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High-frequency structural gene deletion as the basis for functional hemizygosity of the adenine phosphoribosyltransferase locus in Chinese hamster ovary cells

(somatic cell genetics/mutagenesis/gene mapping/azaadenine resistance/Southern blotting technique)

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ABSTRACT **The CHO-AT3-2 Chinese hamster ovary cell line is functionally hemizygous for the adenine phosphoribosyltransferase (APRT; EC 2.4.2.7) locus. Class 1 *APRT* +/− heterozygotes, such as CHO-AT3-2, can be isolated at high spontaneous frequencies from wild-type CHO cell populations. Simon *et al.* [Simon, A. E., Taylor, M. W., Bradley, W. E. C. & Thompson, L. (1982) *Mol. Cell. Biol.* 2, 1126–1133] have proposed that a high-frequency event that inactivates one *APRT* allele might be responsible for both the spontaneous generation of class 1 *APRT* +/− heterozygotes and the high-frequency occurrence of APRT⁻ mutants in class 2 *APRT* +/− heterozygote populations. This event appears to occur at only one of the two *APRT* alleles. To investigate the nature of this high-frequency event, and to determine the genetic basis for functional hemizygosity of the *APRT* locus in CHO-AT3-2 cells, we have mapped the *APRT* locus by using CHO-AT3-2–mouse somatic cell hybrids. Our data confirm that CHO-AT3-2 cells have a single functional *APRT* allele, which is located on the Z7 chromosome. Karyotypic analysis of CHO-AT3-2 revealed an interstitial deletion on the long arm of the Z4 chromosome, in the very region where the other *APRT* allele should be located. To determine whether the Z4q interstitial deletion had resulted in physical loss of the *APRT* gene, DNA from CHO-AT3-2–mouse cell hybrids that had either lost or retained the Z4q⁻ chromosome was analyzed for the presence of CHO *APRT* coding sequences. Our data suggest that allele-specific high-frequency structural gene deletion events involving the long arm of chromosome Z4 are responsible for the spontaneous generation of functional hemizygosity at the *APRT* locus in CHO cells.**

Adenine phosphoribosyltransferase (*APRT*) is a diploid autosomal locus in Chinese hamster ovary (CHO) cells (1). *APRT* +/− heterozygotes can be selected on the basis of their resistance to intermediate levels of adenine analogs such as 8-azaadenine (AzA) or 2,6-diaminopurine (1–7). Two classes of CHO cell *APRT* +/− heterozygotes have been described (2, 3, 8). Class 1 heterozygotes, such as CHO-AT3-2 (1), can be readily isolated at high frequencies from unmutagenized cell populations, have 50% wild-type (WT) adenine phosphoribosyltransferase activity (APRT; EC 2.4.2.7), and give rise to fully AzA- or diaminopurine-resistant, APRT⁻ mutants at a low spontaneous rate ($\approx 3 \times 10^{-7}$ per cell per generation), which is markedly enhanced by treatment with known mutagens (1–7). Class 2 heterozygotes, which have been obtained only from mutagenized cell populations, give rise to fully AzA- or diaminopurine-resistant, APRT⁻ mutant phenotypes at spontaneous rates of 10^{-6} to 10^{-3} per cell per generation; mutagenesis does not significantly increase the frequency of APRT⁻ mutants in class 2 heterozygote cell populations (2, 3). On the basis of these data, Simon *et al.* (2) proposed a two-step model for the generation and expression of recessive mutations at the *APRT* locus in CHO cells. In this model, a high-frequency event results in inactivation of one *APRT* allele, and a low-frequency, classical mutational event at the other allele results in a structurally altered gene product. Either event could occur first, with each resulting in a different class of *APRT* heterozygote.

Simon and Taylor (8) recently presented evidence that the high-frequency event responsible for the generation of APRT⁻ mutant phenotypes in class 2 heterozygote populations is not an inactivation event, but rather involves deletion of the WT *APRT* allele. In this paper, we present evidence that: (*i*) the class 1 heterozygote CHO-AT3-2 has a single, functional *APRT* allele, which is located on the Z7 chromosome, and (*ii*) functional hemizygosity of the *APRT* locus in this cell line reflects the physical loss of the other *APRT* allele by deletion of material from the midregion of the Z4 chromosome.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The derivation and characterization of CHO-AT3-2, the *APRT* heterozygote used in this study, have been described (1, 6). LTAO, an APRT⁻, ouabain (OUA)-resistant subline of LMTK⁻ Cl.1D (9) was used as the mouse parental cell line for the generation of hybrids. Cells were maintained in monolayer culture in α modified minimal essential medium (KC Biological, Lenexa, KS), supplemented with antibiotics (penicillin/streptomycin) and 10% fetal bovine serum.

Selection of Hybrid Clones. Interspecific somatic cell hybrids were generated by polyethylene glycol-mediated cell fusion (9, 10) of CHO-AT3-2 with LTAO. Hybrids were selected in three different selection media: HAT/OUA [100 μM hypoxanthine/2 μM methotrexate (amethopterin)/50 μM thymidine/1 mM OUA], ALA/OUA (50 μM alanosine/100 μM adenine/1 mM OUA), or ASA/OUA (50 μM azaserine/100 μM adenine/1 mM OUA). ALA and ASA selection media both select for APRT⁺ hybrids (11, 12); only those hybrids that retain the CHO-AT3-2 chromosome carrying the functional *APRT* allele will survive in these media.

Selection of APRT⁻ Hybrid Segregants. Each hybrid was grown in nonselective medium for approximately 14 days to allow spontaneous chromosome segregation. APRT hybrid segregants were selected in AzA at 60 μg/ml (9); this selection medium required the use of dialyzed fetal bovine serum (1, 6).

Isozyme Analysis. Isozyme analyses were performed on cell extracts from parental cells, hybrid clones, and AzA-resistant (AzA^r) hybrid segregants. Each extract was screened for the

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Abbreviations: AzA, 8-azaadenine; AzA^r, AzA-resistant; APRT, adenine phosphoribosyltransferase; kb, kilobase; OUA, ouabain; WT, wild-type.

presence of 33 different Chinese hamster and mouse isozyme gene products by vertical starch gel electrophoresis and histochemical staining or autoradiography (9, 10).

Chromosome Analysis. Giemsa-banding analyses were performed on conventional air-dried chromosome preparations (9, 10). At least 10 good-quality banded metaphase spreads were examined per hybrid subclone. Normal (nonrearranged), and rearranged Z-group CHO chromosomes were classified by the standard nomenclature of Ray and Mohandas (13), and Deaven and Peterson (14), respectively.

Southern Blot Filter Hybridization. Hybrid cell DNA was prepared as described by Nairn *et al.* (15). Twenty-microgram samples were digested with restriction endonucleases for 8 hr at 37°C in the buffers recommended by the suppliers (Bethesda Research Laboratories and Boehringer Mannheim) at 3 units of restriction enzyme per μg of DNA. Endonuclease-digested DNAs were electrophoresed for 560 V·hr in 0.8% agarose gels, denatured *in situ* (16), and transferred to nitrocellulose (17). Blots were probed with electroelution-purified 1.5-kilobase (kb) pAS-1 *Hin*dIII/*Pvu* II and 1.8-kb pRG-1 *Pvu* II restriction fragments that had been labeled with [^{32}P]dCTP by nick translation (18). These restriction fragments contain the CHO *APRT* coding sequences (8, 19). After hybridization, blots were washed, air-dried, and exposed to Kodak X-Omat AR film at −70°C for several days, using an intensifying screen (15).

RESULTS

Chromosomal Localization of the APRT Genes in Chinese Hamster and in CHO Cells. We have recently mapped the *APRT* locus (Fig. 1) to the short arm of Chinese hamster chromosome 3, in the region 3p2→pter (9). In the CHO cell line, both chromosome 3 homologs have undergone rearrangement; on the basis of Giemsa banding, the Z3 and Z7 marker chromosomes in CHO appear to be the products of a reciprocal translocation between chromosomes 3 and 4, while the Z4 marker chromosome appears to have arisen by pericentric inversion after a break in the proximal region of the short arm of the other chromosome 3 homolog (14, 20). Thus, on the basis of our regional mapping assignment for *APRT* in Chinese hamster and the chromosomal rearrangements that have taken place during the evolution of the CHO cell line, the CHO *APRT* genes should map to the distal portion of the short arm of the Z7 chromosome and to the midregion of the long arm of the Z4 chromosome, perhaps quite close to the site of inversion–addition of 3q material (Fig. 1). Preliminary segregation results obtained for CHO (*APRT* +/+)–LMTK$^-$ Cl.1D cell hybrids are consistent with this prediction. Chinese hamster APRT was found to be present in each of over 50 such interspecific hybrid clones that retained either a Z4 or a Z7 chromosome, but nine clones that had segregated APRT had segregated both of these chromosomes (unpublished results).

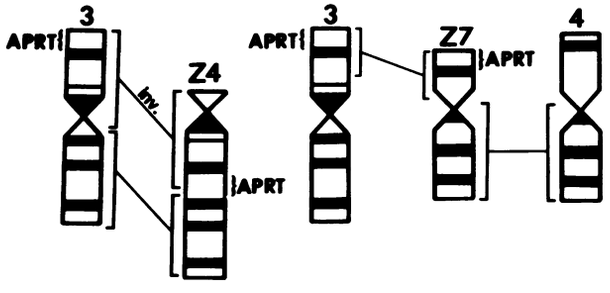

FIG. 1. Ideograms of Chinese hamster chromosome 3 and the CHO Z4 and Z7 chromosomes, showing the provisional regional mapping location of the *APRT* locus on chromosome 3 (9) and the chromosomal rearrangements that have generated the Z4 and Z7 marker chromosomes. inv., Inversion. The ideograms themselves are taken from Deaven and Peterson (14).

Possible Cytogenetic Basis for Functional Heterozygosity of the APRT Locus in the CHO-AT3-2 Cell Line. Cytological analysis of the CHO-AT3-2 cell line revealed a modal karyotype similar to that described by Deaven and Peterson (14), with several minor differences. These differences include additions of material to Xq, 5p, and 6p, and interstitial deletions of material from the long arms of chromosomes 7 and Z4. The Z4q interstitial deletion (Fig. 2) is of considerable interest, because it involves the region of the Z4 chromosome where one of the *APRT* genes is thought to be located. Functional heterozygosity of the *APRT* locus in CHO-AT3-2 might be the result of physical loss or inactivation of the Z4 *APRT* gene as a consequence of the Z4q deletion.

Mapping of the Functional *APRT* Allele in the CHO-AT3-2 Cell Line. In order to map the functional *APRT* locus in CHO-AT3-2, we generated a series of interspecific somatic cell hybrids between CHO-AT3-2 and LTAO, an APRT-deficient subline of mouse LMTK$^-$ Cl.1D. Twenty-two independent hybrid clones were isolated; isozyme analysis confirmed that each hybrid expressed the Chinese hamster form of APRT. AzA^r segregants were obtained from these hybrid populations at frequencies between 4×10^{-4} and 2.4×10^{-2}. Twenty-eight AzA^r clones were isolated; 8 from HAT/OUA-selected hybrids, and 1 from each of the 20 independent ALA/OUA- or ASA/OUA-selected hybrids. APRT activity was confirmed to be absent from each of the 28 AzA^r hybrid segregants analyzed. Cytogenetic examination of 21 of these AzA^r APRT$^-$ hybrid segregants revealed that each was missing the CHO-AT3-2 Z7 chromosome (Table 1). Segregation of Chinese hamster *APRT* from these hybrids was not correlated with the loss of any other CHO-AT3-2 chromosome; only one AzA^r clone segregated the Z4 chromosome (Table 1). Each of the 12 independent APRT$^+$ hybrid clones that were analyzed retained the Z7 chromosome (Table 1). The concordant expression, or segregation, of Chinese hamster APRT with retention, or loss, of the Z7 chromosome strongly suggests that the functional *APRT* allele in CHO-AT3-2 is located on that chromosome. The further implication is that it is the Z4 *APRT* locus that is deleted or inactive in CHO-AT3-2.

Southern Blot Analysis of Interspecific Somatic Cell Hybrids for the Presence of Chinese Hamster *APRT* Gene Sequences. The CHO *APRT* gene has been cloned in the bacterial plasmid pHaprt (19) and is known to reside within a 4.3-kb *Hin*dIII/*Bgl* II restriction fragment (Fig. 3). Two fragments of the cloned *APRT* gene have been subcloned in pBR322; pAS1 contains the 1.5-kb *Hin*dIII/*Pvu* II fragment, and pRG1 contains the 1.8-kb *Pvu* II fragment encompassing most of the coding sequence (8). To determine whether the Z4q interstitial deletion in CHO-AT3-2 had resulted in physical loss of the Z4 *APRT* gene, DNA from CHO-AT3-2–LTAO cell hybrids that had segregated the Z7, Z4q$^-$, or both chromosomes was analyzed for the presence or absence of CHO *APRT* genomic sequences. As shown in Fig. 4, blot hybridization of *Hin*dIII/*Pvu*

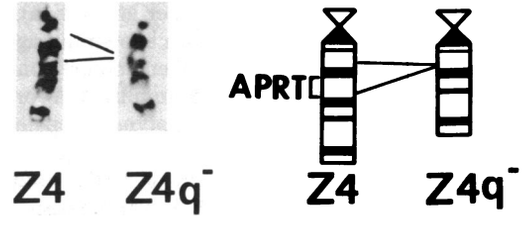

FIG. 2. Giemsa-banded normal CHO Z4 and CHO-AT3-2 Z4q$^-$ chromosomes (*Left*) and ideograms (*Right*) showing the Z4q region that has been interstitially deleted in this class 1 *APRT* +/− heterozygote.

Table 1. Segregation of Chinese hamster APRT and chromosomes in CHO-AT3-2–LTAO hybrids

Hybrid clone	Hamster APRT	CHO-AT3-2 chromosomes segregated	Hybrid clone	Hamster APRT	CHO-AT3-2 chromosomes segregated
R9/L1	+	X, 2, 5, Z1, Z2, Z4	R9/A1	−	X, 1, 2, 5, 7, Z1, Z2, Z4, Z6, **Z7**, Z8
R10/L2	+	X, 7, Z1, Z5, Z8, Z10	R10/A2	−	X, 5, 7, Z1, Z2, Z5, **Z7**, Z8, Z9, Z13
R12/L4	+	X, 1, 5, Z3, Z10	R12/A4	−	X, 1, 5, 7, 10, Z1, Z2, Z3, Z6, **Z7**, Z8, Z10
R13/L5	+	X, 7, Z1, Z2, Z4, Z8	R13/A5	−	X, 1, 5, 7, Z2, Z3, Z6, **Z7**, Z8
R14/L6	+	X, 5, 7, Z8, Z10, Z13	R14/A6	−	X, 5, 7, Z1, Z2, Z3, **Z7**, Z8
R15/L7	+	X, 5, 7, Z1, Z2, Z9	R15/A7	−	X, 5, **Z7**
R19/S1	+	X, 5, Z3, Z6	R19/A1	−	X, 7, Z1, Z2, Z3, Z6, **Z7**, Z9
R20/S2	+	X, 5, Z1, Z2, Z13	R20/A2	−	X, 1, 2, 7, Z1, Z2, **Z7**, Z8, Z9, Z13
R22/S4	+	X, 1, 5, Z3, Z10	R22/A4	−	X, 1, 5, 7, Z3, **Z7**
R23/S5	+	X, 2, 5, 7, Z1, Z2, Z3	R23/A5	−	5, 7, Z1, Z2, Z3, **Z7**
R24/S6	+	X, 5, 7, Z1, Z3, Z5, Z8	R24/A6	−	1, 7, Z1, Z2, Z3, **Z7**, Z8, Z9
R25/S7	+	X, 5, Z1, Z5	R25/A7	−	X, 5, 7, Z1, Z2, Z5, Z6, **Z7**, Z8, Z10, Z13
R26/S8	+	X, 5, 7, Z1, Z2, Z3, Z10	R26/A8	−	X, 1, 5, 7, Z1, Z2, Z3, **Z7**, Z10
R1/H1	+	X, 5, 7, Z1, Z9, Z10	R1/A1-1	−	X, 5, 7, 10, Z1, Z2, Z3, Z6, **Z7**, Z8
			R1/A1-2	−	X, 1, 2, 5, 7, Z2, Z3, **Z7**, Z8
			R1/A1-3	−	5, 7, Z1, Z3, **Z7**, Z9, Z10, Z13
			R1/A1-4	−	X, 5, 7, Z1, **Z7**, Z9, Z10, Z13
R2/H2	+	5, 7, Z1, Z10	R2/A2-1	−	X, 1, 5, 7, Z2, **Z7**, Z8
			R2/A2-2	−	X, 1, 5, 7, 10, Z2, **Z7**, Z8, Z13
			R2/A2-3	−	X, **Z7**
			R2/A2-4	−	X, 5, 7, Z1, **Z7**, Z10

II digested hybrid DNAs with the 1.5-kb pAS1 probe revealed a single 1.5-kb hybridizing fragment in the DNA of each hybrid that retained a Z7 chromosome, whether or not the $Z4q^-$ was retained; a single, Chinese hamster-specific 1.8-kb hybridizing fragment was also observed in the DNA of these hybrids after blot hybridization with the 1.8-kb pRG1 probe. However, hybrids that had segregated the Z7 chromosome but retained the $Z4q^-$ chromosome did not contain Chinese hamster-specific DNA sequences that hybridized with either probe (Fig. 4). These results indicate that CHO-AT3-2 cells contain a single copy of the *APRT* gene, which is located on the Z7 chromosome. The $Z4q^-$ chromosome in these cells does not contain any *APRT* gene sequences. Thus, the Z4 *APRT* locus appears to have been physically lost as a result of the Z4q interstitial deletion and not simply inactivated.

DISCUSSION

The high-frequency occurrence of recessive mutations at autosomal loci in cultured mammalian cells has been attributed to (*i*) the existence of extensive functional hemizygosity, generated during the karyotypic evolution of cell lines such as CHO (21, 22); (*ii*) gene inactivation (23–25); (*iii*) chromosome segregation or loss/reduplication (26); or (*iv*) mutational hot spots (27–29); each of these mechanisms probably contributes. A growing body of evidence suggests that the expression of certain phenotypes in mammalian somatic cells is associated with chromosomal rearrangements (30–33) or deletion events (32–33). Such events may occur at genetically significant frequencies in cultured cells, even in karyotypically stable lines such as CHO.

Several laboratories have reported evidence for the occurrence of structural gene mutations at the *APRT* locus in CHO cells. Mutants with specific restriction-site alterations in *APRT* gene structure (8, 34) or that produce a structurally altered gene product (2) have been isolated. According to the model proposed by Simon *et al.* (2), the occurrence of a "high-frequency inactivation event" results in loss of expression of one WT *APRT* allele, allowing expression of any recessive structural gene mutations that occur at the other allele. After ethyl methanesulfonate treatment of a WT (*APRT* +/+) CHO cell population, Meuth and Arrand (34) obtained several $APRT^-$ mutants that were heterozygous for specific *APRT* gene restriction alterations. However, one mutant appeared to be homozygous for

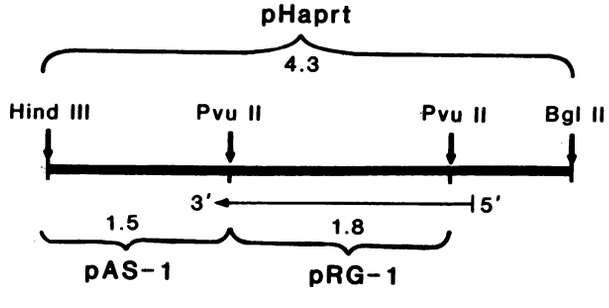

FIG. 3. Restriction map of the CHO *APRT* gene contained in the 4.3-kb *Hind*III/*Bgl* II fragment in the plasmid pHaprt-1, showing the 1.5-kb *Hind*III/*Pvu* II fragment contained in the plasmid pAS-1 and the 1.8-kb fragment contained in the plasmid pRG-1. The restriction sites shown were mapped by Lowy *et al.* (19). The plasmids pAS-1 and pRG-1 were characterized by Simon and Taylor (8).

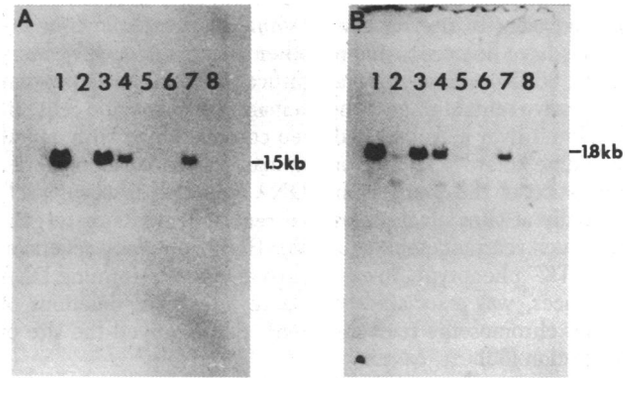

FIG. 4. Southern blot analysis of CHO-AT3-2–LTAO hybrids for the presence of Chinese hamster *APRT* coding sequences. Twenty micrograms of *Hind*III/*Pvu* II-digested DNA from WT *APRT* +/+ CHO (lane 1), *APRT* +/− CHO-AT3-2 (lane 2), mouse LTAO (lane 3), the −Z4/+Z7 hybrid R9/L1 (lane 4), the −Z4/−Z7 hybrid R9/A1 (lane 5), the +Z4/−Z7 hybrid R15/A7 (lane 6), the +Z4/+Z7 hybrid R19/S1 (lane 7), or the +Z4/−Z7 hybrid R22/A4 (lane 8) was electrophoresed through 0.8% agarose, denatured *in situ*, transferred to nitrocellulose, and hybridized to nick-translated ^{32}P-labeled probes derived from pAS-1 or pRG-1 (Fig. 3) restriction fragments.

a restriction alteration involving loss of a *Taq* I/*Xho* I site; this mutant may have resulted from point mutation of the WT *APRT* allele in a spontaneous class 1 *APRT* +/− heterozygote in the WT cell population. In a recent study, using a cloned probe for the CHO *APRT* genomic sequence, Simon and Taylor (8) were able to show that the high-frequency event responsible for the generation of spontaneous APRT$^-$ mutant phenotypes in a class 2 heterozygote is not an inactivation event but rather represents deletion of the WT *APRT* allele. Although the size of the deletion is unknown, it must be at least 3.2 kb, the size of the *APRT* probes used in their study. They did not notice any cytologically detectable chromosomal alterations accompanying deletion of the *APRT* gene.

Our results suggest that similar high-frequency deletion events are responsible for the spontaneous generation of class 1 *APRT* +/− heterozygotes and APRT$^-$ cells in class 2 heterozygote populations. Because none of the class 1 heterozygotes isolated in five different laboratories has been observed to give rise to APRT$^-$ mutants at high spontaneous frequencies like the class 2 heterozygotes (1–8), it appears that these high-frequency deletion events occur at only one of the two *APRT* alleles in CHO. This raises the intriguing possibility that physical differences in the genomic environment of the two alleles, such as close proximity of one allele to a rearranged region, might render that gene highly susceptible to deletion. In this paper, we have shown that the class 1 *APRT* +/− heterozygote CHO-AT3-2 has a single functional *APRT* gene, which is located on the Z7 chromosome. We have also shown that functional hemizygosity of the *APRT* locus in this cell line reflects the physical loss of the other *APRT* allele as a result of an interstitial deletion occurring in the midregion of the long arm of the Z4 chromosome. On the basis of our mapping studies (ref. 9, this paper, and unpublished results), the Z4 *APRT* gene in CHO appears to be located very near the site of inversion–addition of 3q material on the Z4 chromosome, while the Z7 *APRT* allele, which does not appear to be subject to high-frequency deletion, is located on the distal portion of the short arm of the Z7 chromosome, far from any translocation breakpoints. Thus, there may be a chromosomal basis for the observed specificity of high-frequency deletion events for one *APRT* allele in CHO cells. However, this needs to be confirmed by analysis of additional class 1 heterozygotes to determine whether it is always the Z4 *APRT* allele that is deleted.

Chromosomal instability and high-frequency deletion of regions adjacent to sites of chromosomal rearrangements or insertions have been reported for other mammalian cell systems (32, 33, 35, 36) and may play a significant role in the expression of recessive mutant phenotypes in mammalian somatic cells. In Buffalo rat liver cells that had been cotransformed with a viral thymidine kinase (TK) gene and human growth hormone gene, integration of the transforming DNA appeared to occur preferentially at sites of chromosomal rearrangements or to generate such rearrangements (33, 35). High-frequency reversion to the TK$^-$ phenotype, involving loss of the transforming DNA sequences, was associated with large interstitial deletions or further chromosome rearrangements that involved the site of integration (33).

Approximately one-half of the chromosomes in the CHO karyotype reflect translocations and other rearrangements that have occurred during the evolution of the cell line (14, 20). Although the mechanism by which one such rearrangement renders a specific region on the Z4 chromosome (including the Z4 *APRT* allele) highly susceptible to deletion is not known, similar high-frequency deletion or rearrangement events in maize (37), yeast (38), and *Drosophila* (39) have been shown to be associated with the insertion or mobilization of transposable elements.

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