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Assignment of the Gene for Adenine Phosphoribosyltransferase to Human Chromosome 16 by Mouse–Human Somatic Cell Hybridization

(human linkage/chromosome 16/APRT)

JAY A. TISCHFIELD* AND FRANK H. RUDDLE

Department of Biology, Yale University, New Haven, Connecticut 06520

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ABSTRACT **A series of mouse–human hybrids was prepared from mouse cells deficient in adenine phosphoribosyltransferase (EC 2.4.2.7) and normal human cells. The hybrids were made in medium containing adenine and alanosine, an antimetabolite known to inhibit *de novo* adenylic acid biosynthesis. The mouse cells, unable to utilize exogenous adenine, were killed in this medium, but the hybrids proliferated as a consequence of their retaining the human *aprt* gene. The hybrids were then exposed to the adenine analogs 2,6-diaminopurine and 2-fluoroadenine to select for cells that had lost this gene. Before exposure to the adenine analogs, the expression of human adenine phosphoribosyltransferase by the hybrids was strongly associated only with the presence of human chromosome 16, and afterwards this was the only human chromosome consistently lost. This observation suggests that the human *aprt* gene can be assigned to chromosome 16.**

Mouse–human somatic cell hybrids are useful for genetic analysis because they progressively lose human chromosomes and the species origin of the chromosomes and a variety of cellular phenotypes can be distinguished. It is possible, therefore, to assign the genes for certain phenotypes to particular human chromosomes by noting the concordant presence or absence of a particular phenotype(s) and chromosome in many independent hybrid clones. Observations of the loss of the phenotype coincident to the loss of the human chromosome strengthen such assignments. Selective methods have been developed to enrich for hybrids in a mixed population of parental cells. Most notable is the HAT selective system of Littlefield (1), which allows hybrid survival by retention of a gene from each of the parental inputs. This subject has been reviewed by Ruddle (2).

Studies have indicated that the locus for adenine phosphoribosyltransferase (APRT) is autosomally inherited (3), and electrophoretic variants have been reported (4). Kusano, Long, and Green (5) described a selection method where survival of mouse–human hybrids depended upon their retention of the human *aprt* gene. Mouse cells resistant to the adenine analogs 2,6-diaminopurine (DAP) and 2-fluoroadenine (FA), and consequently lacking APRT activity (APRT$^-$), were hybridized to normal human cells in medium containing adenine and alanosine. Alanosine is an inhibitor of *de novo* AMP biosynthesis (6, 7). The mouse cells died, as they could not utilize exogenous adenine to produce AMP. Hybrids, however, survived by retaining the human *aprt* gene which allowed utilization of the exogenous adenine. The hybrids could then be counterselected with FA to obtain cells that were APRT$^-$. This method has enabled us to assign the human *aprt* gene to chromosome 16, providing an additional selectable marker whose human linkage is known.

Abbreviations: APRT, adenine phosphoribosyltransferase (EC 2.4.2.7); *aprt* gene, gene directing synthesis of APRT; DAP, 2,6-diaminopurine; FA, 2-fluoroadenine; AA medium, medium containing alanosine and adenine.

* Present address: Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106.

MATERIALS AND METHODS

Quantitative Assay for APRT. Cells were washed three times *in situ* with cold phosphate-buffered saline (pH 7.2) and collected with a Teflon scraper. They were pelleted and resuspended at about 5×10^7 cells per ml of 0.01 M Tris buffer, pH 7.4, containing 1 mM EDTA. The suspension was then ultrasonically disrupted for 10 sec and centrifuged at 40,000 × *g* to remove cell debris. The supernatant fraction was either assayed immediately or stored at −70°. Storage for 6 months did not significantly affect activity.

The assay mixture for APRT (8) contained [^{14}C]adenine, 25 μM (0.1 μCi, 40.7 mCi/mmol); $MgCl_2$, 5 mM; phosphoribosyl pyrophosphate, 1 mM; and Tris buffer, 55 mM (pH 7.4). TTP (3.3 mM) was added to inhibit breakdown of labeled AMP by 5′-nucleotidase (9). Blank values were obtained with bovine-serum albumin or boiled supernatant.

Assays were performed at 37°, and samples were removed after 15 and 30 min. The reaction was stopped with the addition of EDTA to 0.01 M and immersion in a dry ice–methanol bath. A 20-μl aliquot of each sample was applied to a Whatman DE-81 (DEAE–cellulose) disk which was then washed with 30 ml of 1 mM ammonium formate and 10 ml of absolute alcohol on a Millipore sampling manifold, and dried with an infrared lamp (5). Radioactivity was determined by liquid scintillation. If linearity with time was observed, activity was calculated from the difference between the 15- and 30-min samples.

Qualitative Assay for APRT. Mouse and human APRT were distinguished with an acrylamide gel electrophoresis–autoradiographic method which is described elsewhere (10).

Production and Analysis of Hybrids. The standard medium was Dulbecco–Vogt modified Eagle's medium supplemented with 10% heat-inactivated fetal-calf serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 100 μg/ml of kanamycin, and equilibrated with 10% CO_2–90% air.

Alanosine [L(−) 2-amino-3-nitrosohydroxylamino propi-

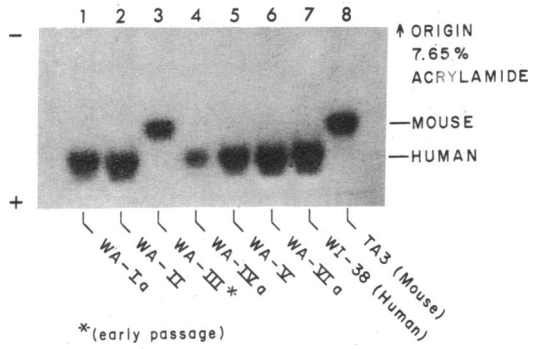

FIG. 1. Autoradiograph (96-hr exposure) of an acrylamide gel incubated for APRT. Extracts of AA-selected clones and mouse and human controls were subjected to electrophoresis. *Slot 3* was an extract of clone WA-III soon after its isolation.

onic acid] was the gift of Dr. P. Sensi of Lepetit, Milan, and FA was donated by Merck, Sharpe and Dohme.

Hybridization was facilitated with Sendai virus inactivated by β-propiolactone (11, 12). Three hybrid series were produced, all of which had cell line A9 (13) as the mouse parent. The *WA* series was produced with the primary diploid human fibroblastic cell strain WI-38, the *JBA* series with leukocytes from an individual heterozygous for a 14/22 centric fusion, and the *JFA* series with cultured fibroblasts obtained from a skin biopsy of the same individual. These hybrids have been utilized in other linkage studies which have been reviewed (2).

Hybridization was done in standard medium. From 2–38 hr after virus treatment, the cells were transferred to multiple flasks containing medium supplemented with alanosine and adenine (see below). Colonies of about 2 mm in diameter were picked 4–8 weeks later. Only one colony per flask was picked to insure their independence. The selective medium should select hybrids expressing human APRT as well as any mouse revertants to the $APRT^+$ phenotype.

Clones isolated from alanosine–adenine medium (AA medium) were assayed for the human forms of 18 enzymes by starch-gel electrophoresis (14). The enzymes were: adenosine deaminase (EC 3.5.4.4), esterase-A4, glutamate oxaloacetate transaminase (EC 2.6.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glucose phosphate isomerase (EC 5.3.1.9), isocitrate dehydrogenase (EC 1.1.1.42), indophenol oxidase-B (tetrameric form), indophenol oxidase-A (dimeric form), lactate dehydrogenase-A (EC 1.1.1.27), lactate dehydrogenase-B (EC 1.1.1.27), malate oxidoreductase decarboxylating (EC 1.1.1.40), malate oxidoreductase (EC 1.1.1.37), mannose phosphate isomerase (EC 5.3.1.8), nucleoside phosphorylase (EC 2.4.2.1), peptidase-A, peptidase-B, peptidase-C, phosphoglycerate kinase (EC 2.7.2.3).

TABLE 1. *APRT activities in cell extracts*

Extracts	Specific activity (cpm in AMP per μg of protein)
Parental cells	
WI-38	151
A9	0
Clones selected in AA medium	
WA-Ia	119
WA-IIa	190
WA-III	99
WA-IVa	96
WA-V	244
WA-VIa	174
WA-VIIa	102
WA-VIIIa	89
WA-IXa	97
JBA-1	55

Chromosome analysis was done with quinacrine mustard banding (15), Giemsa banding (16), and constitutive heterochromatin (17). To facilitate chromosome identification, metaphases photographed with ultraviolet illumination for quinacrine mustard banding were also photographed with darkfield illumination in the visible spectrum. The majority of metaphase cells were prepared as karyotypes and scored independently by two people. A total of 972 metaphases were analyzed, with an average of about 50 per clone.

Human and mouse chromosomes were identified by their unique banding patterns with all three techniques and by their relative lengths. Identifications were made with reference to a series of standard mouse and human karyotypes, and designations of human chromosomes were consistent with the criteria established by the Paris Conference (18). To confirm identifications based on quinacrine mustard banding, the same metaphases were frequently stained for constitutive heterochromatin. This procedure was useful for easily distinguishing between mouse and human chromosomes, especially the biarmed chromosomes (17). Chromosome 16 was particularly striking with Giemsa banding and constitutive heterochromatin staining, as it displayed a large, submetacentric heterochromatic block on the long arm.

RESULTS

During a survey of cell lines, A9, a mouse L-cell variant selected for resistance to 8-azaguanine and lacking hypoxan-

TABLE 2. *Human enzymes expressed by 9 hybrid clones selected for retention of human APRT*

Enzyme	No. of clones expressing enzyme	Human linkage unit (chromosome)
APRT	9	?
ADA	4	20
Es-A4, LDH-A	3	11
GOT-S	1	10
G6PD, PGK	4	X
GPI	2	19
IDH, MOR-S	5	2
IPO-A	4	21
IPO-B, MOD-S	1	6
LDH-B, Pep-B	5	12
MPI	1	7
NP	4	14
Pep-A	3	18
Pep-C	2	1

ADA, adenosine deaminase; *Es-A4*, esterase-A4; *LDH*, lactate dehydrogenase; *GOT-S*, glutamate oxaloacetate transaminase; *G6PD*, glucose-6-phosphate dehydrogenase; *PGK*, phosphoglycerate kinase; *GPI*, glucose phosphate isomerase; *IDH*, isocitrate dehydrogenase; *MOR-S*, malate oxidoreductase; *IPO*, indophenol oxidase; *MOD-S*, malate oxidoreductase decarboxylating; *MPI*, mannose phosphate isomerase; *NP*, nucleoside phosphorylate; *Pep*, peptidase.

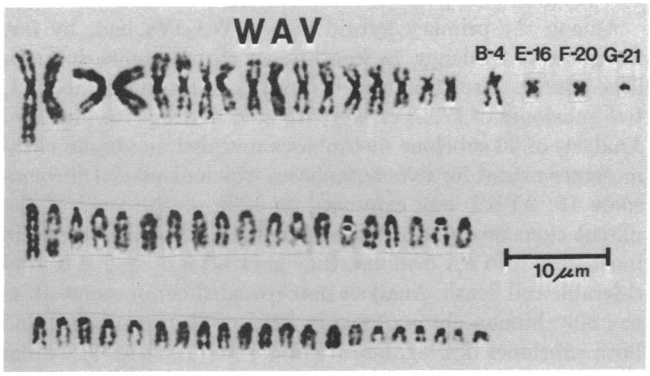

FIG. 2. Karyotype of a cell from hybrid clone WA-V stained for Giemsa banding. Human chromosomes are identified. This clone expressed human APRT.

thine phosphoribosyltransferase activity (13), was noted to be essentially devoid of APRT activity, a finding which has since been confirmed (19). Mixing experiments with mouse cell lines having high APRT activity suggested that the deficiency was not due to an inhibitor of enzyme activity. As expected, A9 was almost completely resistant to 100 μg/ml of DAP and 20 μg/ml of FA.

When a total of 5×10^6 A9 cells were inoculated into AA medium, no survivors were observed. Killing was rapid at a cell density of $1 \times 10^4/cm^2$ in medium containing 1.5 μg/ml of alanosine and 50 μM adenine, whereas WI-38 cells proliferated in this medium at only a slightly reduced growth rate, with the addition of uridine having no discernable effect. WI-38, however, was rapidly killed in medium containing 0.5 μg/ml of FA. Cells were grown in standard medium supplemented with adenine only, and 50 μM seemed optimal as it had no effect on the growth of WI-38, but caused about a 20% reduction in the growth rate of A9. Higher concentrations reduced the growth rate of A9 more drastically and caused some inhibition of WI-38.

Presumptive hybrid clones were distinguished from the sparse monolayer background of human fibroblasts since they grew rapidly and had a different cellular morphology, forming compact masses with a significant degree of cell piling (20). Where the human parent was a leukocyte, identification was simple since the leukocytes did not attach to the substratum or proliferate.

Clones were isolated from medium containing 7 μg/ml of alanosine and 50 μM adenine, where they had decreased viability as compared to their growth in drug-free medium. When the concentration of alanosine was lowered to 1.75 μg/ml, their growth approximated that observed in drug-free medium. Since this lower concentration of alanosine could effectively kill $APRT^-$ cells, the clones were switched to it after several passages at the higher concentration.

Ten clones were randomly chosen for analysis. All had high APRT activity (Table 1). Nine of the clones were judged to be mouse–human hybrids because: (*i*) They expressed human APRT (Fig. 1); (*ii*) each clone expressed a different combination of human enzymes in addition to all of the mouse enzymes (Table 2); and (*iii*) human chromosomes were observed in their metaphases (Fig. 2 and Table 3). One clone, WA-III, was probably an A9 revertant to the $APRT^+$ phenotype, since it expressed mouse APRT (Fig. 1), did not express any of the human enzymes tested, and had a karyotype similar to that of A9. WA-III was assayed soon after its isolation and again after 6 months of growth in AA medium; only mouse APRT was detectable, suggesting that its expression was not secondary to the expression and subsequent loss of the human enzyme (21).

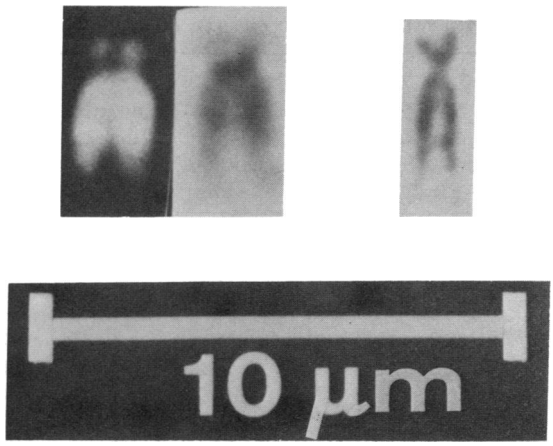

FIG. 3. An example of chromosome T_1 from hybrid clone WA-VIa stained for quinacrine mustard fluorescence (*left*) and constitutive heterochromatin (*middle*); another example stained for Giemsa banding (*right*).

Analysis of the hybrid clones revealed no association between the expression of any of the 18 human enzymes tested and APRT, suggesting that the genes for these enzymes are asyntenic (not linked) to the *aprt* gene (Table 2). Since the human chromosome assignments of these genes are known (2), it is unlikely that *aprt* could be linked to any of these chromosomes (Table 2). The chromosome analysis of the hybrids is shown in Table 3. The expression of human APRT was associated most strongly with the presence of chromosome 16. In only one clone, WA-VIa, was chromosome 16 not detected. This clone, however, displayed several chromosomes that were morphologically unique and had never been observed in either the mouse or human parents. One of these chromosomes, designated T_1 (Fig. 3), was present in 93% of the metaphases examined. All but one of the hybrids were rela-

TABLE 3. *Human chromosomes in 9 hybrid clones selected for retention of human APRT*

Human chromosome	No. of clones detected in	Average frequency in those clones (%)	Human chromosome	No. of clones detected in	Average frequency in those clones (%)
1	2	81	13	1	31
2	2	57	14	4	17
3	2	34	15	0	—
4	5	41	16	8	88
5	0	—	17	1	49
6	1	37	18	3	52
7	2	50	19	2	42
8	3	9	20	4	47
9	0	—	21	4	60
10	2	28	22	4	36
11	3	61	X	4	19
12	5	55	T_1*	1	93

* Unique chromosome observed only in primary hybrid clone WA-VIa.

TABLE 4. *Human chromosomes in 12 counter-selectants*

Human chromosome	No. of clones detected in	Average frequency in those clones (%)	Human chromosome	No. of clones detected in	Average frequency in those clones (%)
1	2	80	13	2	52
2	2	68	14	2	11
3	1	31	15	0	—
4	5	67	16	0	—
5	0	—	17	1	23
6	0	—	18	4	66
7	2	43	19	2	43
8	2	11	20	5	53
9	0	—	21	6	67
10	2	38	22	6	47
11	1	63	X	3	17
12	2	81	T_1*	0	—

* Unique chromosome observed only in primary hybrid clone WA-VIa.

tively stable, since they retained all of their human enzymes after 6 months of serial cultivation.

After one passage in drug-free medium five hybrid clones and WA-III, the putative mouse revertant, were inoculated at several densities into medium containing 100 μg/ml of DAP or 10 μg/ml of FA. No colonies grew in FA, but the average frequency of clones in DAP for the five hybrids was 9.6×10^{-4}. The frequency of colonies in DAP for WA-III was 4.8×10^{-5}. The same experiment was repeated after the clones had been maintained in drug-free medium for 14 passages. In this instance, the average frequency of colonies in DAP for the hybrids was 5.1×10^{-2} and for WA-III, 1.1×10^{-4}. Furthermore, those two hybrids with the highest frequency of colonies in DAP (an average of 1.1×10^{-1}) also showed colonies in FA at about the same frequency. Cells isolated from 100 μg/ml had similarly high plating efficiencies (21–56%) when inoculated into 100 μg/ml of DAP, 10 μg/ml FA, or drug-free medium.

Density-dependent differences in the frequency of colonies in FA were noted for the two hybrids that could be counter-selected in both DAP and FA. The number of colonies in DAP increased linearly with inoculum size, whereas there was a decrease in the number of colonies in FA at cell densities above $6.6 \times 10^3/cm^2$. When the density was doubled, there was an average decrease of 51% in the frequency of colonies, and at 10 times this density the decrease was about 99%.

Ten hybrid subclones derived from four primary clones and an uncloned mass population from a fifth primary clones were isolated by transfer from 100 μg/ml of DAP to 10 μg/ml of FA. None had detectable APRT activity and all were killed in AA medium. The subclones continued to express most of their other human enzymes, and where enzymes were lost, there was no consistent pattern. The chromosome analysis of the counter-selectants is shown in Table 4. Since these clones lacked APRT activity, those human chromosomes detected are unlikely sites for the *aprt* gene. Chromosome 16 was conspicuously absent from all of the counter-selectants. Also, chromosome T_1 was absent from all three of the counter-selected subclones of WA-VIa, while other human chromosomes were retained.

Among the primary hybrid clones, WA-IVa had, by far, the greatest tendency to lose human chromosomes (unpublished data). After 7 months of growth in drug-free medium, two subclones of WA-IVa were isolated in drug-free medium. Analysis of 36 subclone metaphases revealed no human chromosomes except for two metaphases which displayed chromosome 16. APRT was expressed at 12% of the level of the parent clone grown in AA medium. When the subclones were inoculated into AA medium, they grew after a lag due to considerable cell death. Analysis now revealed chromosome 16 as the only human chromosome in 82% of 27 metaphases, and both subclones now expressed human APRT at levels similar to that of the parental clone. When these subclones were counter-selected with DAP and the surviving colonies transferred to FA, they had no detectable APRT activity and human chromosomes were not observed in 64 metaphases.

DISCUSSION

The presence of chromosome 16 correlated most strongly with the expression of human APRT and it was the only human chromosome consistently lost with the enzyme, suggesting assignment of the *aprt* gene to chromosome 16. Failure to detect chromosome 16 in one hybrid clone can be explained by postulating a chromosome rearrangement. Chromosome T_1 could have carried the *aprt* gene in this clone, since its presence correlated with the expression of human APRT and it was lost with the enzyme. This unique chromosome might have arisen by rearrangement of chromosome 16 or translocation of the part bearing the *aprt* gene to a mouse chromosome. Translocation of the long arm of chromosome 17 carrying the thymidine kinase locus to a mouse chromosome has been reported. The hybrid possibly expressed human thymidine kinase but did not have a morphologically distinct chromosome 17 (22). Other workers described a hybrid in which the X chromosome was not detected although one of two X-linked enzymes was expressed (23). *In situ* annealing experiments with mouse satellite DNA (24) and chromosome T_1 might be informative.

Kusano, Long, and Green (5) suggested the rearrangement of a biarmed chromosome bearing the *aprt* gene in their clones or assignment to a human acrocentric chromosome. Our data argue against assignment to any human chromosome other than chromosome 16 because of a lack of positive association between APRT and other human enzymes whose chromosome assignments are known, the presence of particular human chromosomes in $APRT^-$ counter-selectants, and the absence of particular chromosomes in a significant number of primary clones. Thus, assignment to a human acrocentric chromosome is unlikely.

Dr. Howard Green kindly provided two of their clones, and our analysis confirmed the expression of human APRT. No other human enzymes were observed, and we were unable to detect any human chromosomes in these clones using the highly discriminatory banding techniques which were previously unavailable. We favor, therefore, the suggestion of a rearrangement in their clones.

Kusano, Long, and Green (5) reported the rapid loss of human chromosomes from their hybrids, whereas our hybrids were relatively stable. This result may reflect differences in the mouse parents since, for example, their mouse parent had an essentially all-acrocentric karyotype after long-term serial cultivation while A9 displays many biarmed chromo-

somes. We believe that in the highly reduced clones they chose to examine, there was very strong selection against human chromosomes. Since the medium selected for retention of the *aprt* gene, the probability of translocation of the part of the human chromosome bearing the *aprt* gene to a mouse chromosome was greatly increased. Alternatively, a rearrangement might have occurred that made it more compatible with the mouse genome, but disguised its identity.

Counter-selection of the hybrids and the presumptive mouse revertant reveals an interesting contrast. After 13 additional passages under nonselective conditions, the hybrids averaged a 53-fold increase in the frequency of colonies in DAP whereas the increase for the revertant was only about 2-fold. This result is consistent with the idea that $APRT^-$ cells arose due to the loss of a human chromosome from the hybrids while a different mechanism, possibly mutation, operated in the revertant. Our experience with mouse–human hybrids indicates that loss of human chromosomes is far more frequent than the mutation of particular genes (unpublished data).

Failure to initially counter-select the hybrids with FA and the observation that the eventual counter-selection in FA was density dependent, suggests that metabolic cooperation may be of greater significance when FA, rather than DAP, is the selective agent. Metabolic cooperation involves the transfer of a substance(s) from normal to enzyme-deficient cells, causing the latter to become phenotypically normal (19). Although it is known that the mouse parent does not engage in metabolic cooperation (19), Pitts has demonstrated that hybrids between A9 and cells that are able to metabolically cooperate, can cooperate (25). For hypoxanthine phosphoribosyltransferase-deficient cells, the extent of cooperation has been related to the density of normal cells (26), with higher densities resulting in impaired recovery of enzyme-deficient cells with 8-azaguanine (27, 28). The observation that only those two clones that displayed the highest frequency of counter-selectants in DAP could also be counter-selected with FA, may indicate that the frequency of $APRT^+$ cells in these clones was reduced to a point at which metabolic cooperation was less significant.

The alpha-haptoglobin locus has been assigned to chromosome 16 (29), and one report places it on the long arm (30). It would be interesting to find families variant for both alpha haptoglobin and APRT and thereby test their linkage. Inability to demonstrate linkage would not necessarily void the assignment of *aprt* to chromosome 16, but linkage would suggest its assignment to the long arm.

Subsequent to the preparation of this manuscript, Dr. Robert DeMars (personal communication) has informed us that work in his laboratory with mouse–human hybrids confirms the assignment of *aprt* to chromosome 16.

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