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Genetics

High-frequency mutation at the adenine phosphoribosyltransferase locus in Chinese hamster ovary cells due to deletion of the gene

(Southern blotting technique/diaminopurine resistance/somatic cell genetics/hereditary cancer)

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ABSTRACT **Evidence for a two-step model to explain the high-frequency expression of the recessive phenotype at the autosomal adenine phosphoribosyltransferase (APRT; EC 2.4.2.7) locus in Chinese hamster ovary (CHO) cells was given by Simon *et al.* [Simon, A. E., Taylor, M. W., Bradley, W. E. C. & Thompson, L. (1982) *Mol. Cell. Biol.* 2, 1126–1133]. This model proposed a high-frequency event, leading to allelic inactivation or a loss of gene function, and a low-frequency event, causing a structural alteration of the APRT protein. Either event could occur first, resulting in two classes of heterozygotes. We have analyzed the low-frequency event that gave rise to the class 2 *aprt* heterozygote D416 and the high-frequency event that led to APRT$^-$ cells derived from D416. Genomic Southern blots of *Msp* I- or *Hpa* II-digested DNA from wild-type CHO, *aprt* heterozygote D416, and two APRT$^-$ cell lines derived from D416 indicate a loss of a specific *Msp* I/*Hpa* II recognition sequence at one *aprt* locus in the heterozygote that correlates with the production of the electrophoretically altered APRT protein found in D416. The APRT$^-$ mutants are homozygous for the loss of this *Msp* I/*Hpa* II site. By using an additional CHO gene as an internal control, it was determined that the APRT$^-$ mutants contain only a single copy of the altered *aprt* gene. Thus, the high-frequency event that produces APRT$^-$ mutants derived from D416 is not an inactivation event but rather a deletion of the wild-type *aprt* gene.**

Two classes of heterozygotes have been isolated at the adenine phosphoribosyltransferase (*aprt*) locus in Chinese hamster ovary (CHO) cells after selection with low amounts of 2,6-diaminopurine (1, 2). Class 1 heterozygotes, the most abundant class, are selected as resistant to diaminopurine at 7 μg/ml. This class arises spontaneously and appears to result from an event that causes inactivation or loss of one of the two autosomal *aprt* loci. We have shown that such heterozygotes have 50% of the parental adenine phosphoribosyltransferase enzyme (APRT; EC 2.4.2.7) activity, and 50% of the immunoprecipitable APRT protein. This protein migrated to the wild-type position on two-dimensional polyacrylamide gels and was as thermally stable as wild-type protein. These class 1 heterozygotes give rise to fully diaminopurine-resistant (diaminopurine at 30 μg/ml) APRT$^-$ cells at a low rate (3×10^{-7}) (1). Class 2 heterozygotes, also selected as resistant to diaminopurine at 7 μg/ml, arise at a very low frequency, and have been isolated only after treatment of the parental cell line with the mutagen ethyl methanesulfonate. This class of *aprt* heterozygotes appears to result from a mutational event at one of the *aprt* alleles. One characteristic of class 2 heterozygotes is that they give rise at a high rate (10^{-3} to 10^{-6}) to fully DAP-resistant APRT$^-$ cells. Initial biochemical analysis of one class 2 heterozygote, D416, indicated that this cell line produces two types of APRT proteins, one wild-type and the other an electrophoretic variant. All six fully DAP-resistant APRT$^-$ mutants derived independently from D416 produce only the electrophoretic variant APRT protein (2). We proposed a model, consistent with the available data, in which we hypothesized that the high-frequency event that gave rise to the APRT$^-$ mutants derived from D416 was due to inactivation or loss of the wild-type allele.

In this paper, we report the identification of a specific alteration in the CHO *aprt* gene that correlates with the production of the electrophoretic variant APRT protein in D416. Furthermore, results from the present study indicate that the high-frequency event that led to isolation of APRT$^-$ cells from D416 was not an inactivation event but rather a deletion of the wild-type allele.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. D416 is an *aprt* heterozygote isolated in the presence of diaminopurine at 7 μg/ml after ethylmethane sulfonate treatment of the wild-type CHO (1). APRT$^-$ spontaneous mutants D416d^{r}c25 and D416d^{r}c26 were derived from D416 by selection in diaminopurine at 30 μg/ml (1). All cells were grown as monolayer cultures and transferred weekly. Cells were maintained in Hams nutrient mixture F12 (GIBCO) supplemented with 10% newborn calf serum (Biocell Laboratories, Carson, CA). APRT activity measurements and two-dimensional gel electrophoresis were performed as described (2).

Extraction and Endonuclease Digestion of DNA. Cells grown in 32-oz (950-ml) prescription bottles were lysed by addition of 5 ml of 0.15 M NaCl/0.1 M EDTA, pH 8.0/0.5% sodium dodecyl sulfate. The lysate was treated with proteinase K at 20 μg/ml for 30 min followed by Pronase at 60 μg/ml for 5 hr, all at 55°C. The temperature was lowered to 37°C and the lysate was treated with pancreatic ribonuclease at 50 μg/ml and phage T1 ribonuclease at 40 units/ml for 90 min. The lysate was reincubated with Pronase at 6 μg/ml at 55°C for 1 hr, then extracted with phenol and isoamyl alcohol/chloroform, 1:24 (vol/vol). Two volumes of cold ethanol was added, and the clumped DNA was removed and resuspended in 10 mM Tris·HCl, pH 8.0/0.5 mM EDTA. Endonuclease digestion was performed in universal buffer (33 mM Tris acetate/66 mM potassium acetate/10 mM magnesium acetate/100 μg of bovine serum albumin per ml/0.5 mM dithiothreitol, pH 7.9), at a ratio of 1 unit of restriction enzyme (Bethesda Research Laboratories) to 1 μg of DNA. Digestion was carried out for at least 4 hr at the supplier's recommended temperature.

Filter Hybridization. Twenty micrograms of endonuclease-digested DNA was electrophoresed through 0.8% or 1.5% agarose, denatured *in situ*, transferred to nitrocellulose (3) or diazo-

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Abbreviations: APRT, adenine phosphoribosyltransferase; kb, kilobase(s).

benzyloxymethyl-paper (4), and probed with ^{32}P-labeled plasmid DNA (4).

RESULTS

Migration of Mutant and Wild-Type APRT on Two-Dimensional Polyacrylamide Gels. As we have previously shown, APRT protein can be immunoprecipitated by antibody (raised in rabbits immunized with purified Syrian hamster liver APRT) and analyzed by two-dimensional polyacrylamide gel electrophoresis (2). Sections of gels showing the position of APRT immunoprecipitated from wild-type CHO, heterozygote D416, and one APRT$^-$ mutant derived from D416, D416d^{r}c25, are shown in Fig. 1. D416 produced two types of APRT protein, one that comigrated with wild type. All six APRT$^-$ cell lines derived at a high frequency from D416 had lost the wild-type protein and retained the mutant protein.

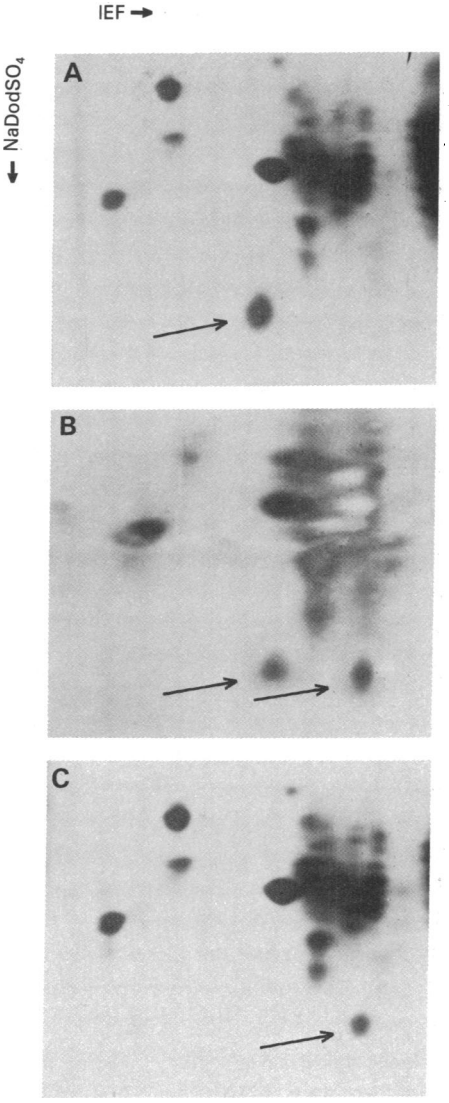

FIG. 1. Two-dimensional gel electrophoresis of immunoprecipitated APRT. APRT was immunoprecipitated from crude cell extracts and electrophoresed as described (2). The first-dimension isoelectric focusing (IEF) gel contained a gradient of pH 4.2–6.2. The second-dimension sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel contained 11% acrylamide. Protein in the gels was visualized with a silver stain (5). (*A*) CHO wild type, (*B*) D416, (*C*) D416d^{r}c25. Arrows denote positions of wild-type and mutant APRTs. The wild-type protein migrated with purified CHO APRT and was absent from gels with only antibody and all gels of immunoprecipitates from cell lines lacking APRT crossreacting material. All other protein staining spots and regions could be attributed to antibody.

Southern Blot Analysis of the *aprt* Gene from Mutant and Wild-Type Cell Lines. The CHO *aprt* gene has been cloned and is known to reside within a 4.3-kilobase (kb) *Hin*dIII/*Bgl* II fragment (6). A restriction map of this fragment is shown in Fig. 2. Fragments of the original cloned *aprt* gene were subcloned in pBR322 for use in the present study. pRG-1 contains the 1.8-kb *Pvu* II fragment and pAS-1 contains the 1.5-kb *Hin*dIII/*Pvu* II fragment. All restriction sites except *Msp* I sites were previously mapped (6). The *Msp* I sites were mapped by restriction endonuclease digestion of pAS-1 and pRG-1.

The *aprt* genes from CHO wild type, heterozygote D416, and APRT$^-$ cell line D416d^{r}c25 were analyzed by genomic Southern blots. DNA was extracted from these cell lines, digested with *Pvu* II, *Hin*dIII and *Eco*RI, or *Msp* I, electrophoresed through 0.8% or 1.5% agarose gels, blotted onto nitrocellulose or diazobenzyloxymethyl-paper, and probed with pRG-1 alone or pRG-1 and pAS-1 (Fig. 3). As expected, the Southern blot of DNAs digested with *Pvu* II and probed with pRG-1 showed a single hybridizing fragment at 1.8 kb for both wild-type and mutant cell lines (Fig. 3, lanes A–C). The blots of DNAs digested with *Eco*RI and *Hin*dIII showed two bands that hybridize to pRG-1, one at 2.6 kb and the other at 6.4 kb with no visible differences between wild-type and mutant cell lines (Fig. 3, lanes D–F). These results indicate that the mutations in D416 and D416d^{r}c25 are not due to massive rearrangements or large (>50-base-pair) insertions or deletions. However, when the same DNAs were digested with *Msp* I, differences in restriction patterns were discernible (Fig. 3, lanes G–I). Wild-type CHO DNA digested with *Msp* I and probed with pRG-1 and pAS-1 gave two major bands at 3.1 and 1.15 kb (Fig. 3, lane G). Two faint bands at 0.52 and 0.35 kb were also visible with longer exposures. D416 had a new band at 1.5 kb as well as the 1.15-kb band (Fig. 3, lane H), D416d^{r}c25 had lost the 1.15-kb band and retained the band at 1.5 kb (Fig. 3, lane I). Our data are consistent with the starred **Msp* I site in Fig. 2 being lost in one chromosome of D416, whereas D416d^{r}c25 is homozygous or hemizygous for the loss of this *Msp* I site.

Determination of the Number of *aprt* Genes in Wild-Type and Mutant Cell Lines. From the DNA and protein data, it is evident that the high-frequency event that leads to APRT$^-$ mutants derived from D416 causes a loss of the wild-type allele. Because the karyotype of the APRT$^-$ cells is identical to that of D416 (1), the event is not simply loss of the chromosome containing the wild-type *aprt* gene. The loss of the wild-type allele could occur by loss of the wild-type allele and duplication of the mutant allele. This could result from a second independent alteration at the *Msp* I site of the wild-type allele, or mitotic recombination followed by gene conversion, or physical loss of the wild-type chromosome and duplication of the mutant chromosome. These possible mechanisms would result in two copies of the mutant gene per genome. Alternatively, the loss of the wild-type gene could occur by simple deletion of the wild-type allele without otherwise greatly affecting karyotype. This would leave a single copy of the mutant gene per diploid genome. In order to differentiate between the possibilities, we used the cloned CHO *cad* cDNA (7) as an internal control in our Southern blots. It was possible to differentiate between genomes that contained one copy or two copies of the *aprt* gene by scanning with a densitometer the intensity of the *aprt*-specific bands relative to the *cad*-specific bands. It was assumed that all cell lines had the same number of *cad* genes. We digested mutant and wild-type DNAs with the restriction endonuclease *Hpa* II. This restriction enzyme recognizes the same sequence, C-C-G-G,

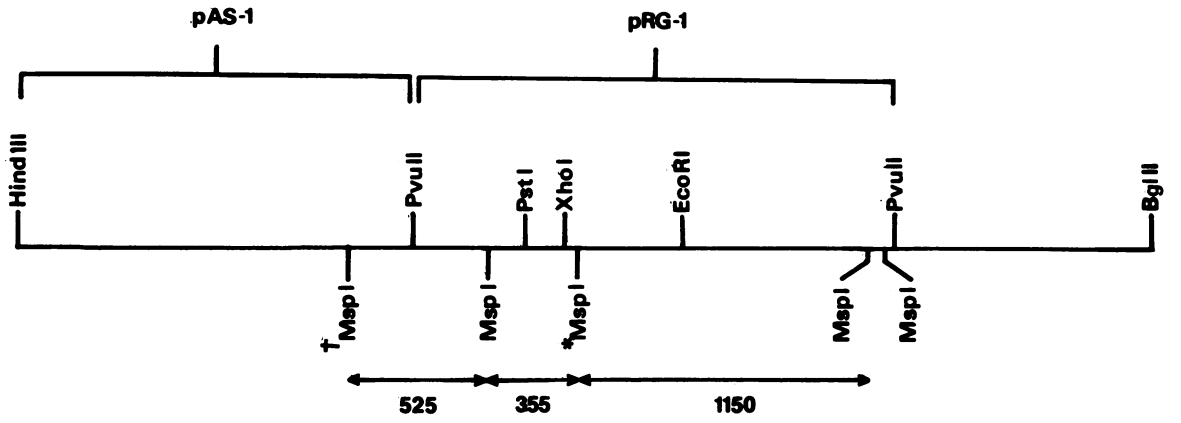

FIG. 2. Map of the CHO *aprt* gene. Restriction sites indicated above the line were mapped previously (6). The *Msp* I sites were mapped by single, double, and triple digestions of pRG-1 and pAS-1. Fragment sizes are indicated in base pairs. The *Msp* I site missing in the heterozygote D416 and the APRT⁻ cell lines derived from D416 (indicated by **Msp* I) was determined by Southern blots of DNA from D416 digested with *Msp* I, *Xho* I + *Msp* I, *Pst* I + *Msp* I, or *Eco*RI + *Msp* I (data not shown). All data were consistent with the **Msp* I site being the one that is lost. The plasmid pAS-1 contains a 1.5-kb *Hin*dIII/*Pvu* II fragment inserted in the *Hin*dIII and *Pvu* II sites in pBR322. pRG-1 contains a 1.8-kb *Pvu* II fragment inserted into the *Pvu* II site in pBR322. †*Msp* I indicates the only *Msp* I site known to be resistant to *Hpa* II digestion.

as *Msp* I, but it will not cleave the sequence if the cytosine preceding the guanosine is methylated (8). *Hpa* II-digested wild-type DNA probed with the *cad* cDNA gives hybridizing fragments of approximately 23 and 6 kb (see Fig. 4). The same *Hpa* II-digested DNA probed with pRG-1 gives hybridization bands at 3.7, 1.15, and 0.35 kb. D416 DNA digested with *Hpa* II and probed with pRG-1 gives the same bands as well as the 1.5-kb band. This indicates that only the *Msp* I site closest to the *Hin*dIII end of the *aprt* gene is methylated (see Fig. 2). In order to determine the number of copies of *aprt* in the mutant cell lines, DNA from wild-type CHO, D416, D416d^{r}c25, and D416d^{r}c26 was digested with *Hpa* II and probed with pRG-1 and the *cad* cDNA fragment (Fig. 4 *Left*). Densitometer scannings of the blot are shown in Fig. 4 *Right*. The results, summarized in Table 1, show that wild-type CHO has two *aprt* wild-type genes, D416 has one copy of both the wild-type and mutant *aprt* genes, and DAP-resistant cell lines D416d^{r}c25 and D416d^{r}c26 have only a single copy of the mutant *aprt* gene. The experiment was repeated and the normalized gene copy values of D416d^{r}c25 and D416d^{r}c26 were again found to be approximately 1 (1.0 and 0.8, respectively).

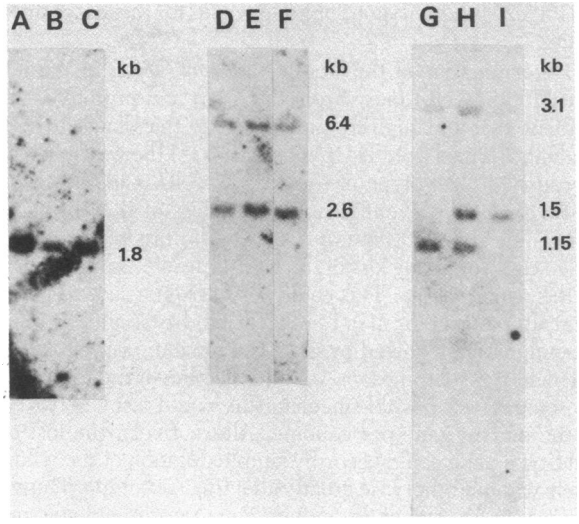

FIG. 3. Southern blots of the *aprt* gene in wild-type and mutant cell lines. Twenty micrograms of DNA from CHO wild type (lanes A, D, and G), heterozygote D416 (lanes B, E, and H), and the APRT⁻ cell line D416d^{r}c25 (lanes C, F, and I) was digested with restriction endonuclease *Pvu* II (lanes A–C), *Hin*dIII and *Eco*RI (lanes D–F), or *Msp* I (lanes G–I). The DNA was electrophoresed through 0.8% agarose (lanes A–F) or 1.5% agarose (lanes G–I), denatured *in situ*, transferred to nitrocellulose (lanes A–F) or diazobenzyloxymethyl-paper (lanes G–I), and hybridized to nick-translated ^{32}P-labeled probe consisting of pRG-1 (lanes A–F) or pRG-1 and pAS-1 (lanes G–I).

DISCUSSION

One of the most puzzling findings in somatic cell genetics has been the high-frequency occurrence of recessive mutation at autosomal loci (9). In a recent paper we presented a two-step model to explain this phenomenon at the *aprt* locus in CHO cells (2). This model suggested that "true" mutation did occur at one allele of the diploid *aprt* locus, and some type of high-frequency inactivation event occurred at the homologous allele. Either event could occur first, resulting in two classes of heterozygotes. The major class of heterozygotes (class 1) arise as a result of a high-frequency inactivation event and are thus functionally hemizygous. These cell lines give rise to fully DAP-resistant APRT⁻ cells only by a subsequent low-frequency mutational event. Class 2 heterozygotes are isolated only after mutagenesis and are true heterozygotes. These subsequently give rise to identical APRT⁻ mutant cell lines by high-frequency inactivation of the wild-type allele. Because no class 1 heterozygote gave rise at a high frequency to APRT⁻ cells, we hypothesized that the high-frequency event could occur only once at the diploid *aprt* locus.

In this paper we confirm our model at the level of the gene for the class 2 heterozygote D416. D416 exhibits an alteration of a C-C-G-G sequence (loss of a *Msp* I/*Hpa* II site) in the DNA, which correlates with the appearance of an aberrant but slightly active protein. It should be noted that this particular mutant protein (as seen in two-dimensional gels) may be the result of a mutational "hot spot," because it has been observed in several other independently isolated APRT⁻ mutant cell lines. Southern blots have revealed that they have each lost the same *Msp* I/*Hpa* II site as D416 (unpublished data). This alteration in the DNA could be due to a single base change, a small deletion, or an insertion so small as to be undetectable when other restriction enzymes were used. Another possibility is that both cytosine residues are now methylated at this *Msp* I/*Hpa* II site and therefore it is not cut by either enzyme (8). The location of this *Msp* I site is known to be within a small 126-base-pair exon (I. Lowy and R. Axel, personal communication). A change

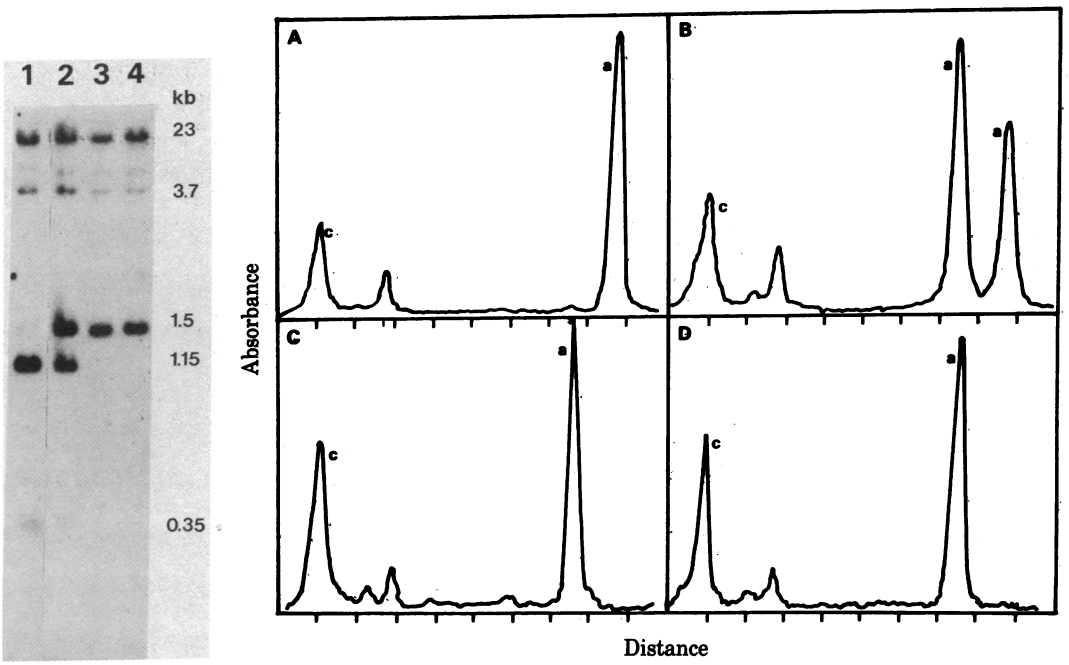

FIG. 4. Determination of the number of *aprt* copies in wild-type and mutant cell lines. (*Left*) Twenty micrograms of DNA isolated from CHO wild type (lane 1), D416 (lane 2), D416d^{r}c25 (lane 3), and D416d^{r}c26 (lane 4) was digested with *Hpa* II, electrophoresed through a 1.5% agarose gel, denatured *in situ*, transferred to nitrocellulose, and probed with the pRG-1 and the *cad* cDNA clone pCAD$_{142}$. *aprt*-specific bands are at 3.7, 1.5, 1.15, and 0.35 kb. *cad*-specific bands are at 23 and 6 kb. (*Right*) The autoradiogram in *Left* was scanned with a densitometer and the intensities of the *aprt*- and *cad*-specific bands were determined by cutting out and weighing the individual peaks. (*A*) CHO wild type, (*B*) D416, (*C*) D416d^{r}c25, (*D*) D416d^{r}c26. c, *cad*-specific peak; a, *aprt*-specific peak.

in the site could therefore alter the amino acid sequence of the protein and cause the electrophoretic shift seen on the two-dimensional gels.

The second event, which occurs at high frequency (10^{-3}) in D416 and leads to cells fully resistant to DAP, is the loss of the wild-type band at the 1.15-kb position in blots of *Msp* I-digested DNA. Our results indicate that the APRT$^-$ mutants D416d^{r}c25 and D416d^{r}c26 have only a single copy of the mutant *aprt* gene. Thus it would appear that the high-frequency event that gave rise to D416d^{r}c25 and D416d^{r}c26 is not an inactivation event but rather a deletion of the *aprt* gene. The size of the deletion is unknown, although it must be at least 3.2 kb, the size of the *aprt* probes used in this study. Chromosome banding of D416 and four APRT$^-$ mutants revealed no detectable differences (1). However, a small change may have been missed because the location of the gene in CHO cells is still unknown.

Table 1. Number of *aprt* gene copies in wild-type and mutant cell lines

Cell line	*aprt*/*cad* ratio: 1.1-kb *aprt*	*aprt*/*cad* ratio: 1.5-kb *aprt*
CHO wild-type	2.2	—
D416	1.0	1.0
D416d^{r}c25	—	0.98
D416d^{r}c26	—	0.92

aprt and *cad* peaks from densitometer scans of the autoradiogram in Fig. 4 *Right* were cut out and weighed and the ratios were determined. The *aprt*/*cad* ratios for D416 were normalized to 1. Because the intensity of the 1.5-kb *aprt* band was greater than that of the 1.15-kb band in D416 (Fig. 4 *Left*, lane 2), the ratio of the *cad* band to the wild-type CHO 1.15-kb band (Fig. 4 *Left*, lane 1) was compared to the ratio of the *cad* band to the lower *aprt* band (1.15 kb) in D416. Similarly, the ratio of the *cad* band to the 1.5-kb band in the two APRT$^-$ cell lines (Fig. 4 *Left*, lanes 3 and 4) was compared with the ratio of the *cad* band to the 1.5-kb band in D416.

Another example of a high-frequency mutational event that appears to involve gene deletion is found in toyocamycin resistance, which results in a defect in adenosine kinase (10, 11). Eves and Farber (12) noted that, in 50% of the adenosine kinase mutants found in the mouse cell line CAK, only one allele of the linked *ES-10* allele was expressed. They concluded from this observation that the occurrence of autosomal recessive mutants was associated with loss of part of chromosome 14. However, CAK cells appear to undergo a large amount of chromosomal rearrangement in culture, unlike CHO cells, which are very stable (13). Robbins *et al.* (14) have found that in a BRL liver cell (BRLtk$^-$) line cells cotransformed with the viral *tk* gene and human growth hormone gene become thymidine kinase negative by a mechanism involving deletion of segments of the *tk* gene, but without any loss of chromosome number. Although this is an artificial situation resulting from cell transformation, it does appear that deletion may be a common mechanism in the expression of recessive phenotypes in cultured cells.

Our findings could also explain some phenomena related to the expression of hereditary cancer in humans. It has been postulated that predisposed heterozygous persons develop cancer when a somatic event renders a target cell homozygous (15). In retinoblastoma the germinal event that created the heterozygous state is usually an invisible mutation, but it is sometimes a deletion. However, the mean number of tumors in this latter group is lower than it is in the first group (16), suggesting that deletion may often constitute one of the events, but not both.

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