Epigenetic modulation of the mouse HGPRT gene in interspecific reconstituted cells and cybrids

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Reconstituted cells *Cybrids* *APRT* *HGPRT* *Epigenetic modulation*

1. INTRODUCTION

Nuclear–cytoplasmic interactions present basic problems in research on eukaryotic cells. The enucleation technique with cytochalasin B (CB) makes it possible to isolate viable reconstituted cells or cytoplasmic hybrids (cybrids) by fusing karyoplasts (or intact cells) to cytoplasts. Chloramphenicol-resistant (CAP^r) cytoplasts from the double-mutant cells which are sensitive to a medium containing hypoxanthine, aminopterin and thymidine (HAT medium) and resist chloramphenicol (CAP) can be fused with HAT-resistant, CAP-sensitive cells (or karyoplasts). Such fusions have led to the isolation of CAP^r-reconstituted cells or cybrids essentially free of contaminating parental cells that were eliminated by selection with both HAT and CAP [1]. We have thus isolated many CAP^r-interspecific reconstituted cells and cybrids by fusing karyoplasts (or intact cells) of HAT-resistant, CAP-sensitive mouse melanoma B_{16} cells with cytoplasts of HAT-sensitive, CAP-resistant rat $L_6TG \cdot CAP^r$ cells. Expression of HGPRT activity and CAP-resistance are essential for the survival of cells in this selection medium. This paper describes the epigenetic modulation of the mouse HGPRT gene in cells reconstituted by fusing rat cytoplasts with mouse karyoplasts. By gel electrophoretic analysis, we found 2 components of HGPRT, a main and a minor component, in several mouse cell lines. Epigenetic modulation of the mouse HGPRT gene occurred in some of the reconstituted cells or cybrids isolated and grown as clones. In these clones the main band of HGPRT migrated much faster than the HGPRT of the parental B_{16} cells.

Abbreviations: APRT, adenine phosphoribosyl-transferase; HGPRT, hypoxanthine-guanine phosphoribosyl-transferase

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2. MATERIALS AND METHODS

2.1. *Cell lines*

Mouse melanoma B_{16} cells were used as whole cells or as karyoplast donors. This cell line was derived from a C57 B1 mouse, and is HAT-resistant and CAP-sensitive. As cytoplast-donor cells, HGPRT-deficient, CAP-resistant rat myoblastic cells, $L_6TG \cdot CAP^r$, were used. This cell line was derived from HGPRT-deficient rat L_6TG cells by mutagenesis with ethylmethane sulfonate (200 μg/ml for 18 h) and stepwise selection in medium with CAP. The cells are resistant to CAP at 150 μg/ml [2]. L_6TG cells were provided by Professor N.R. Ringertz (Karolinska Institute, Stockholm). The other mouse cell lines used were L_{929}, a thymidine kinaseless subline B_{82} and an HGPRT-deficient subline A_9. These cell lines were provided by Professor H. Harris (Sir William Dunn School of Pathology, Oxford University). All cell lines were cultivated in Eagle's MEM containing 10% fetal calf serum.

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2.2. *Isolation of interspecific reconstituted cells and cybrids*

Interspecific reconstituted cells and cybrids were isolated by cloning according to the principle [1] employing both nuclear marker HAT-resistance and cytoplasmic marker CAP-resistance to exclude contaminating parental cells.

Karyoplasts were prepared from mouse B_{16} cells while cytoplasts were prepared from rat $L_6TG \cdot CAP^r$ cells as in [3] with cytochalasin B. The karyoplast preparation obtained by centrifugation contained karyoplasts, some intact cells (usually, 10–15%), and considerable amount of cytoplasmic debris. This crude karyoplast material was purified by sedimentation on a sterile linear gradient of 1–3% bovine serum albumin (BSA, Armour fraction V) [4]. The extent of contamination of the karyoplast preparation with intact cells was reduced by this purification to < 1% of that of the untreated preparation. The enucleated cytoplasts ($2–3 \times 10^5$/disc) attached to glass discs were fused with the same number of purified karyoplasts or B_{16} cells (cybridization) using UV-inactivated hemagglutinating virus of Japan (HVJ). After fusion (24 h) the cells on discs were cultivated in double selection medium HAT • CAP (150 μg CAP/ml). During this double selection, parental cells were unable to grow or form colonies. Unfused rat cytoplasts on the glass discs were gradually attenuated. A few colonies appeared on some discs 10 days after fusion, and 2–3 weeks after fusion they were isolated by cloning by the cylinder method [5].

2.3. *Karyological studies*

The chromosome analysis was carried out on these clonal cells by staining with quinacrine mustard combined with Hoechst 33258 [6]. Mouse chromosomes were identified by their bright centromeres and characteristic banding patterns.

2.4. *Isozyme analysis of APRT and HGPRT*

Samples of 2×10^7 cells were harvested with a rubber policeman and centrifuged. The packed cells were washed 3 times with phosphate-buffered saline (PBS) (pH 7.0) and then with 0.5 M Tris–HCl buffer (pH 6.8) and suspended at 2×10^7 cells/ml in the latter buffer. The cells were lysed by sonication using a model W-220F Sonicator (Ultrasonic, New York NY) with the probe intensity set at 60 for 2 min. Sonicated samples were centrifuged at 12 000 × *g* for 30 min at 4°C to remove cell debris. The clear supernatants were retained for the assays. Electrophoretic separations of APRT and HGPRT were carried out as follows. The method in [7] was used to prepare separating gels of 7% acrylamide and stacking gels of 4% acrylamide. Electrophoresis was carried out at 4°C at a constant 1 mA/cm until the band of dye reached the bottom of the gel. The APRT or HGPRT was located on the gel as in [8,9] with some modifications. After electrophoresis, the gels were incubated at 37°C in 10 ml solution of 0.01 M Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 mM 5-phosphoribosyl-pyrophosphate (PL Biochemicals) and 5 μCi [8-^{14}C]adenine (40.5 mCi/mM, New England Nuclear) or 5 μCi [8-^{14}C]hypoxanthine (42.5 mCi/mM, New England Nuclear). After 1 h, these substrate solutions were decanted and the gels were rinsed with deionized water. The products, adenosine-5-monophosphate or inosine-5-monophosphate, were precipitated in the gels by immersing in a solution of 0.1 M Tris–HCl (pH 7.0) and 0.1 M $LaCl_3$ at 4°C for 2 h. The radioactive substrates adenine or hypoxanthine were removed by rinsing the gels with 2 l deionized water for 30 min. The gels were then dried on filter paper and autoradiographed by contact with an X-ray film for several days.

2.5. *Mycoplasma test*

Possible contamination of cell cultures with mycoplasm was examined as in [10] with Hoechst 33258 staining.

3. RESULTS

3.1. *Clonal isolation of interspecific reconstituted cells and cybrids*

So far 41 reconstituted clones have been isolated in 16 independent reconstruction experiments in each of which 2.5×10^6 purified mouse karyoplasts were used for fusion. In addition, 25 cybrid clones have been isolated in 10 independent cybridization experiments in each of which 2.5×10^6 mouse cells were used for fusion. Chromosome analysis was carried out on cells of these clones. The rat cells, $L_6TG \cdot CAP^r$, were characterized by the presence of 37–42 chromosomes (mode 40). The mouse B_{16} cells were characterized by a modal number of 41 chromosomes (range 38–42), 3 of which were biarmed. Karyotype analysis of two reconstituted

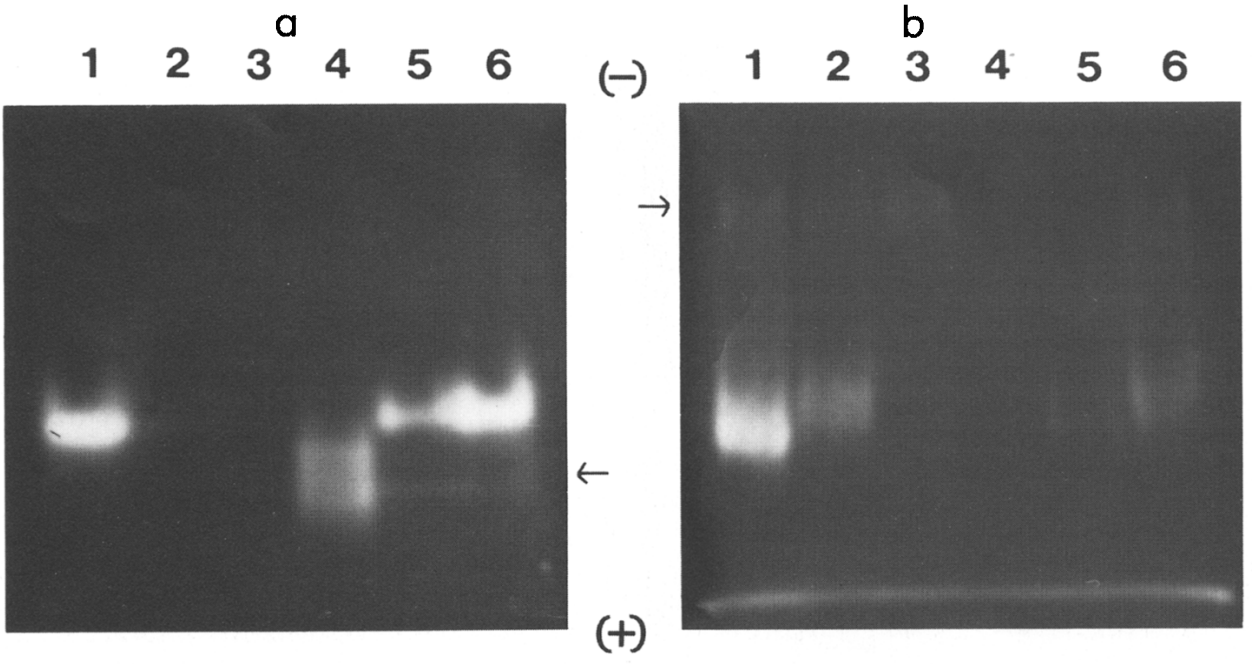

Fig.1. Electrophoretic separation of APRT and HGPRT isozymes of several mouse cell lines and rat cell line: (a) zymogram of APRT; (b) zymogram of HGPRT. Channels: (1) mouse L_{929} cells; (2) mouse TK-B_{82} cells; (3) mouse HGPRT deficient A_9 cells; (4) rat HGPRT-deficient $L_6TG \cdot CAP^r$ cells; (5) reconstituted clone, RC 13-11 cells; (6) mouse B_{16} cells. A minor component of APRT or HGPRT is marked with arrows.

clones, RC 6-7-8-5 and 14-4-6, showed that they did not contain any rat chromosomes; only mouse chromosomes were present. Details of karyotypic analysis of these cell lines have been reported [5,11].

3.2. *Appearance of a minor component and a main band of APRT in the mouse cell lines*

The APRT isozyme pattern of L_{929} cells had a predominant mouse isozyme. In contrast its thymidine kinase (TK)-deficient subline B_{82} cells and its HGPRT-deficient subline A_9 cells revealed a complete lack of the enzyme (fig.1a). In addition to the mouse main band, there was a minor component in B_{16} cells (fig.1a). This minor component was weak and had a faster mobility than that of the main band. This minor component also appeared in reconstituted clones and cybrid clones (i.e., RC 6-7-8-5, 14-4-6, 13-11, and cybrid clone 15-1-1 cells) (fig.2a). The APRT isozyme pattern of HGPRT-deficient rat cells, $L_6TG \cdot CAP^r$ cells had a strong and broad band. The band was faster than the APRT main band of the mouse cell lines (fig.1a).

3.3. *Appearance of a minor component and a different main band of HGPRT in the mouse cell lines*

Gel electrophoretic analysis of HGPRT isozymes, showed that L_{929} cells expressed 2 bands of HGPRT isozymes, a main and a minor component (fig.1b). The minor component was weak and had a very low mobility. This minor component appeared in L_{929}, in its TK-deficient subline B_{82} and in B_{16} cells. A_9 cells, an HGPRT-deficient subline of L cells, expressed only the minor component. HGPRT-deficient rat cells, $L_6TG \cdot CAP^r$ cells, expressed neither the main nor the minor component (fig.1b).

3.4. *Modulation of the HGPRT gene in reconstituted cells and cybrids*

Electrophoretic separation of the HGPRT isozyme of isolated reconstituted clones and cybrid clones, showed that some clones (i.e., RC 6-7-8-5 and 14-4-6 cells) expressed a similar pattern of HGPRT isozymes to that of the parental mouse B_{16} cells (fig.2b). In contrast, other clones (i.e., RC 13-11, and cybrid clone 15-1-1 cells) expressed a different

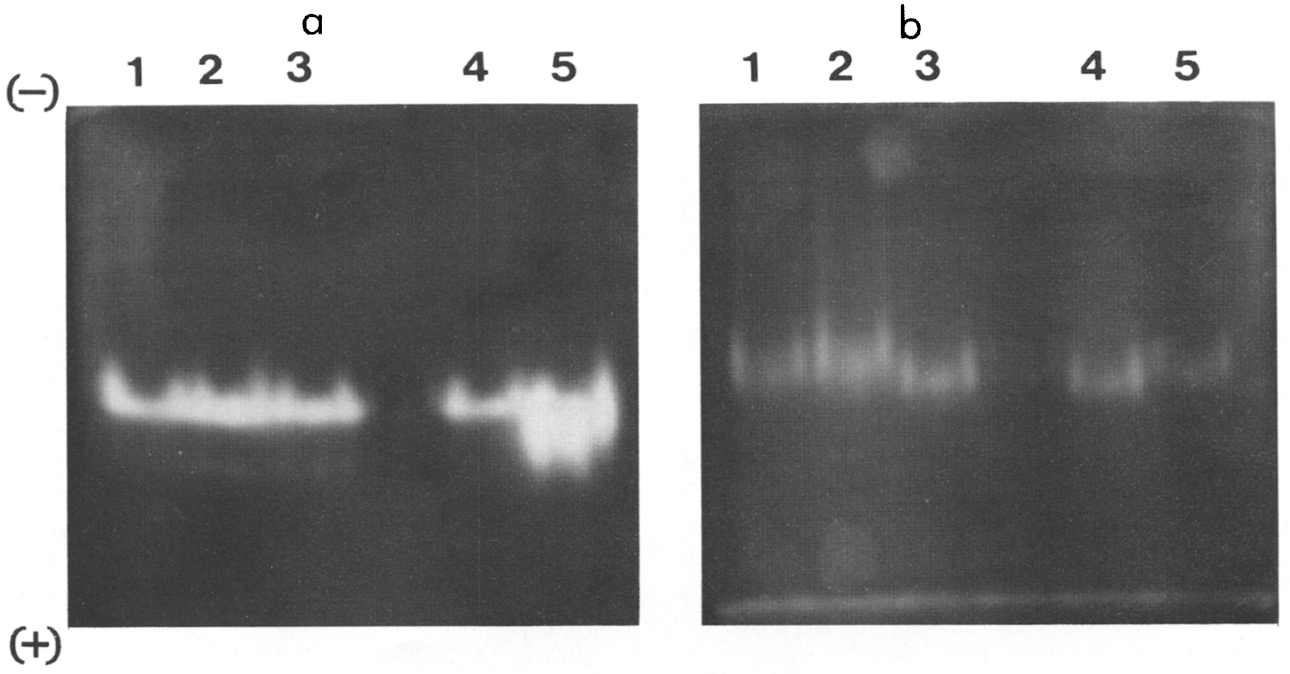

Fig.2. Electrophoretic separation of APRT and HGPRT isozymes of reconstituted cells and cybrid: (a) zymogram of APRT; (b) zymogram of HGPRT. Channels: (1) reconstituted clone, RC 6-7-8-5 cells; (2) reconstituted clone, RC 14-4-6 cells; (3) reconstituted clone, RC 13-11 cells; (4) cybrid clone 15-1-1 cells; (5) hybrid clone 1-1 cells (mouse B_{16} × rat $L_6TG \cdot CAP^r$).

main component of HGPRT which migrated faster than that of the parental B_{16} cells. The minor component in all these clonal cells produced a very weak band (fig.2b). Thus the reconstituted cells and cybrid clones fall into 2 groups, an HGPRT-modulated group and an HGPRT-unmodulated group. Repeated examination of the HGPRT isozyme patterns of these clonal cells during >6 months in culture has shown that the modulation of expression of the HGPRT gene in these clones is stable.

4. DISCUSSION

4.1. *Appearance of a minor component and a main band of APRT in mouse cell lines*

The present results demonstrate the existence of 2 isozymes of APRT, a main and a minor isozyme of different electrophoretic mobilities. The minor band is expressed in mouse melanoma B_{16} cells, reconstituted clones and cybrid clones whose nuclei are derived from B_{16} cells. It is unlikely that the appearance of a minor component is due to degradation of APRT molecule, since B_{16} cells have a minor component, on the other hand L cells treated by the same condition have no minor component. No mycoplasm contamination could be detected; thus it is unlikely that the minor component was due to mycoplasm contamination. Our studies show that the minor component of APRT is regulated by nuclear gene of B_{16} cells, since not only B_{16} cells but also every clone of reconstituted cells of nuclei derived from B_{16} cells has a minor component. It would be interesting to know the location of the gene encoding the minor component of APRT.

HGPRT-deficient subline of L cells, A_9 cells had no APRT activity [12]. Similarly TK-deficient subline of L cells, B_{82} cells have no APRT activity. It would be interesting to know the relation between APRT deficiency and purine metabolism.

4.2. *Appearance of a minor component and a different main component of HGPRT in mouse cell lines*

These results demonstrate the existence of 2 isozymes of HGPRT, a main and a minor isozyme of different electrophoretic mobilities. Both are enzy-

matically active molecules and are expressed in several mouse cell lines. Hitherto, only the main component of HGPRT has been detected in mouse cell lines by gel electrophoresis [13], or by DEAE-cellulose chromatography [13]. A_9 cells, an HGPRT-deficient subline of L cells, expressed only the minor component. There are 2 components of HGPRT in mutant Chinese hamster cells [14,15]. These isozymes were detected by immunoprecipitation and SDS–polyacrylamide gel electrophoresis. These 2 components were both antigenically active, but one of them was an enzymatically inactive, mutant molecule. It is known that thymidine kinase-deficient mouse L cells, LK(TK-) Cl 1d cells, are HAT-sensitive cells but possess normal levels of mitochondrial thymidine kinase activity [16]. This fact indicates that the mitochondrial TK activity in Cl 1d cells is not enough for survival of the cells in HAT medium. HGPRT-deficient mouse L cells, A_9 cells, are HAT-sensitive cells but they do possess a small amount of HGPRT activity that appears as the minor component after electrophoresis. The minor isozyme is therefore not sufficient to permit survival of A_9 cells in HAT medium.

It is unlikely that the appearance of a minor component or a different main component of HGPRT in these mouse cell lines is due to degradation of the HGPRT molecule since the results were reproducible, and the cells were harvested with a rubber policeman, not with trypsin or ethylenediamine tetraacetate. No mycoplasm contamination could be detected in these cultured cells; thus it is unlikely that the minor component was due to mycoplasm contamination. Some clones of HGPRT-deficient Chinese hamster cells [17] or mouse cells can complement each other [18]; thus, some clones of 8-azaguanine resistant mouse cells complemented all the clones of 6-thioguanine-resistant mouse cells tested, and produced an active, but altered, HGPRT molecule [18]. It would be interesting to know which HGPRT component is lacking in these 8-azaguanine- or 6-thioguanine-resistant mouse cells, and what kind of HGPRT molecule is produced in these complemented cells. It would be also interesting to know the location of the gene encoding the minor component of HGPRT, whether on the X chromosome or an autosome or in the mitochondrial genome.

4.3. *Epigenetic modulation of the HGPRT gene in reconstituted cells and cybrids*

These results also demonstrate that some clones of isolated reconstituted cells or cybrids express a modified HGPRT isozyme, that migrates as a fast main band. The difference in the mobility of HGPRT main band was a little; however, the result was reproducible. The mobility of APRT main band separated with the same apparatus at the same time as analyzing HGPRT was identical. Thus we believe firmly that the difference is significant. There are several reports of correction or modulation of the HGPRT gene in mammalian somatic cells as observed after cell-to-cell hybridization. When mouse A_9 cells were fused with chick erythrocytes, clonal cells were isolated which expressed the HGPRT isozyme of the chick species [19]. A similar phenomenon was reported in [20]. Chick HGPRT was expressed in HGPRT-deficient Chinese hamster cells after fusion of the latter with chick erythrocytes. In these heterokaryons, however, no chick chromosomes could be detected, and it was assumed that a small fragment of the chick genome, including the HGPRT gene, had been integrated into the genome of these heterokaryons. However, in [22] mouse HGPRT gene in A_9 cells or RAG cells was reported after treatment with DNA isolated from several sources including HGPRT-deficient cells. Activation of the TK gene in TK- mouse cells was reported after fusion with chick erythrocytes in [21]. A factor inherited from the chick was claimed to correct the TK gene in the TK-mouse cells, but no conclusions were drawn on whether or not the factor was of cytoplasmic origin. Here, no rat chromosomes could be detected in the reconstituted cells or cybrids, and the expression of an HGPRT gene must be attributed to the rat cytoplasts. A few examples of epigenetic activation of gene expression in reconstituted cells or cybrids have been reported. The expression of the liver-specific enzyme, tyrosine aminotransferase, by mouse fibroblast nuclei which had been transplanted into rat hepatoma cytoplasts, was reported in [23]. Epigenetic activation of phenylalanine hydroxylase in mouse erythroleukemia cells by fused cytoplasts of rat hepatoma cells was reported in [24]. A remarkable finding here is that the modified expression of the HGPRT gene in some reconstituted cells or cybrids has been stable for >6 months. Detailed studies on the stable

cytoplasmic regulation of gene expression in these clonal cells will be reported elsewhere (submitted).

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