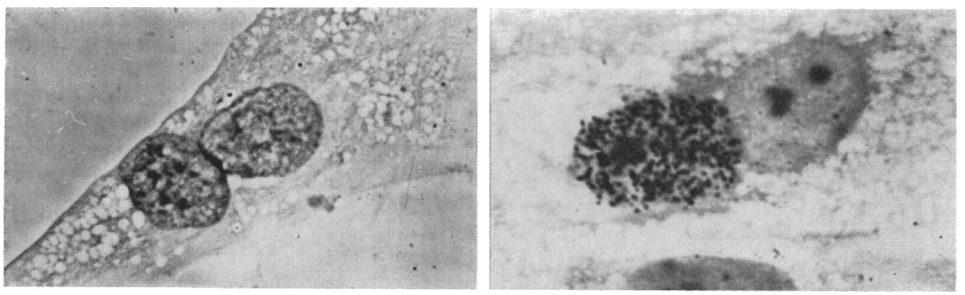
Sex chromatin and chromosome preparations: For determination of sex chromatin frequencies, replicate slides from each experiment were removed at 1, 2, 3, and 5 days after fusion. The slides were fixed in 95% ethyl alcohol, stained according to the Feulgen procedure, and counterstained with 1% light green coloring. Chromosome preparations were made from replicate cultures at various intervals.

Chemical selection against HGPRT-deficient cells: In a selective medium containing 1, 3, or 10γ/ml azaserine (a glutamine analogue: 6-(1′-methyl-4′-nitro-5′-imidazolyl) serine) all the HGPRT-deficient cells, and some nondeficient cells, died within approximately 10 days.[7] However, if medium containing the selective drug was replaced every third day, only the HGPRT-deficient cells were eliminated, with no detectable effect on the nondeficient cells even after 30 days.

Nomenclature of fused cells: The terms homokaryon and heterokaryon will be used to describe the fusion product of cells with the same genome or different genomes, respectively. The term hybrid cell will refer to the mononucleated fusion product possessing characteristics of both parental strains, and not to binucleated cells.

Cellular phenotypes: Gd(+), formazan precipitates; positive for g-6-pd activity. Gd(−), no or very few formazan precipitates; negative for g-6-pd activity. P(+), heavy silver grain deposits all over the cell in autoradiographs indicating incorporation of ^{3}H-hypoxanthine; positive for HGPRT activity. P(−), no or few silver grains after similar treatment; negative for HGPRT activity.

Results.—Autoradiographic studies: In fusion experiments between approximately equal numbers of the cells of the two deficient strains, one unlabeled and the other ^{3}H-thymidine treated (92% of nuclei labeled), about half of the cells had a single labeled nucleus, 42 per cent had a single unlabeled nucleus, and 7–8 per cent had two nuclei (Fig. 1*a*)—either both labeled, both unlabeled, or with one of each type (Fig. 1*b*). Less than 0.1 per cent were tri- or multinucleated. There was remarkable variation in nuclear size, and several of the binucleated cells had their nuclei very close together (Fig. 1*a*).



(*a*) (*b*)

FIG. 1.—(*a*) Binucleate cell alcohol-fixed 24 hr after the fusion experiment. Phase contrast, Feulgen-light green stain.
(*b*) Autoradiograph of a binucleate cell fixed 24 hr after fusion of a ^{3}H-thymidine labeled strain with an unlabeled strain. Only one nucleus is labeled.

Chromosome and sex chromatin studies: Within the first week after fusion 10–32 per cent of the cells had tetraploid chromosome complements. The random distribution of the chromosomes at metaphase and the absence of multipolar anaphases suggest that the tetraploid cells were derived from normal single spindle divisions. In the fusions between male and female diploid strains, unquestionable 92,XXXY metaphases could be found.

Sex chromatin bodies were not found in cells after male-male fusions, and not more than two were found after female-female fusions. These results are in accord with the sustained functional activity of the *X* chromosomes in hybrid cells as described in the following section.

Localization of gene products in the hybrid cells: Hybrid formation from fusion of cells Gd(−)P(+) and Gd(+)P(−) was further supported by the finding that, 48 hours after fusion in equal numbers, binucleate cells were found with formazan precipitated around only one of the two nuclei. As expected, other binucleate cells had either no deposits or deposits around both nuclei. These observations indicate that the g-6-pd deficient nucleus remains deficient after fusion and suggest also that true cytoplasmic fusion had occurred. Similar evidence could not be obtained for HGPRT since the autoradiographic method for its demonstration does not permit the study of nuclear morphology.

In order to demonstrate that hybrid cells produce both gene products, a selective procedure against HGPRT-deficient cells involving treatment with azaserine was applied to the culture after fusion.[7] HGPRT-deficient cells were fused with g-6-pd deficient cells in a 9:1 ratio, permitting the elimination of most of the

parental cells by the chemical selection procedure. The selective efficiency of this system was enhanced by the fact that the g-6-pd deficient strain grew more slowly than the HGPRT-deficient strain, particularly at low cell densities. Control fusions were also performed within each of the two parental strains to evaluate the possibility that fusion itself might have a nonspecific effect on the rate of cellular synthesis of the two enzymes. The results (Table 1 and Fig. 2) may be summarized as follows:

1. Gd(+)P(−) cells which fused with each other grew into a confluent monolayer in which all cells were Gd(+) and P(−), and these did not survive the addition of azaserine.

2. Gd(−)P(+) cells which fused with each other grew into a confluent monolayer, in which all cells were Gd(−) and P(+), and their growth was unaffected by azaserine under the conditions of these experiments.

3. Without azaserine, cells derived from the 9:1 fusion rapidly grew into a confluent monolayer, and nearly all were found to be Gd(+) and P(−). With azaserine (1,3 and 10γ/ml), the majority of the cells detached from the floor of the culture flask within 6–15 days, leaving only about 10 to 20 circumscribed areas which were P(+) (Fig. 3). These presumably were clones derived from resistant cells, either surviving Gd(−)P(+) cells originally incubated or mononucleated hybrids in which the HGPRT activity was expressed. When these were allowed to form confluent cultures, trypsinized, and distributed in replicate cultures for parallel tests for enzyme activity, all were P(+) and about 90 per cent were Gd(+), indicating that they were in fact hybrids.

Evolution of fused lines: Azaserine-selected lines after fusions in a 9:1 ratio (cf. above), as well as two cultures obtained after fusion of the same parental strains in a 1:1 ratio, were examined at intervals during an eight-week period involving ten culture passages and approximately 23 cell divisions. During this period the proportion of Gd(+) and/or P(+) cells decreased progressively in all three hybridized lines. This occurred also in both parental diploid strains,

TABLE 1. *Data suggesting intergenic complementation between g-6-pd and HGPRT loci in fused cell strains.*

Code no. of fusion experiment	Phenotypes of cells used in fusions	Medium Supplemented with: Azaserine*	Medium Supplemented with: ^{3}H-hypoxanthine (10 μc/ml for 24 hr)	Per cent of Positive Cells: g-6-pd	Per cent of Positive Cells: HGPRT (incorporation of ^{3}H-hypoxanthine)
2–4 a		+		No survival	
b	Gd(+)P(−)	+		No survival	
c		0	0	100	—
d		0	+	100	Absent
2–10 a		+	0	Absent	—
b	Gd(−)P(+)	+	+	Absent	100
c		0	0	Absent	—
d		0	+	Absent	100
1–4 a		+	0	90 ±	−
b	Gd(+)P(−) × Gd(−)P(+)	+	+	90 ±	100
c	9:1	0	0	100	—
d		0	+	100	1–2 ±

* 10γ/ml for 1 month after fusion as selective procedure.

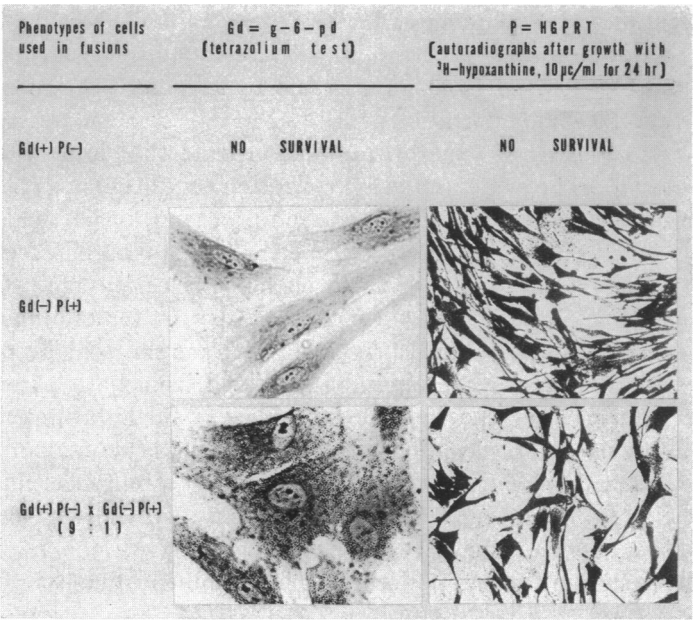


FIG. 2.—Evidence of intergenic complementation between g-6-pd and HGPRT loci in fusion product of Gd(+)P(−) and Gd(−)P(+) cells. Cultures treated with 10γ/ml azaserine for 1 month after fusion prior to test.

which had undergone a total of 20–30 passages and approximately 40–60 cell divisions by the time the selection experiments had been completed.

Chromosome studies of the cell cultures after fusion showed the persistence of tetraploid metaphase plates over a period of several months and up to 15

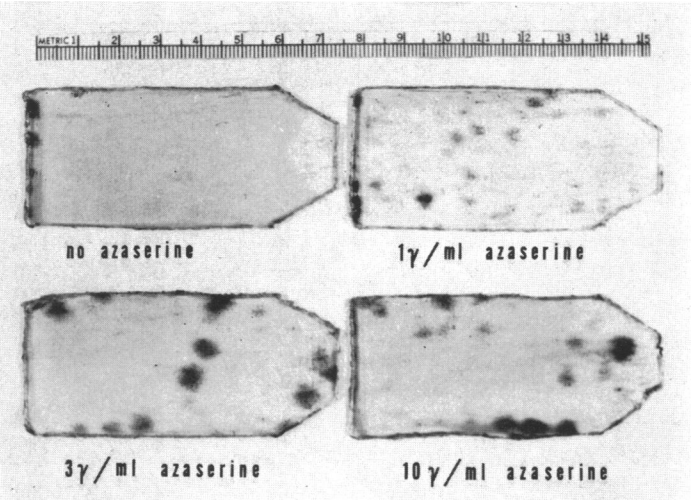


FIG. 3.—Appearance of HGPRT-positive clones in culture flasks after fusion of Gd (+)P(−) with Gd(−)P(+) cells in a ratio of 9:1, followed by selective treatment with azaserine for 1 month. Autoradiographs after ³H-hypoxanthine incorporation.

culture generations at a frequency of 6 to 66 per cent (Table 2). Since very few (<0.5%) chromosomally aberrant cells were found, it seems unlikely that the loss of the biochemical markers was due to loss of X chromosomes or to recognizable chromosome aberrations.

Discussion.—The present experiments demonstrate that human diploid cells can be hybridized. The evidence for hybridization consists of: (1) the presence of large numbers of tetraploid cells after the fusion and, in particular, the fact that, in male-female fusions, some of these cells have a 92,XXXY chromosome complement; and (2) the fact that, after chemical selection against one of the parental strains, one can identify hybrid cells that persist for a number of culture generations and that are capable of producing both normal gene products. The last finding indicates that X chromosomes derived from both parental strains are functionally active in the hybrid cell. This is further supported by the observation that no sex chromatin was found in cells after male-male fusions, and no more than two sex chromatin bodies in tetraploid cells after female-female fusions. The presence of binucleate cells with g-6-pd activity concentrated around only one nucleus suggests that in heterokaryons the mutant g-6-pd allele remains inactive just as it does in the mononucleated hybrids obtained

TABLE 2. *Illustrative estimations of tetraploid metaphases in chromosome preparations of some of the cultures before and after fusion.*

Code no. of fusion experiment	Phenotypes of cells used	Selection with azaserine	Days in culture after fusion†	Total no. of metaphases counted	Tetraploids (%)
Controls	Parental strain: Gd(+)P(−)	−	—	500	1.4
	Parental strain: Gd(−)P(+)	−	—	500	0.8
With Sendai virus					
2-14-I	Gd(+)P(−) × ^{3}H,				
	Gd(−)P(+)	0	47	200	6
2-14-D		0	53	200	8
2-14-F	1:1	+	54	550	18
2-14-6X		+	102	200	20
2-14-7Y		+	102	200	10
2-14-6T		+	103	200	22
2-15-I	Gd(+)P(−) × ^{3}H,				
	Gd(−)P(+)	0	47	250	16
2-15-J		0	66	100	63
2-15-B	9:1	+	52	200	29
2-15-C		+	52	200	22
2-15-E		+	53	300	41
2-15-H		+	60	200	58
2-15-L		+	68	200	40
2-15-I		+	68	200	46
Without Sendai virus					
2-16-2B	Gd(+)P(−) × Gd(−)P(+)	0	17	200	19
2-16-I	1:1	0	47	200	57
2-16-J		0	68	200	66
2-16		+	46	200	28
2-16-D		+	54	350	34

* Cultures frozen for 126–212 days at −170°C prior to subculture and analysis.

† Does not include frozen storage time; i.e., *in vitro* growth time only. Cultures divided two- to fourfold at 1 to 2 week intervals.

through the fusion of g-6-pd deficient cells with each other. Although we have demonstrated that in hybrid cells two different genes on different X chromosomes are both expressed, the experimental conditions do not permit the demonstration that both gene products are produced simultaneously in a single cell. Nevertheless, we feel justified in assuming that the synthesis of both gene products in the hybrid cell is due to true intergenic complementation, rather than to a nonspecific effect of fusion on the rate of RNA synthesis at the respective loci.

Since the products of the two most common mutants at the g-6-pd locus have been shown to differ by a single amino acid substitution,[16] there is no doubt that glucose-6-phosphate dehydrogenase is a primary gene product. Cells from subjects with complete g-6-pd deficiency because of a defect at different sites within the g-6-pd locus might prove useful for studies of allelic complementation in hybrid cells. Similar studies may be possible at the HGPRT locus. The potentialities of the system here described for studies of somatic recombination and of X linkage would obviously be greatly improved if there were a selective procedure for each of the parental types. This can perhaps be achieved with a guanine analog which is lethal only for P(+) cells.[17]

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