TRANSFER OF HUMAN CHROMOSOMES VIA HUMAN MINISEGREGANT CELLS INTO MOUSE CELLS AND THE QUANTITATION OF THE EXPRESSION OF HYPOXANTHINE PHOSPHORIBOSYLTRANSFERASE IN THE HYBRIDS

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SUMMARY

The behaviour of human cells arrested in mitosis can be severely perturbed so as to generate numerous small minisegregants containing very few chromosomes. These cells can be separated according to size and DNA content and fused with intact cells. In this paper we describe the production and some properties of proliferating cell hybrids generated by fusion of human minisegregant cells derived from a HeLa strain with mouse A6 cells deficient in hypoxanthine phosphoribosyltransferase (HPRT, EC 2.4.2.8). The hybrids were shown to contain up to 10 human chromosomes including a single X. Independently derived hybrid clones were quantitatively characterized and compared with the parental phenotypes with respect to HPRT. Human isozymes of each of the 3 enzymes HPRT, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and phosphoglycerate kinase (EC 2.7.2.3) were found. Tests to evaluate both structure and function of HPRT were utilized. The specific activity of HPRT of more than 10 hybrids tested was approximately 10% that of the HeLa parent. Structural characterization of HPRT from hybrid cells as evidenced by heat inactivation and electrophoretic mobility results in a 'human-like' phenotype. Functional characterization of parental HPRT results in kinetic constants for cofactor and substrate which do not permit distinction of human and of human and mouse enzymes; HPRT from the minisegregant hybrids had normal kinetic constants. The reduced specific activity of HPRT in the hybrids is discussed in terms of the inability of the mouse environment to regulate the full expression of the human structural gene.

INTRODUCTION

Fusion between somatic cells followed by segregation of phenotypic markers is a powerful genetic tool and has permitted the assignment of numerous traits to particular chromosomes and to regions of chromosomes (see Ruddle, 1972). The mechanism of chromosome segregation in hybrid cells is, however, poorly understood, and although

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some successful efforts have been made to direct chromosome loss in hybrids (Pontecorvo, 1971), there is as yet no general means for controlling the chromosome constitution of hybrid cells. The ability to manufacture hybrid cells containing different proportions of parental genomes would undoubtedly permit a more precise and rapid analysis of gene localization and function, and recently there have been a number of reports demonstrating that somatic cells can be phenotypically transformed by isolated metaphase chromosomes. In no case, however, could the transforming chromosome(s) or parts of chromosomes be found in the hybrid cells beyond the first few generations (McBride & Ozer, 1973; Willeke & Ruddle, 1975; Wullems, van der Horst & Bootsma, 1975, 1976).

We have described a technique for the production of human cells containing small amounts of DNA and few chromosomes. These minisegregants are obtained by perturbing cell division in HeLa and diploid human cells to obtain abnormalities of both cytokinesis and chromosome segregation. The outcome of these disturbances is a highly aberrant process during which the mitotic cell subdivides into a cluster of minisegregants varying in size and DNA content. Minisegregants can be isolated according to size, which is in turn related to DNA content, and they are readily fused with whole cells (Johnson, Mullinger & Skaer, 1975; Schor, Johnson & Mullinger, 1975; Mullinger & Johnson, 1976; Johnson, Mullinger & Downes, 1978).

It should be noted that eukaryotic microcells can be produced by a completely different method (Ege & Ringertz, 1974). In this procedure the micronuclei of rodent cells, produced by previous exposure to colcemid, are removed from the main body of the cell (as microcells) by means of cytochalasin B. Human cells, however, do not respond to colcemid in this way and human microcells must therefore be produced by an alternative method.

In this paper we demonstrate that human minisegregant cells can be hybridized with hypoxanthine phosphoribosyltransferase (HPRT, EC 2.4.2.8)-deficient mouse A9 cells and proliferating hybrids maintained in HAT (hypoxanthine-aminopterin-thymidine) selective medium. A selected number of independently cloned hybrid cell lines, resulting from such fusions and possessing HPRT activity, have been systematically examined for chromosomal constitution and for quantitative and physicochemical relationships of the expressed HPRT with 3 objectives. First, a rigorous quantitation of the expression of a heterospecific genome in a host environment may reveal previously unknown genetic mechanisms controlling the expression of a given phenotype. Second, since no single physicochemical method of enzyme characterization can distinguish all of the subtle protein structural and functional differences that may be present (Paigen, 1971; Langridge, 1968), tests to evaluate both structure and function were utilized, to determine the best method of distinguishing human from mouse HPRT, and to ascertain the functional normality of the human enzyme operating in a largely mouse environment. Third, we have examined the stability of the human HPRT locus in these hybrids. Removal of the selective pressure which favours retention of the donor genome in the hybrid environment was followed temporally by a quantitative procedure to select for segregation of HPRT expression.
MATERIALS AND METHODS

Cell culture, preparation of minisegregants and hybridization

L cells (Earle, 1943) and their HPRT-deficient A₀ derivative cell strain (Littlefield, 1964) were grown in Eagle’s Minimal Essential Medium (Eagle, 1959) and Dulbecco-modified Minimal Essential Medium respectively, supplemented with 10% foetal bovine serum in 75-cm² Nunc plastic flasks in a humidified CO₂ incubator. Diploid human fibroblasts were obtained from Flow Laboratories (Irvine, Scotland), and the American Type Culture Collection. HeLa cells with a modal chromosome number of 64 (Waldren & Johnson, 1974) were used to prepare minisegregants by the method of Johnson et al. (1975) and fractionated using a Ficoll density gradient in the manner described by Schor et al. (1975).

The gradients were collected in 50-ml fractions and numbered 1 to 20 starting from the top of the gradient. The minisegregants used for the 2 series of hybridizations were on both occasions obtained from fraction 4 from the top of standard Ficoll gradients. Previous examination of HeLa minisegregants fractionated in a similar manner has confirmed their homogeneity within a given fraction from experiment to experiment with respect to (a) size and (b) the presence and amount of DNA (Johnson et al. 1978). Feulgen staining of the minisegregants from fraction 4 of each gradient revealed that 20 and 26% of the cells respectively contained DNA. Previously, quantitative densitometry of pooled fractions 4 and 5 showed that 97% of those cells containing DNA had less than 2C HeLa value and over 80%, much less than 1C (Schor et al. 1975). The size distribution of 100 minisegregants from each fraction was assessed by means of an eyepiece micrometer. In 6 gradient fractionations including the 2 reported in this paper no cell with a diameter greater than 12 μm was observed in fraction 4. Routinely 80% of the minisegregants in this fraction had a diameter of 7.5 μm or less.

For cell fusion about 10⁷ minisegregants recently obtained from the gradient and counted by means of haemocytometer were mixed with 2.5 x 10⁶ A₀ cells in Hanks balanced salt solution with 250 haemagglutinating units of u.v.-inactivated Sendai virus. Agglutination at 4 °C for 5 min preceded a 30-min incubation at 37 °C during which fusion occurred. The fusion mixture was plated into a series of 60-mm plastic dishes (Nunclon), in Dulbecco-modified Minimal Essential Medium supplemented with 10% foetal bovine serum. After 24 h the medium was removed and the cells selected in HAT (Littlefield, 1964). Within 3 weeks colonies were visible and were picked from the dishes by cloning rings. Two hybrids arose from the first hybridization of which A₁ survived. About 50 hybrids arose from the second hybridization, and 4 genetically independent series were picked, A₁, B₁, C₁ and D₁, each series from a different plate. A number of independent colonies was picked from each of the B₁, C₁ and D₁ plates (θ₁, θ₂, θ₃, θ₄, θ₅, θ₆, θ₇, 0₁, 0₂, 0₃) and grown up for analysis.

To assess the capacity of fractionated minisegregants to proliferate and to assess whether they were contaminated by whole cells, the remaining cells from fraction 4 (about 10⁷ cells) were incubated in parallel with each fusion mixture. In these and other experiments with fraction 4 no cells were found to survive and proliferate. The plating efficiency of whole HeLa cells is routinely 35% after mitotic synchronization.

Cell extract preparation

The cell extracts were prepared from cultures that were approximately 75% confluent and partially purified by the method of Sharp, Capecchi & Capecchi (1973). Briefly, extracts were prepared at 4 °C with all of the buffers at 4 °C. Flasks were washed twice with 10 ml of cold buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.5) and drained for 5 min. One millilitre of extraction buffer (10 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 30 mM KCl, 0.5% Triton X-100, 1 mM dithiothreitol) was added. The flasks were shaken every 5 min for 20 min.

The flasks were tilted on their base for 5 min followed by removal of the cell extract with a Pasteur pipette; 2 mM KCl was then added to bring the final KCl concentration to 0.1 mM for increased stability of HPRT.

The extracts were centrifuged at 1000 g for 5 min to remove the debris. The supernatants
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were then centrifuged at 250,000 g for 1 h, dispensed into 1-ml aliquots and frozen at −80 °C. Such extracts were stable for longer than 4 weeks although the specific activity of hybrid and parental culture was routinely determined on freshly prepared extracts.

**HPRT assay**

HPRT activity was measured by the DEAE filter disk assay of Beaudet, Roufa & Caskey (1973) as modified by Wahl, Hughes & Capecchi (1975) except that the standard assay contained 0.1 μCi of [3H]hypoxanthine (2 Ci/mmol) in a final 60 μM concentration in 50 μl volume. The incubation time was limited to 5 min at 37 °C. The reaction mixture with no added enzyme gave 300 cpm, and addition of heat-inactivated enzyme did not increase this count. The accumulation of product ([3H]inosine monophosphate, 3H-IMP) was linear for substrate concentrations in the range 0.68 to 60 μM for both parental and hybrid cells; at high concentrations the rate was linear for 20 min, and at low concentrations it was linear for more than 5 min. The HPRT-deficient cell line A9 did not accumulate any 3H-IMP under the standard conditions of the assay. The enzyme activity increased linearly with increase in extract concentration from 3 to 30 μg (2 to 20 μl) of extract. The standard assay contained 20 μl of extract.

To prevent inactivation of the Sephadex G-200 filtered enzyme, 1 mg/ml albumin (final concentration) was added to the collecting tubes prior to filtration so that the eluted enzyme was never exposed to dilution after gel filtration. The enzyme was used approximately 2 h after filtration and immediately after concentration in a Minicon A25 (Amicon Corporation) for all the kinetic and 8-azaguanine (8AG) studies.

The Sephadex-filtered HPRT from HeLa, L or hybrid cell line was purified approximately 6-fold was stable and did not inactivate after 45 min of 65 °C heating. The saturation studies are all at initial rate recording for each substrate concentration, with an approximately ten-fold variation in each direction from the apparent \( K_m \) values. 3H-IMP counts for the lowest substrate concentration are at least three times the background for most of the curves.

Protein was determined by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951). A unit of enzyme activity is defined as that amount of enzyme required to convert hypoxanthine to one nanomole of IMP per hour under standard assay conditions.

**[3H]Hypoxanthine incorporation into nucleic acids**

Cells were grown in 35-mm plastic plates in the standard medium with HAT. At zero time 1 ml of fresh Dulbecco’s medium supplemented with 10% foetal calf serum and 0.5 μM [3H]hypoxanthine (2 Ci/mmol) was added. The cells were incubated at 37 °C in a CO₂ atmosphere for an additional 6 h. The cells were then washed twice with phosphate-buffered saline and lysed in 0.3 ml 0.5 M KOH, and an aliquot of 100 μl was withdrawn for protein determination. The nucleic acid was precipitated with trichloroacetic acid (TCA) and collected on GM filters and washed a further 3 times. The radioactive product was quantitated by scintillation counting. For autoradiographic analysis of [3H]hypoxanthine uptake into nucleic acid, cells were grown on glass coverslips at low density (essentially no cell-to-cell communication), and incubated for 20 h in medium containing 12 μCi/ml [3H]hypoxanthine (2 Ci/mmol). After cold 5% TCA extraction, the preparations were dipped in Ilford G5 emulsion and exposed for 3 days.

**Cellogel electrophoresis**

Extract preparation and Cellogel electrophoresis for HPRT, glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), and phosphoglycerate kinase (PGK EC 2.7.2.3) were done by the method of Meera Khan (1971). For HPRT the electrophoresis time was extended to 4 h and activity was directly measured on 3-mm Cellogel strips incubated for 30 min at 37 °C in a 200-μl standard reaction mixture.
Phenotypic reversion studies

HAT was withdrawn from the standard culture medium, and at the indicated times thereafter, cells were grown in the presence of 20 µg/ml of 6-thioguanine (6TG) and surviving colonies were counted after 2 weeks. (A9 cells grow in up to 40 µg/ml of 6TG). A number of 6TG-resistant colonies were picked and grown up for enzyme and chromosome characterization.

Chromosomal constitution of hybrid and revertant cultures

Air-dried metaphase preparations were obtained as usual after a 2-h application of colcemid and treatment with hypotonic KCl solution. At least 5 metaphases of each hybrid were analysed after Giemsa-i1 staining using a slightly modified procedure from Bobrow & Cross (1974), and after treatment of the cultures for 24 h with the benzimidazole compound '33258 Hoechst' in combination with G-banding (Kim & Grzeschick, 1974). Both techniques allow clear discrimination between murine and human chromosomes. In the first case human chromosomes are pale blue while the mouse chromosomes stain dark magenta; in the latter case the pericentric regions of most A9 chromosomes are elongated while the HeLa chromosomes are normally condensed.

RESULTS

HPRT specific activity determination of HeLa, L cell, A9, and hybrid cultures

The specific activity of HPRT of hybrid, HeLa, and L and A9 cultures was evaluated by 2 independent methods. All of the hybrid cultures, which represent at least 5

![Fig. 1A. HPRT specific activity of parental, hybrid and revertant cultures. HPRT specific activity was determined by pooling the contents of two culture flasks and processing the cells as described under Materials and methods. Each point represents the mean of duplicate determinations in separate experiments, on freshly prepared extract. The average specific activities ± S.E.M. of HPRT for HeLa, L and various minisegregant hybrids expressed as nmol mg⁻¹ h⁻¹ are as follows, where n = no. of experiments and the figure in parentheses is the percentage of HeLa activity: HeLa, 75.48 ± 0.60, n = 9; L, 71.10 ± 0.60, n = 8; A9, 80.04 ± 0.90 (11), n = 9; A8, 6.69 ± 1.10 (9), n = 6; O, 7.92 ± 1.50 (9), n = 6; ?, 5.99 ± 0.61 (8), n = 10; ω, 5.82 ± 2.10 (8), n = 6.

![Fig. 1B. HPRT activity determination by [3H]hypoxanthine incorporation into nucleic acids by cell cultures in vitro. Each point represents a separate experiment as described under Materials and methods.](image-url)
genetically independent clones, A₃, α₃, and the δ, θ and ω series, have a range of specific activity which is approximately 10% that of HeLa or L cell lines (Fig. 1A).

Additionally, when the nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity of the extracts was inhibited by thymidine triphosphate (TTP) (Fujimoto & Seegmiller, 1970), there was no observable increase in HPRT activity of the hybrid or parental cell lines, which argues against an accelerated IMP utilization by hybrid extracts. However, there was a rise of $5.7 \pm 0.68$ to $44.27 \pm 2.18$ nmol mg$^{-1}$ h$^{-1}$ ($n = 4$) in HPRT specific activity when GM-6 (a human fibroblast cell line) was used which indicates that the apparent initial low specific activity in this human fibroblast cell line results from a very high 5'-nucleotidase activity and can be corrected for by TTP inhibition.

[3H]hypoxanthine incorporation into nucleic acids in culture in vivo was also directly measured (Fig. 1B). The hybrid cultures incorporated less than a third as much as HeLa or L cell cultures. A₃, and all of the hybrid revertants tested, did not
Table 1. Properties of hypoxanthine phosphoribosyltransferase from L, HeLa, human fibroblast and hybrid cultures

<table>
<thead>
<tr>
<th>Cell type</th>
<th>PRPP</th>
<th>Hypoxanthine</th>
<th>8AG inhibition</th>
<th>Heat inactivation</th>
<th>Electrophoretic mobility</th>
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<tr>
<td></td>
<td>Kᵦ, μM</td>
<td></td>
<td>Slope</td>
<td>r²</td>
<td>t₀₅ min</td>
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<td>L</td>
<td>11.53 ± 1.64</td>
<td>1.23 ± 0.18</td>
<td>-2.13 ± 0.26</td>
<td>0.80</td>
<td>28.06</td>
</tr>
<tr>
<td>HeLa</td>
<td>10.27 ± 3.20</td>
<td>1.96 ± 0.69</td>
<td>-2.13 ± 0.15</td>
<td>0.94</td>
<td>10.91</td>
</tr>
<tr>
<td>Human fibroblast</td>
<td>6.04 ± 0.90</td>
<td>3.67 ± 2.44</td>
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<tr>
<td>A₁</td>
<td>10.25 ± 2.04</td>
<td>2.66 ± 0.69</td>
<td>-2.47 ± 0.43</td>
<td>0.73</td>
<td>7.82</td>
</tr>
<tr>
<td>δf</td>
<td>10.50 ± 0.97</td>
<td>2.57 ± 0.97</td>
<td>-1.49 ± 0.20</td>
<td>0.80</td>
<td>11.98</td>
</tr>
<tr>
<td>ωo</td>
<td>7.05 ± 1.20</td>
<td>2.86 ± 0.23</td>
<td>-1.89 ± 0.24</td>
<td>0.83</td>
<td>8.52</td>
</tr>
</tbody>
</table>

**Determination of kinetic constants.** Enzyme filtered through Sephadex G-200 was used to measure the reaction velocity. Hypoxanthine saturation experiments were done with the 9 Ci/mmole substrate. The concentrations of [3H]hypoxanthine used were 0.068 μM (0.03 μCi), 0.137 μM (0.06 μCi), 0.275 μM (0.12 μCi), 0.55 μM (0.25 μCi), 1.1 μM (0.5 μCi), and 2.2 μM (1.0 μCi). Higher concentrations, between 7.2 and 52 μM, were prepared by dilution of 1.0 μCi of the radioactive hypoxanthine. The background counts for the lowest concentration were 50 cpm, for the intermediate concentrations 100, and 350 for the 2.2 mM (1 μCi) concentration and above. Values are shown ± S.E.M.

The PRPP saturation experiments were done with 0.2 μCi (9 Ci/mmole) in a final 60 μM of [3H]hypoxanthine, with the appropriate concentrations of PRPP. The incubation time was limited to 2 min to obtain strict linearity in time for all hypoxanthine concentrations and to 5 min for all PRPP concentrations, and sufficient enzyme added to obtain a minimum of 1500 cpm 3H-IMP for the lowest substrate concentration.

The Kᵦ and Vₘₐₓ were computed by the Cleland computer program (Cleland, 1967). The Vₘₐₓ in nmol IMP min⁻¹ was 70 for L, 307 for HeLa, 107 for A₁, 69 for δf and 153 for ωo.

8-azaguanine inhibition was determined on enzyme filtered through Sephadex G-200. The concentration of hypoxanthine was 0.275 μM, and of 8AG 0.5, 1.0, 1.5 or 2.0 mM. Triplicate determinations were done for each concentration. The 100% counts were: HeLa 6500 cpm, L cell 7000 cpm, A₁ 6800 cpm, δf 4000 cpm, and ωo 4000 cpm. The background count was 140 cpm. The slope of inhibition and the S.E.M. by 8AG was determined by linear regression. M and H designate mouse (L cell) and human (HeLa) respectively.
incorporate [3H]hypoxanthine during a 12-h period. Thus there was excellent agreement between in vitro and in vivo methods of measurement of HPRT activity in tissue culture cell lines.

The low HPRT activity in the hybrid populations could be the result of either a decrease in the fraction of cells expressing HPRT or a large decrease in HPRT activity in every cell. The first possibility can be eliminated by scoring the proportion of cells capable of utilizing [3H]hypoxanthine in autoradiograms. Hybrids A1, δ7 and ωc were examined in this manner, and 100% of cells in each population found to incorporate radioactivity into acid-insoluble material after plating at very low cell density to avoid metabolic cooperation.

**Physicochemical characterization of HPRT of hybrid and parental cell lines**

Three hybrid clones, A1, δ7 and ωc, were selected for a more detailed study of the physicochemical properties and the stability of the HPRT locus and for comparison with L and HeLa cell lines.

**Heat inactivation of HPRT**

Heat inactivation at 80 °C of partially purified HPRT from HeLa and L cell results in complete and unambiguous separation of the human from the mouse HPRT (Fig. 2A). A1, δ7 and ωc inactivate with a time constant in the range characteristic of human HPRT. A mixture of ωc and L cell HPRT inactivates with a biphasic slope indicating the independence of ωc from L HPRT inactivation (Fig. 2B and Table 1). Partially purified HPRT from HeLa, L or ωc was heated at 65 °C for 45 min. There was no inactivation whatsoever for any of the phenotypes (not shown). Thus the HPRT from the hybrids has no unusual heat lability.

**Determination of Michaelis constants and 8AG inhibition of HPRT**

Saturation of partially purified HPRT from HeLa, human fibroblasts, L and hybrid cells with the cofactor phosphoribosyl pyrophosphate (PRPP) or the substrate hypoxanthine results in no significant difference in the Michaelis constant for any of the cell lines (Table 1).

$K_m$ determinations for PRPP from human red blood cells have been reported as 200 μM (Krenitsky & Papaioannou, 1969) or 130 μM (Richardson, Ryckman, Komarnicki & Hamerton, 1973). Using methods identical to those described in these papers, we obtained a value of 10 μM for human HPRT from partially purified RBC. Similarly the $K_m$ for hypoxanthine from human RBC has been reported to be 25 μM (McDonald & Kelley, 1971), and 4.5 μM (Richardson et al. 1973). Our results with partially purified HPRT from HeLa and human fibroblasts are similar to that of Richardson et al. (1973) but an order of magnitude different from that of Krenitsky et al. (1969). The mouse (L cell) $K_m$ for hypoxanthine determined by us is similar to the result reported by Sharp et al. (1973), 1.13 μM (Table 1).

Analysis of 8AG inhibition of partially purified HPRT activity in the hybrids and in both HeLa and L cells did not reveal statistically significant differences between HeLa, L or hybrid enzyme when a wide range of inhibitor concentrations was used (Table 1).
Our results do not permit a differentiation of human from mouse HPRT by the determination of kinetic constants, or by 8AG inhibition. In addition, $A_1$, $\delta_1$, and $\omega_c$ hybrid HPRT kinetic constants are not statistically significantly different from either HeLa or mouse.

**Electrophoretic mobility of HPRT**

Cellogel electrophoresis resulted in separation of human (HeLa) from mouse (L) HPRT (Fig. 3). The 3 hybrids, $A_1$, $\delta_1$, and $\omega_c$, were repeatedly tested with the parentals as controls to determine whether electrophoretic mobility could be used to distinguish hybrid HPRT as either human or mouse consistently and unequivocally. There was some variation in the electrophoretic mobility of the hybrid enzyme from that of the

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**Fig. 3.** Cellogel electrophoresis of HPRT. Cellogel electrophoresis was done by the method of Meera Khan (1971) except that the electrophoresis time was extended to 4 h for $A$ and 6 h for $B$. $A$, equal amounts of extract from L and HeLa were diluted by 1 vol. of buffer, and 7 $\mu$l were applied to the Cellogel strip. $B$, 3-5 $\mu$l from L or HeLa and 7 $\mu$l of $\omega_c$ were mixed and applied to the Cellogel strips and treated identically to $A$, except that the electrophoresis was run for 6 h.
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parents and from experiment to experiment under identical conditions (not shown). However, when hybrid extracts were mixed with either L or HeLa there was clear separation of hybrid from L but none from HeLa (Fig. 3).

**G6PD and PGK activity in hybrids determined by Cellogel electrophoresis**

Cellogel electrophoresis of L, A9, and HeLa results in a clear separation of the mouse phenotype for G6PD and PGK from human and hybrid cell lines. HeLa, αα, δ3, θα, θγ, θβ, θγ, and θα and θγ, all had the human bands of G6PD and PGK on Cellogel electrophoresis (Fig. 4). The hybrids had an additional G6PD intermediate band which has been argued to represent a heteropolymer of human and mouse G6PD enzyme molecules (Nabholz, Miggiano & Bodmer, 1969; Miller et al. 1971). All of the 6TG revertant hybrid cultures lost the human band of PGK and both the human and intermediate heteropolymeric bands of G6PD.

Fig. 4. Cellogel electrophoresis of PGK (top panel) and G6PD (bottom panel), done by the method of Meera Khan (1971). Freshly prepared extract was used for all of these studies. Representative electrophoretic patterns are shown. Channels are designated by arabic numerals: 1, L cell; 2, HeLa; 3, A9; 4, δ3; 5, θγ; and 6, revertant A9, which has lost the HeLa phenotype. The human (HeLa) band of G6PD is not well visualized in this photograph except in channel 4 even though it was present on all of the Cellogel strips: however, the heteropolymeric band of human x mouse enzyme is visible for all of the hybrids.

**Stability of HPRT expression in hybrids in the absence of selection**

Hybrids which had been maintained in HAT selective medium for 6 months from clonal isolation were transferred into non-selective medium and after various intervals 10⁵ cells were plated in medium containing 20 μg/ml 6TG. For each hybrid the frequency of 6TG-resistant clones increased as a function of the time in non-selective medium (Fig. 5). After a further 1 year of continuous growth in selective medium the
stability of HPRT in the absence of selection was retested in 2 hybrids (A1 and ωc) and found not to have changed substantially.

A number of the 6TG-resistant clones from different hybrids were isolated, expanded and examined for the expression of human HPRT. None of the 6TG-resistant cell clones expressed HPRT activity (Fig. 1A, B), and radioautographic examination of [3H]hypoxanthine incorporation confirmed that all cells in each population were negative in this respect. Loss of human HPRT in these clones was also associated with loss of human G6PD and PGK isozymes (Fig. 4).

Chromosomal analysis after differential staining of hybrid cells

Each hybrid had a single identifiable human X chromosome and various numbers of human chromosomes from all other groups (Table 2). For simplicity, the different HeLa marker chromosomes and some non-identifiable elements are included in this table according to their length and arm ratio. Only the large submetacentric HeLa marker chromosome is listed separately. No X chromosome could be found in the 6TG-selected clones.

The total number of chromosomes in each hybrid exceeds that of the A9 parent, although only a few of the extra chromosomes are human. In 6TG-selected clones the total chromosome number decreases, although of the human chromosomes only the X is regularly lost from these cells (Fig. 6).
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It is possible that the chromosome constitution of the hybrids reflects the initial selection of the minisegregants for fusion from the top of the gradient, although some post-fusion segregation must have occurred because of variation in the number of human chromosomes present among cells of cloned hybrids. Initial chromosome selection in the minisegregants may help to explain why, for example, the E group element No. 17 was regularly found in the hybrids but no chromosome No. 9 though both elements are present in multiple copies in the HeLa set. Moreover, the large submetacentric HeLa marker chromosome which is partially derived from human chromosome No. 5 (Heneen, 1976) and is present only once in the HeLa complement was retained in most of the hybrids. If it was missing, as in $\alpha_n$, $\delta_1$, $\theta_6$ and $\omega_c$, a normal B group chromosome (No. 5) could be found.

Table 2. Human chromosomal constitution of HeLa minisegregants $\times A_9$ hybrids

<table>
<thead>
<tr>
<th>Chromosomal group</th>
<th>X</th>
<th>A</th>
<th>M$^a$</th>
<th>B</th>
<th>C</th>
<th>D</th>
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The data represent the average human chromosome constitution of each hybrid analysed from a minimum of 5 metaphase spreads. M$^a$ is the large submetacentric HeLa marker chromosome (Heneen, 1976). Each hybrid prefixed with an R represents an independently derived 6TG-resistant subclone.

DISCUSSION

A variety of methods now exists whereby genetic information can be transferred between somatic mammalian cells. These include whole cell fusion and chromosome-mediated transfer of genetic information. To this list can now be added the transfer of genetic information by means of rodent microcells (Ege & Ringertz, 1974; Fournier & Ruddle, 1977) and human minisegregant cells. There are now 2 methods for the production of mammalian cells containing small quantities of DNA. When rodent cells are exposed to colcemid a common consequence is the production of karyomeres (Stubblefield, 1964). The micronuclei can be removed as microcells by means of cytochalasin B (Ege & Ringertz, 1974) and fused with whole cells to complement genetic deficiencies (Fournier & Ruddle, 1977). Human cells do not form karyomeric nuclei in the presence of colcemid and mini human cells must therefore be produced.
Human minisegregant cell hybrids

Fig. 6. Total chromosome number in metaphase cells of A9 parent and minisegregant hybrids. At least 30 chromosome spreads were scored for each cell line. RA1, Rωc, and Rθ1b were isolated as 6TG-resistant subclones of HPRT+ hybrids A1, ωc and θ1 respectively.

by means of extrusion subdivision (Mullinger & Johnson, 1976). In this paper we describe the production and some properties of mouse hybrids obtained by fusion of human minisegregant cells containing small numbers of chromosomes and mouse A9 cells deficient in HPRT activity.

Although we cannot formally exclude the possibility that the hybrid cells reported in this paper were derived from fusion between whole HeLa or HeLa minisegregants with a complete genome and A9 cells we consider that this is extremely unlikely for the
following reasons. First, the minisegregants were taken from a region of the gradient which is not contaminated with large cells; we have found by quantitative DNA densitometry of 200 minisegregants from this region of the gradient that each cell had a reduced DNA content compared with the parental mitotic cells (Schor et al. 1975). Second, analysis of 200 prematurely condensed chromosome sets from minisegregants from pooled fractions 1 to 8 showed that less than 1% contained as many chromosomes as whole cells (Schor et al. 1975).

HeLa minisegregants are readily fused with whole cells (Schor et al. 1975). In the experiments reported here $10^7$ minisegregants were used in each fusion and $2.5 \times 10^6$ A9 cells, yielding 2 and about 50 hybrids. Since approximately 25% of minisegregants used in the fusions contained DNA a multiplicity of 1:1 was obtained. The hybridization indices (the numbers of hybrid clones produced by a given concentration of virus/total cells employed at a 1:1 multiplicity (Klebe, Chen & Ruddle, 1970)) for the minisegregant/A9 fusions are therefore $2.5 \times 10^{-6}$ and $10^{-8}$.

Complementation for the HPRT locus using intraspecific and heterospecific hybridization has been used as a powerful tool to gain insight into somatic cell genetics (Weiss & Ephrussi, 1966; Schwartz, Cook & Harris, 1971; Kahan & de Mars, 1975). In this study, the HPRT specific activity of hybrids was consistently a fraction of that of the parentals. In previous whole cell hybridization studies of A9 x human (Shin, Meera Khan & Cook, 1971) and rat hepatoma x human fibroblast (Croce, Bakay, Nyhan & Koprowski, 1973), HPRT activity has been reported to be no different from that of parentals. Similarly human x mouse hybrids can have the same amounts of G6PD as parentals (Miller et al. 1971), and A9 x WI-38 human fibroblasts have normal thymidine kinase activity (Migeon, Smith & Leddy, 1969).

Functional and physical characterization of HPRT from hybrid cultures based on kinetic constant determination, heat denaturation and electrophoretic mobility argues in favour of a structurally normal enzyme. Additionally [3H]hypoxanthine incorporation in vivo by these cultures argues against a functionally defective HPRT and there is no evidence to suggest either enhanced utilization or dephosphorylation of IMP product in the hybrids. A number of possibilities can be considered in seeking to explain the low specific activity of hybrid HPRT. First, hybrids generated by the minisegregant introduction of human chromosomes into A9 cells and subjected to the selective pressure of HAT retain the obligatory X chromosome but require additional human chromosomal loci for the full expression of the HPRT locus as a general problem of gene balance. The first hypothesis is unlikely since in addition to the human X chromosome the hybrids contained human chromosomes from all groups either singly or in combination. In spite of this array of combinations of human chromosomes all of the hybrids possessed similar HPRT specific activity. A second possibility, decreased synthesis or accelerated degradation of the ‘human’ HPRT in a mouse cell environment, is unlikely because whole cell heterospecific hybrids have been reported to have the same HPRT and G6PD activity as the parentals (Miller et al. 1971; Shin et al. 1971; Croce et al. 1973). Additionally, in man x mouse hybrids with up to 35 human chromosomes only mouse 28-s type ribosomal genes are repressed in heterospecific hybrids produced by whole cell fusion (Eliceiri & Green, 1969).
Third, it is possible that a more specific human HPRT regulatory locus not located on the X chromosome, and having specificity of its gene product is necessary for the full expression of HPRT. In hybrids formed after transfer of human metaphase chromosomes from heteroploid HPRT+ cell strains to HPRT− mouse or Chinese hamster cells, the specific activity of human HPRT was always found to be uniformly lower than that of the human parental cell and, in addition, was variable from one hybrid clone to another (Willeke & Ruddle, 1975; Wullems et al. 1975). In this context it is of interest that transfer of metaphase chromosomes from near-diploid HPRT+ to HPRT− Chinese hamster cells resulted in the production of hybrids not only with uniform HPRT specific activity but also having identical activity to the Chinese hamster HPRT+ parent (Wullems et al. 1975). The possibility still exists, therefore, that a human regulatory element for HPRT cannot be fully actuated by a heterospecific signal.

It is also possible that the low activity of HPRT in the hybrids is at least partly related to gene dosage. The HeLa cells used in the present study to obtain minisegregants are nearly triploid and there is evidence that many HeLa strains contain 2 euchromatic X chromosomes (Czaker, 1973). Each of the minisegregant hybrid clones contains only 1 human X chromosome and HPRT activity should theoretically be half that of the HeLa parent. In all cases however, the specific activity of the hybrid HPRT is much less than half that of HeLa.

Perhaps the greatest benefit to be derived from the transfer of cells or particles containing less than the diploid quantity of DNA is predetermined control of chromosome segregation, which normally occurs very slowly in intraspecies crosses (Kao, Johnson & Puck, 1969), or in a rapid and directional manner in interspecies combinations (Kao & Puck, 1970). Attempts to direct chromosome loss in hybrids have met with limited success (Pontecorvo, 1974). In addition, since minisegregants (Schor et al. 1975) and microcells (Fournier & Ruddle, 1977) can be separated according to size and DNA content, it should be possible to control the amount of DNA introduced by the microcells more carefully than is possible in metaphase chromosome transfer.

In the future, it will be necessary to undertake comparative studies of hybrids formed after metaphase chromosome transfer and by fusion with minicells containing various amounts of DNA. Elimination of considerable portions of the genome before fusion takes place will help to clarify any analysis of gene regulation in somatic cells.

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