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Characterization of Hypoxanthine-Guanine Phosphoribosyl Transferase in Man–Mouse Somatic Cell Hybrids by an Improved Electrophoretic Method

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The hypoxanthine-guanine phosphoribosyl transferase (HGPRT) activity in a group of man–mouse somatic cell hybrids, produced by Sendai virus–mediated cell fusion and HAT selection, has been analyzed by a new electrophoretic technique. Evidence is presented which shows that the hybrid lines derived from fusion of a mouse fibroblast deficient in HGPRT with various human cell strains have an HGPRT activity that is characteristic of the human enzyme, whereas a hybrid line derived from a mouse fibroblast which is deficient in thymidine kinase has an HGPRT activity characteristic of the mouse. This new technique involves electrophoresis of cell extracts on cellulose acetate gel, followed by the localization of the enzyme activity by autoradiography.

INTRODUCTION

When somatic cells of human and murine origin are fused together, the resultant hybrid cells show a progressive and preferential loss of the human chromosome complement, while apparently retaining that of the mouse (Weiss and Green, 1967). Such hybrids provide a potentially useful tool for the genetic analysis of man on the cellular level (see Davidson, 1969; Siniscalco, 1970). Weiss and Green selected their hybrids

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from a fusion between a diploid human fibroblast strain and a mutant strain of mouse L-cells which lacked the enzyme thymidine kinase (Kit *et al.*, 1963), by use of the HAT selective medium of Littlefield (1964). The presence of aminopterin in this selective medium limited the growth of cells to those which had a functioning thymidine kinase. This system therefore permitted in principle the isolation of hybrid cells which had selectively retained the human chromosome which carries the structural gene for this enzyme.

Later studies by Matsuya *et al.* (1968) and Migeon and Miller (1968) established in this way that the thymidine kinase activity in such man–mouse hybrids could be correlated with the presence of a human chromosome of the group E. In a series of similar experiments, we have analyzed a group of seven somatic cell hybrids which were produced by fusion of two mutant mouse cell lines with various diploid human cells. The mouse parent of the first six hybrids was the A_9 cell line of Littlefield (1963), an L-cell derivative deficient in hypoxanthine-guanine phosphoribosyl transferase (HGPRT; E.C. 2.4.2.8). The seventh hybrid was derived from mouse fibroblast strain B82, another L-cell subline lacking in thymidine kinase (TK; E.C. 2.7.1.21) and similar to the mouse fibroblast originally used by Weiss and Green (1967). Details of the production and analysis of these seven hybrid lines have been reported elsewhere (Miller *et al.*, 1970).

Following the selective elimination of the parental cell strains after the fusion, the man–mouse hybrids grown in HAT medium were expected to have retained (1) in the first group derived from the A_9 cell, at least that part of the human genome which coded for the HGPRT activity, and (2) in the last hybrid descended from B82, at least the chromosomal segment bearing the human thymidine kinase locus. This expectation forms the theoretical basis on which various genetic analyses, particularly the current efforts to study the linkage of human genetic markers by interspecific somatic cell hybrids, are founded. However, since the precise nature of the genetic defects in the mutant mouse cells is still not known, a direct proof that our man–mouse hybrids do indeed depend for their survival in Littlefield's system upon the selective retention and functional expression of the particular human genes concerned was considered necessary.

In this paper, we present evidence that the A_9–man hybrids in fact contain an HGPRT activity which has the electrophoretic characteristics of the human type, while the B82–man hybrid has only the mouse-type HGPRT, thus confirming the expectation. The improved electrophoretic technique reported here has certain advantages over those hitherto available, and may have general usefulness.

MATERIALS AND METHODS

The various human cell strains used, and the procedures for cell fusion and selection of the hybrids, have been described in detail (Miller *et al.*, 1970). Here we shall describe only the method for the electrophoretic characterization of HGPRT on cellulose acetate gel.[5] This method is an adaptation of the methods of Der Kaloustian *et al.*

[5] "Cellogel," from Chemetron, Via G. Modena 24, Milan, Italy.

(1969) and Cook (1970), and is based on the selective adsorption of the reaction product (inosinic acid) on an anion exchange paper (DEAE-cellulose paper, Whatman DE81). After the unbound excess substrate (^{14}C-hypoxanthine) is removed by chromatography, the product is assayed directly by liquid scintillation counting or localized on the DEAE paper by autoradiography.

Preparation of Cell Extract

Cells growing as a monolayer were collected by trypsinization or by scraping with a rubber policeman, and washed twice with isotonic saline. Usually, 1 to 3×10^6 cells from a confluent Falcon tissue-culture flask (250 ml) were used for each assay. Human leukocytes were obtained from 20 ml fresh blood by the differential sedimentation technique in dextran as described by Böyum (1968), and were washed as above. The cells were resuspended in 0.1–0.2 ml of 0.02 M phosphate buffer, *p*H 7.0, and sonicated for 10 sec in an ice bath. Extreme care was taken not to overheat the suspension The Sonocone sonicator[6] was used with a narrow-tipped probe ("needle probe," 5/32-in. diameter) at energy level setting 7. Sonicates were then centrifuged at $27{,}000 \times g$ for 30 min at 4 C. The homogenates could be stored at 4 C for up to 1 week, and for several months when frozen at -20 C, without any appreciable loss of activity or change in the electrophoretic mobility.

Electrophoresis on Cellogel

The general procedure for Cellogel electrophoresis described by Rattazzi *et al.* (1967) and Meera Khan and Rattazzi (1968) was followed throughout. On a standard sheet (16 by 17 cm, 0.5 mm thick), 10 samples can be assayed conveniently. Cell homogenates are applied on the gel as a narrow streak 10 mm long in 2–3 μl aliquots, at 5mm from the cathodal shoulder piece. Electrophoresis is carried out for 3 hr at room temperature in 0.02 M phosphate buffer, *p*H 7.0, keeping the current constant at a level which is obtained with an initial potential difference of 200 v. After the run the gel sheet is cut along the shoulder pieces, quickly blotted with filter paper, and used immediately for enzyme assay.

Enzyme Assay by Autoradiography

For each assay, a minimum practical volume of reaction mixture is prepared immediately before use; for a standard sheet containing 10 samples, 7 ml is sufficient. The reaction mixture contains the following: 0.55×10^{-3} M 5-phosphoribosyl pyrophosphate (Sigma), 2.5 μc/ml hypoxanthine-8-^{14}C (New England Nuclear Corp.; specific activity 4.13 mc/mmole), 5.0×10^{-3} M $MgSO_4$, and 55×10^{-3} M tris–HCl buffer, *p*H 7.4 (Krenitsky *et al.*, 1969).

The gel sheet is first soaked on both sides in 1.5 ml reaction mixture on a dry glass plate and then placed with the porous side up, on a second glass plate (20 by 20 cm). Care is taken not to trap air bubbles under the gel. During this process, the gel should not be allowed to dry by evaporation. A DEAE-cellulose paper strip (11 by 16 cm),

[6] Powertron Division, Giannini Control Corp., Plainview, N.Y.

which has been marked with a soft pencil to indicate the origin and the side which will come into contact with the gel, is carefully placed over the presoaked gel. By applying evenly from the center outward, the remainder of the reaction mixture is now used to soak the DEAE paper. Air bubbles that may be formed under the paper should be removed. The glass plate carrying the Cellogel and DEAE paper is put into a humidified airtight chamber and incubated for 1 hr at room temperature.

At the end of the incubation, the DEAE paper is peeled off the gel, washed twice in two successive 800 ml baths of 0.1 M tris buffer, *p*H 9.5, and air-dried overnight. A strip of Whatman No. 1 filter paper is then sewed on to each end of the DEAE paper—a 20 by 18 cm strip to the anodal end and a 5 by 18 cm strip to the cathodal end. The whole sheet is then washed by a descending chromatography. The smaller filter paper strip, attached to the cathodal end (which corresponds to the place of origin on the Cellogel), is serrated to facilitate an even flow and placed at the bottom during the chromatography. Each sheet is washed by allowing 300 ml of the same alkaline buffer, *p*H 9.5, to run through the paper. This removes more than 99% of the unreacted substrate. After drying, the Whatman No. 1 filter paper strips are carefully removed, and the DEAE-cellulose paper is again sewed on to a sheet (18 by 24 cm) of 3 mm Whatman filter paper. A 24 by 18 cm medical X-ray film (Kodak Royal Blue) is now tightly sandwiched with the DEAE paper between two heavy glass plates, exposed for 2–5 days at room temperature, and developed for 5 min in Kodak D-19 developer. To orient the film after development, it is useful to put markers on the DEAE paper with radioactive ink before exposure.

Direct Enzyme Assay

Alternatively, the HGPRT activity within the Cellogel strip may be assayed directly. Immediately after electrophoresis, and without allowing the gel to dry, the Cellogel sheet is cut into 1 cm strips along the channels of each sample from the point of origin to anode. Each strip is again cut into consecutive 4 mm sections and assayed for HGPRT activity, by incubating with hypoxanthine-8-C^{14} for 2 hr at 37 C using the conditions of Harris and Cook (1969). Radioactive inosinic acid, the product of the reaction, is then separated from the unreacted substrate by selective adsorption onto DEAE paper discs. Under the conditions of the assay, the enzyme activity is proportional to the number of cpm bound.

RESULTS

The electrophoretic mobility of HGPRT from seven different sources is shown in Fig. 1. It is clear that the human leukocytes from a normal control male (sample b) and human diploid fibroblast in culture (d) both have an HGPRT activity similar in mobility to those from two man–mouse hybrid lines derived from the mouse fibroblast mutant A_9 (c and g). The A_9 cell, which is deficient in HGPRT, shows no detectable enzyme activity in this assay system. It is noteworthy that human red cells, though obtained from the same blood sample from which the leukocytes (b) are derived, exhibit a broader band (a). This behavior of the human red cell enzyme is a

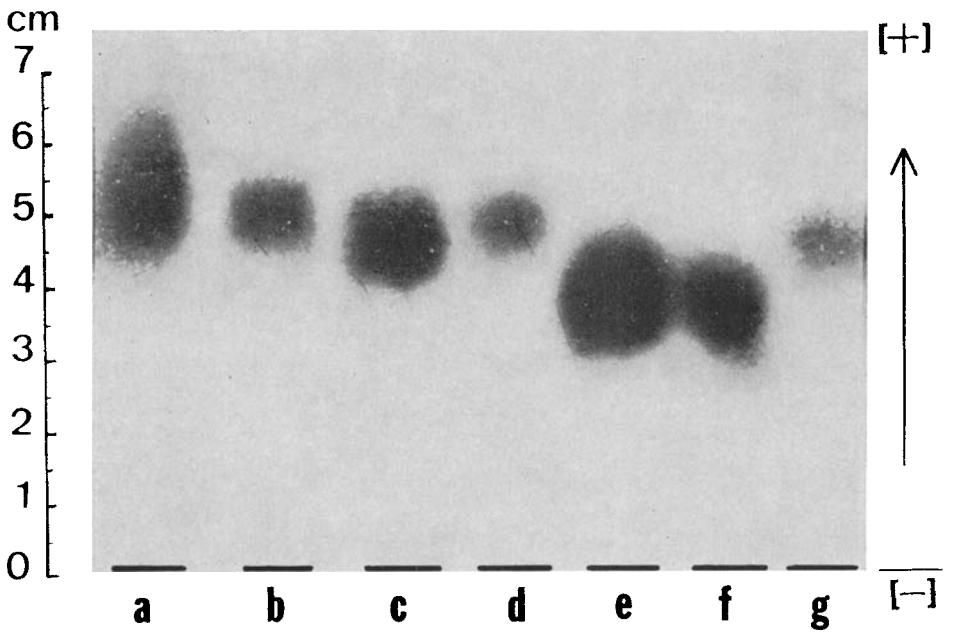

Fig. 1. Electropherogram of hypoxanthine-guanine phosphoribosyl transferase (HGPRT), revealed by autoradiography. (The incubation mixture contained 5 μc hypoxanthine-8-^{14}C per milliliter, and the film was exposed for 2 days.) Samples (a) and (b): Control human red blood cells and leukocytes, respectively, from a normal male donor. (c) Man–mouse hybrid A_9/HRBC(2), derived from the HGPRT-deficient mouse fibroblast A_9 and nucleated cells from the blood of an erythroblastotic male infant with red cell precursors making up 50 percent. (d) Diploid human fibroblast culture. (e) Man–mouse hybrid B82/HF2, from mouse fibroblast B82 (HGPRT normal, TK deficient) and a human embryonic fibroblast culture. (f) Normal mouse fibroblast L-929. (g) Man–mouse hybrid A_9/4XY, from A_9 and a male human fibroblast culture carrying multiple X chromosomes.

reproducible phenomenon, and does not appear to be due to storage of the hemolysate before electrophoresis. Whether this reflects the progressive senescence *in vivo* of the red cells in the peripheral blood, or is an indication of red cell specific polymorphism of the enzyme, is still not determined. However, all A_9–man hybrid cells have HGPRT activity which is electrophoretically indistinguishable from that of the human leukocyte itself (b). The parental human cell types in this group included (1) fibroblasts from a normal diploid control and an adult with the sex chromosomal complement of XXXXY (4X,Y), (2) peripheral blood lymphocytes from two female and one male adults, and (3) nucleated cells from the blood of an erythroblastotic newborn male infant with red cell precursors making up 50 percent.

On the other hand, the HGPRT activity of the hybrid line B82/HF2 (Fig. 1e), a product of fusion between the mouse line B82 ($HGPRT^+$, TK^-) and a human embryonic fibroblast culture, is similar to that of the wild-type mouse fibroblast L–929 (f). The human-type enzyme is notably absent from the hybrid B82/HF2.

In order to determine with greater sensitivity whether the two peaks representing the human and the mouse HGPRT activities overlap significantly and whether additional minor activity peaks are present but not detected by autoradiography, the

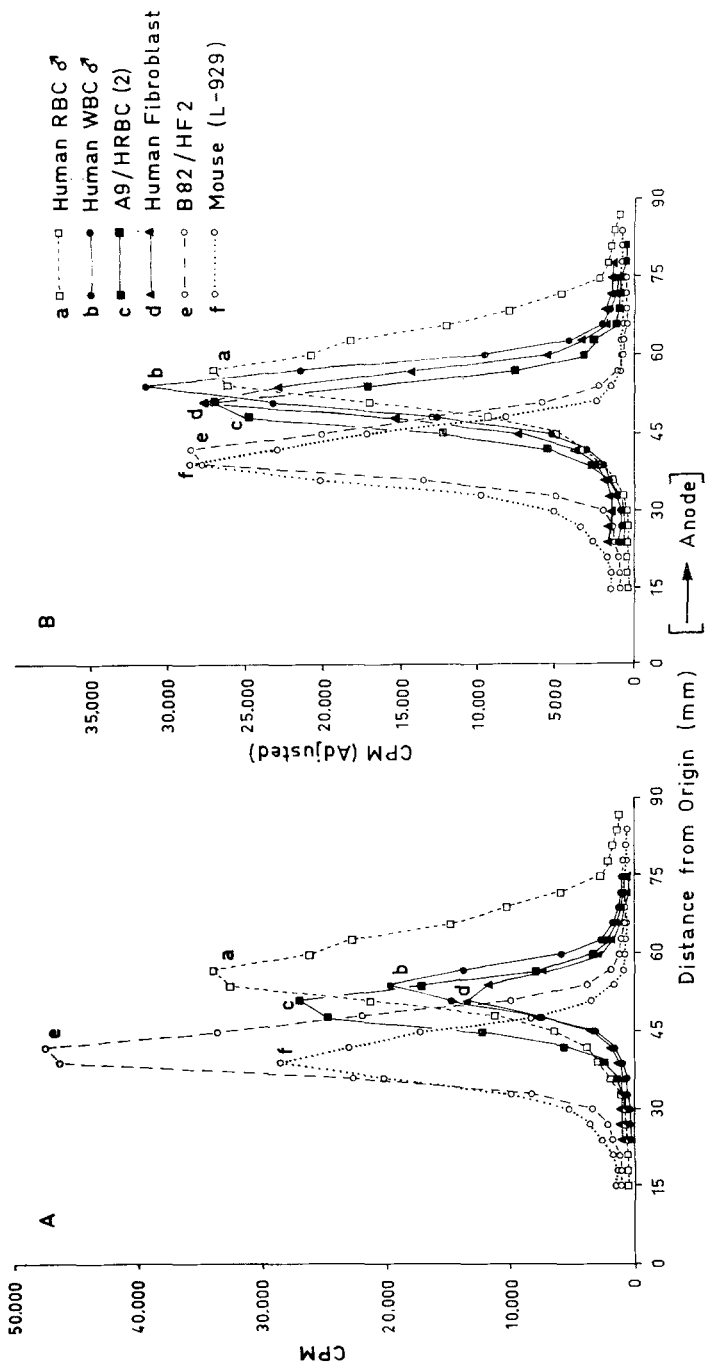

Fig. 2. Radioactivity of DEAE-cellulose paper sections cut out from the electropherogram. The letters identifying the samples correspond to those given in Fig. 1. In A, the actual cpm is given; in B, the activity for each sample is normalized to the level of sample (f) so that the peak heights are comparable. (See text.)

following test was carried out. The DEAE-cellulose paper, from which the autoradiograph reproduced in Fig. 1 was made, was cut into 1 cm strips from the origin to the anode along the center of each sample channel. Each strip was again cut transversely into consecutive 1 cm by 3 mm sections and counted in a liquid scintillation counter. The results are summarized in Fig. 2. In Fig. 2A, the height of the peak (cpm) is a measure of the HGPRT activity in that particular cell extract. Differences in the total enzyme activity originally applied to the Cellogel resulted in peaks of different heights. However, if the enzyme activities are normalized to the activity of the control mouse fibroblast (sample f in Fig. 2A), the resolution of the HGPRT into two clearly different, nonoverlapping areas becomes apparent (Fig. 2B). It can also be seen that there are no other activity peaks present which are not detected by autoradiography.

The direct assay of HGPRT, in which cut-out sections of the Cellogel were incubated directly in the reaction mixture, produced results identical to those shown in Fig. 2. This provided additional evidence that the results of the indirect assay using autoradiography do represent the true HGPRT activity of the cells. We have now used this autoradiographic method to analyze the nature of the HGPRT in more than 150 clonal sublines of various man–mouse hybrids. We have also found that the mobilities of HGPRT of man, mouse, rat, and hamster can be distinguished from each other (Meera Khan and Shin, unpublished data).

DISCUSSION

Cellogel has been used successfully for the past few years in the Leiden laboratory in the survey of human populations for variants in several polymorphic enzymes, such as glucose 6-phosphate dehydrogenase (Rattazzi *et al.*, 1967) and 6-phosphogluconate dehydrogenase (Meera Khan and Rattazzi, 1968). It is supplied and stored in a ready-to-use form, and lends itself to easy and fast handling for routine microassay. Our results indicate that it can also be used in the characterization of HGPRT in smaller quantities than is possible with the conventional starch gel technique. We believe that the improved method reported here could be a useful tool for the characterization of kinases and phosphorylases for which a direct method of electrophoretic assay is not yet available.

While our work was in progress, Migeon *et al.* (1969) reported that the thymidine kinase activity in man–mouse somatic cell hybrids derived from a TK^- mouse cell line had the electrophoretic mobility in starch gel similar to that of human fetal liver and fibroblasts. Since the hybrid B82/HF2 in our series (Fig. 1e) represents the same kind of combination of parental genotypes as the hybrids studied by these authors, we have omitted the characterization of the thymidine kinase in our hybrid line. The fact that this line has retained its mouse-type HGPRT is as expected, and suggests that its thymidine kinase would indeed be of the human type as this is required for its survival in HAT medium.

After it was shown by Seegmiller *et al.* (1967) that HGPRT deficiency is a basic biochemical defect responsible for the X-linked recessive disease known as the Lesch–Nyhan syndrome (Lesch and Nyhan, 1964), this enzyme has become a most useful genetic marker for the study of X-linkage in man (Siniscalco *et al.*, 1969).

Production of man–mouse somatic cell hybrids specifically designed for this purpose has been reported recently by several independent groups (Nabholz *et al.*, 1969; Boone and Ruddle, 1969; Miller *et al.*, 1970). The underlying assumption that the human HGPRT gene is retained and functionally expressed in these hybrids, thus enabling them to proliferate selectively in the HAT medium, is strengthened by our data. In a collaborative study, we are presently investigating the hybrids from these three laboratories to determine the degree of association of the human HGPRT activity with other human X-linked markers among clonal subpopulations.

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