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Genetics

Chromosome segregation is frequently associated with the expression of recessive mutations in mouse cells

(adenosine kinase deficiency/toyocamycin/esterase-10 electrophoresis/chromosome analysis)

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ABSTRACT **The genes coding for adenosine kinase (ADK; ATP:adenosine 5'-phosphotransferase, EC 2.7.1.20) and esterase-10 (ES-10; carboxylesterase, carboxylic-ester hydrolase, EC 3.1.1.1) are both located on chromosome 14 in the mouse. The near-diploid mouse cell line CAK is heterozygous for two electrophoretic variants of ES-10. Recessive *Adk*$^-$ mutants of CAK have been isolated and analyzed for Es-10 phenotype and karyotypic abnormalities. Two classes of mutants were found with approximately equal frequencies: those that remained heterozygous in the expression of Es-10 and those that expressed only one Es-10 allele. Of the mutants that lacked one form of ES-10, approximately half were missing most or all of one copy of chromosome 14; the others contained two copies of 14, frequently in the form of an isochromosome. There were no abnormalities of this chromosome found among the mutants that were Es-10 heterozygotes. These results suggest that the expression of an autosomal recessive mutation in near-diploid mouse cells is frequently associated with events that result in the segregation of a physically linked marker and part or all of a chromosome.**

The observed frequencies of recessive mutation in cultured mammalian cells are generally much higher than would be predicted by comparisons with the frequencies of dominant mutations in the same cell lines or with recessive mutations in haploid organisms (1–3). Also, the differences in recessive mutation frequencies for a specific locus are not as great as expected when cell lines of different ploidies are compared (4–6). As the result of observations of this type, questions have been raised regarding the nature of heritable changes in cultured somatic cells.

It is possible that some variants expressing recessive mutations arise by mutation in one of the two copies of a locus, followed by a second event that results in the loss or inactivation of the remaining normal copy of the locus. Among the types of secondary events that could occur are (*i*) loss of the homologue carrying the wild-type allele, (*ii*) loss of the wild-type homologue and duplication of the mutant homologue, (*iii*) deletion or inactivation of a region including the wild-type locus, and (*iv*) mitotic recombination. Preexisting hemizygosity would also permit the expression of a single, recessive mutation. There is evidence that such hemizygosity occurs at some loci in Chinese hamster ovary (CHO) cells (7, 8).

We have used a near-diploid mouse cell line to assess the relative contributions of some of these possible mechanisms to high frequencies of recessive mutants. We have isolated a number of mutants of mouse CAK cells deficient in adenosine kinase (ADK) and examined their esterase-10 (ES-10) phenotypes. The CAK line is heterozygous for Es-10, and the genes for both the esterase and ADK are located on chromosome 14 (9). Our results suggest that the frequencies of expression of a recessive mutation in near-diploid cultured cells can be enhanced by secondary genetic events that involve whole chromosomes or chromosomal regions encompassing more than a single locus.

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MATERIALS AND METHODS

Cell Lines and Culture Conditions. The mouse cell lines used were derivatives of the CAK-A32-G11 embryonic fibroblast line established by Farber and Liskay (10). CAK-A32-G11-D9-B3 is the result of two clonings of CAK-A32-G11 and will be referred to simply as CAK-B3.

The CHO cells used for hybrid construction (CHO-TG12-OB1) were deficient in hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) and resistant to 3.0 mM ouabain.

Cells were grown in Eagle's minimal essential medium supplemented with penicillin (50 units/ml) and either 10% fetal calf serum or 5% fetal calf serum plus 5% horse serum.

Mutant Isolation. CAK-B3 cells were treated with ethyl methanesulfonate (500 μg/ml) for 16 hr. Approximately 50% of the cells survived this treatment. After removal of the ethyl methanesulfonate, the cells were maintained and subcultured in nonselective medium for 10 days to allow for mutation expression. At the end of this period, cells were plated into 100-mm Petri dishes (2×10^5 cells per dish) in medium containing toyocamycin (25 ng/ml), an adenosine analogue that becomes cytotoxic when phosphorylated by ADK (2); wild-type CAK cells are killed by toyocamycin at 5 ng/ml. Drug-resistant colonies were isolated after 2–3 weeks. The toyocamycin was generously provided by J. Dauros (National Cancer Institute) and by Gist-Brocades (Delft, The Netherlands).

Cell Hybridization. Hybrids between CHO-TG12-OB1 and CAK-B3 cells were produced by treatment with polyethylene glycol according to the methods of Davidson and Gerald (11) and Davidson *et al.* (12). The selective medium contained 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16.0 μM thymidine (12) and 3.0 mM ouabain (14). Hybrids between CHO-TG12-OB1 and *Adk*$^-$ mutants of CAK-B3 were tested for growth in toyocamycin at 25 ng/ml and in medium containing 50.0 μM alanosine, 0.1 mM adenosine, and 0.5 mM uridine. This medium was developed to select for *Adk*$^+$ cells (2, 15). The alanosine was donated by L. Coronelli (Gruppo Lepetit, Milan, Italy) and by L. Kedda (National Cancer Institute).

Enzyme Assays and Electrophoresis. Cell extracts were prepared at a concentration of 5×10^7 cells per ml by the method

Abbreviations: ADK, adenosine kinase; ES-10, esterase-10; CHO, Chinese hamster ovary; NP-1, nucleoside phosphorylase; Toy^r, toyocamycin resistant; iso, isochromosome; HPRT, hypoxanthine phosphoribosyltransferase; emt, emetine; G6PD, glucose-6-phosphate dehydrogenase.

of Nichols and Ruddle (16), and hemolysates were prepared by the method of Peters and Nash (17). Extracts were analyzed for ES-10 and nucleoside phosphorylase (NP-1; purine-nucleoside phosphorylase, purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) activities on starch gels. The horizontal starch gel electrophoresis protocol of Peters and Nash (17) was followed except that 12% starch (Connaught) was used, and gels were run for 16–17 hr at 4–5 V/cm (measured in the gel). All gels were run at 4°C.

ES-10 activity in the gels was identified by the methods of Peters and Nash (17, 18). The procedure of Womack *et al.* (19) was used to detect NP-1 activity.

The procedure of Rabin and Gottesman (3) was followed for assays of ADK activity, except that a single time point (15 min) was used. This method readily identified extracts with significant deficiencies of ADK activity.

Chromosome Preparation and Banding. Cells were treated with Velban (vinblastine sulfate, 0.015 μg/ml) for 2 hr and then harvested with trypsin/EDTA. The hypotonic treatment and fixation have been described (20). Bands were produced by a modification of the trypsin-Giemsa banding technique of Seabright (21). Suspensions of fixed cells were dropped onto moist slides, air-dried, and baked for 10 min in a 90°C oven (22). When the slides had cooled, they were immersed in trypsin/EDTA for 1–5 sec, rinsed in phosphate-buffered saline, and stained for 4–6 min in Gurr's R66 Giemsa (1:50 in Gurr's buffer, pH 6.8). Stained slides were rinsed first in Gurr's buffer and then in water and were air-dried.

RESULTS

Near-diploid mouse CAK-B3 cells were treated with mutagen and plated in toyocamycin for the selection of Adk^- mutants. The karyotype of this line is shown in Fig. 1. Two separate mutagenesis experiments were performed. Ten groups of 2×10^5 cells each were used in the first experiment, and 1.6×10^6 cells from each group were finally plated into selective medium. Seven groups each produced from one to six clones resistant to toyocamycin at 25 ng/ml; three groups produced no toyocamycin-resistant (Toy^r) clones. For all but two groups, 4 and 7, Toy^r clones within a group were identical with respect to Es-10 phenotype and very similar in karyotype. Given these results and considering the long expression time allowed, multiple clones arising within the same groups were considered to be duplicates (with the exceptions of groups 4 and 7). In the second experiment, 20 groups of 2×10^5 cells were used, and 1.2×10^6 cells from each group were plated into selective medium. A single Toy^r clone was isolated from each of 13 groups; in 7 groups, no Toy^r clones were found. The mutation frequency was $5–10 \times 10^{-7}$ per surviving cell in both experiments.

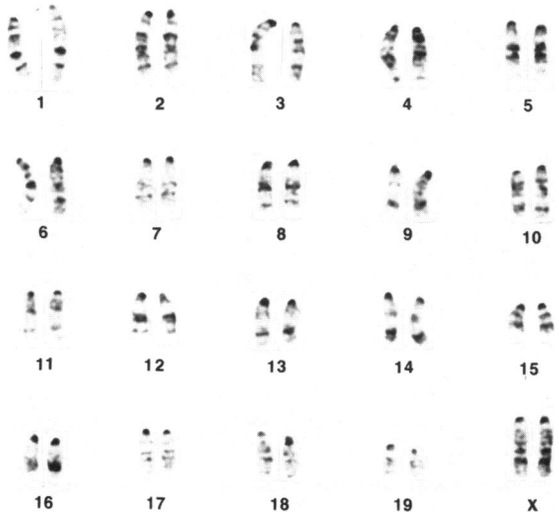

FIG. 1. CAK-B3 karyotype. The only deviation from the normal female mouse karyotype is the additional material on the end of the chromosome 16 on the right.

The ADK activities of the Toy^r mutants were compared with that of the wild-type CAK-B3 cells. All extracts were prepared at identical cell concentrations. The enzyme activities per cell of 14 of the 18 mutant groups assayed were less than 1.0% that of the wild type; the range of activities for all 18 mutants was 0.12–3.32% of the CAK-B3 activity. Therefore, all of the Toy^r clones that were examined were greatly deficient in ADK activity.

To confirm that the Adk^- mutations were recessive, we constructed hybrids between CHO-TG12-OB1 and CAK-B3 Toy^r-10. Hybrids were isolated by their ability to grow in hypoxanthine/aminopterin/thymidine medium containing 3.0 mM ouabain. Four hybrids were tested for their responses to selective media. All of them were sensitive to toyocamycin at 25 ng/ml and capable of growth in alanosine/adenosine/uridine medium (2), indicating that they contained ADK activity. In addition, extracts of these hybrids had levels of enzyme activities resembling that of CHO-TG12-OB1 cells rather than that of CAK-B3 Toy^r-10. Therefore, at least one of the Adk^- mutations that was recovered behaved as a recessive in hybrids. Toy^r-10 had a normal karyotype and expressed both allelic forms of the ES-10 enzyme. Based on their Es-10 phenotypes, two of the hybrids contained two copies of mouse chromosome 14 and the other two contained at least one copy of this chromosome.

The CAK-B3 cells were derived from the CAK-A32-G11 line established by Farber and Liskay (10) from (C57BL/6J × AKR/J) F_1-hybrid embryonic cells. On the basis of the genotypes of the parental inbred mouse strains, the CAK-B3 cells were expected to be heterozygous for the *a* and *b* alleles of the Es-10 electrophoretic marker. [C57BL/6J mice are $Es\text{-}10^a$ in genotype, and AKR/J mice are $Es\text{-}10^b$ (17, 19).] CAK-B3 cells appeared to be heterozygous for an esterase activity that exhibited the precise substrate and inhibitor specificities of ES-10 described by Peters and Nash (17, 18). The CAK-B3 pattern consisted of three bands having the relative positions expected of two homodimers and a heterodimer; however, the mobilities of the cathodal band and the heterodimer band were significantly less than those predicted by comparison with hemolysates obtained from (C57BL/6J × AKR/J) F_1-hybrid mice. The anodal band did correspond to the Es-10B band observed in AKR/J blood. The CAK-B3 cathodal band was also different from ES-10C (see Fig. 2G), which is an uncommon variant found in only a few inbred mouse strains (19, 23). The unusual CAK-B3 band consistently ran slightly anodal to the ES-10C band.

An examination of hybrids between CAK-B3 and CHO-TG12-OB1 showed that both ES-10B and the CAK variant form of the enzyme could form active heterodimers with the corresponding Chinese hamster esterase and that the two mouse enzyme types could segregate independently in hybrids. Of the eight hybrids analyzed, one expressed ES-10B, three expressed the variant band, and two expressed both mouse forms of the enzyme. The remaining two had no mouse ES-10 activity. All of the hybrids expressed the hamster activity and, where expected, mouse-hamster heterodimers. The mouse gene for nucleoside phosphorylase (NP-1) has been shown to be linked to the Es-10 locus (19, 23). The levels of mouse NP-1 activity in the hybrids, as visualized on starch gels, correlated with the

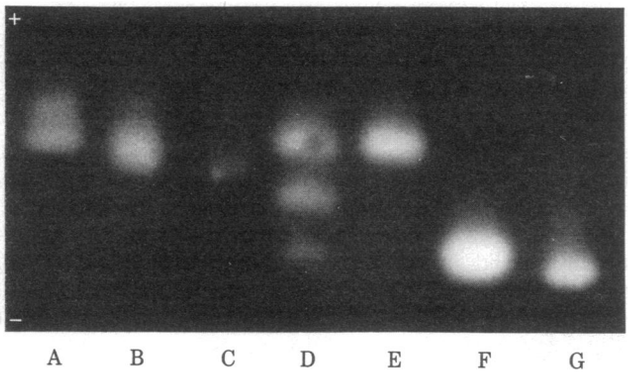

FIG. 2. Starch gel stained for Es-10 activity. (*A*) AKR/J hemolysate (*Es-10*^{b}). (*B*) (C57BL/6J × AKR/J) F_1-hybrid hemolysate (*Es-10*^{ab}). (*C*) C57BL/6J hemolysate (*Es-10*^{a}). (*D*) CAK-B3 cells (*Es-10*^{bv}). (*E*) Toy^{r}-2 (*Es-10*^{b}). (*F*) Toy^{r}-8 (*Es-10*^{v}). (*G*) BUB/BnJ hemolysate (*Es-10*^{c}).

apparent number of Es-10 alleles. Hybrids expressing both forms of ES-10 had the highest amount of mouse NP-1 activity, those expressing only one form of ES-10 had lower mouse NP-1 activities, and those expressing only the hamster esterase had no discernable mouse NP-1 activity (Table 1). Therefore, the behavior of the variant allele was consistent with a location on chromosome 14.

In view of these findings, it is reasonable to regard the CAK-B3 cathodal band as a previously undescribed variant, ES-10V. This variant probably arose during the early development of the CAK line or was present in the mouse from whose cells the line was derived. Earlier-passage clones of CAK cells [CAK-A39, CAK-A32, CAK-A32-G11 (10), and CAK-A32-G11-D9] all had the same Es-10 phenotype as CAK-B3.

The ES-10 gel patterns of 20 *Adk*$^{-}$ mutants of CAK-B3 were examined. Eleven mutants were heterozygous for Es-10 expression. Nine expressed only one of the allelic forms of Es-10. One of these expressed ES-10B, and the remainder expressed ES-10V. (The significance of the relatively high frequency of loss of the B form of the enzyme is not known.) This segregation at the Es-10 locus was observed only in *Adk*$^{-}$ mutants. The ES-10 patterns of 14 *Adk*$^{+}$ clones of CAK were examined, in addition to the early-passage clones mentioned above. These included three lines selected for resistance to 2,6-diaminopurine, four lines resistant to 6-thioguanine, and one line resistant to bromodeoxyuridine (all after treatment with ethyl methanesulfonate) and six CAK-B3 subclones. All of these had heterozygous ES-10 patterns identical to that of CAK-B3.

Eight of the nine independent *Adk*$^{-}$ mutants that segregated an Es-10 allele were karyotyped. Four of the mutants were missing a copy of chromosome 14 in many or all of the cells examined (Table 2). All of the cells in group 2 and most of those in group 17 had only one chromosome 14; most of these cells

Table 1. Mouse enzyme expression in mouse–hamster hybrids

Hybrid	ES-10 forms expressed: B	V	NP-1 activity
3	+	+	++
7	−	−	−
8A5	−	+	+
9A	+	+	++
13	−	+	+
14	−	−	−
16	+	−	+
19	−	+	+

Table 2. Chromosomal abnormalities in *Adk*$^{-}$ mutants expressing only one Es-10 allele

Group	Clone	Es-10 phenotype	Karyotypic abnormalities
		Experiment 1	
2	2,5,12	B	−14, + marker
4	7	V	None
	8	V	Mixture of cells with no abnormalities and cells with iso-14
5	13	V	−14, 1+, 8+
6	15, 20	V	iso-14, many metacentrics
7	16	V	Mixture of cells with no abnormalities and cells missing one 14
		Experiment 2	
9	30	V	heteroploid
13	34	V	ND
17	32	V	−14, −X, + one or more of several unidentifiable chromosomes

At least 10 cells of each mutant were karyotyped. ND, not done.

had at least one unidentifiable (marker) chromosome. The marker was the same in every cell in group 2 (Fig. 3A). Group 17 cells contained one to three small unidentifiable chromosomes. *Adk*$^{-}$ group 5 was also monosomic for 14; in all of these cells, one copy each of chromosomes 1 and 8 contained extra material (Fig. 3B). Finally, in group 7, clone Toy^{r}-16 consisted of some cells that were monosomic for chromosome 14 and some cells that had two normal copies of 14. The cells that had only one 14 had neither marker chromosomes nor obvious additions to identifiable chromosomes.

The remaining four *Adk*$^{-}$ mutants that expressed only form of ES-10 and were karyotyped had two copies of chromosome 14. Cells from group 6 and clone Toy^{r}-8 from group 4 contained an isochromosome 14 (iso-14). In group 6, all of the cells contained a single iso-14 (Fig. 4). Other isochromosomes were found frequently in group 6, but unlike the iso-14, these other isochromosomes were almost always accompanied by a normal homologue, resulting in trisomy for that chromosome. Clone Toy^{r}-8 was a mixture of cells having a single iso-14 and cells having two normal copies of 14. Clone Toy^{r}-7 from group 4 contained two normal copies of 14 in all cells. (We have considered Toy^{r}-8 and Toy^{r}-7 to be different mutants because their karyotypes were different; however, these clones could have been derived from the same *Adk*$^{-}$ mutant with the karyotypic difference having arisen subsequent to the mutation.) Group 9 cells were heteroploid. The mean number of chromosomes per cell was 71, and the line appeared to be relatively deficient for chromosomes 14. It averaged 2.0 copies of 14 per cell; averages for the other chromosomes ranged from 3.0 to 4.2.

Eight of the 11 *Adk*$^{-}$ mutants that remained heterozygous for Es-10 were karyotyped. None of these lines had consistent aberrations involving chromosome 14 (Table 3). Four had karyo-

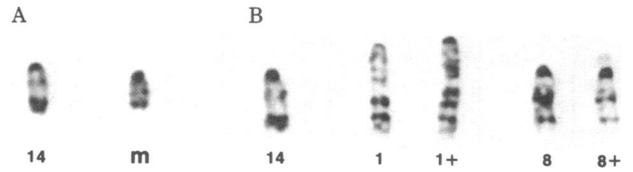

FIG. 3. (*A*) Single chromosome 14 and marker (m) chromosome found in group 2 cells. (*B*) Single chromosome 14; chromosomes 1, 1+, 8, and 8+ from group 5.

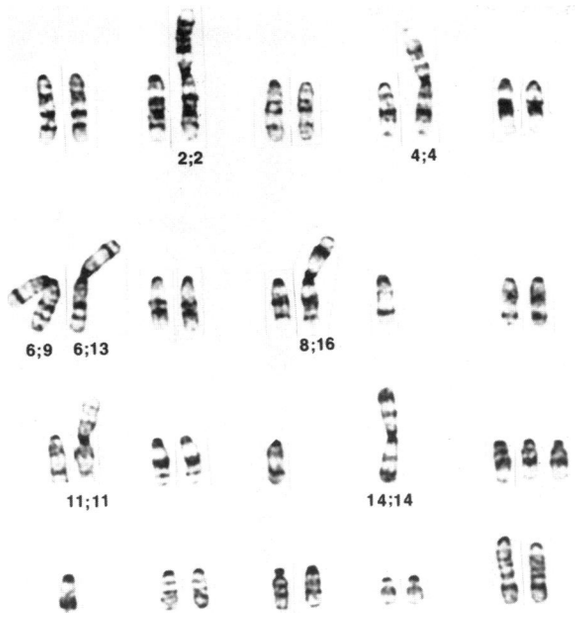

FIG. 4. Karyotype of an Adk^- cell from group 6.

types identical to CAK-B3 cells, and one was a mixture of diploids having an iso-X and tetraploids having two iso-Xs (group 3); because the tetraploids faithfully doubled the aberration seen in the diploid, these two clones were considered to have arisen from the same mutant. Most of the abnormalities in the remaining lines consisted of metacentric chromosomes that appeared to have resulted from the fusion of two normal acrocentric chromosomes at the centromeres. Very few alterations in the amount of chromosomal material were observed in any of the nonsegregant clones.

DISCUSSION

We have analyzed 20 different Adk^- mutants of the near-diploid CAK-B3 mouse line. Nine of these mutants expressed only one allele of Es-10, a locus that is on the same chromosome as Adk and for which normal CAK-B3 cells are heterozygous. These results suggest that recessive mutations can frequently be expressed in diploid cultured cells by a two-step or multistep process.

Table 3. Chromosomal abnormalities in Adk^- mutants heterozygous for Es-10

Group	Clone	Karyotypic abnormalities
	Experiment 1	
1	1,3,4,9,10,11	None
3	6	4n, 2 copies of iso-X
	14	iso-X
7	18	(5; 9) metacentric
	Experiment 2	
8	22	None
10	27	None (eight cells)
11	25	None
12	24	2+ (six cells)
15	26	ND
16	23	(6; 8) metacentric; (10; 15) metacentric
18	33	ND
20	28	ND

Except where noted, at least 10 cells of each mutant were karyotyped. ND, not done.

In some of the mutants, the segregation of an Es-10 allele, and presumably an Adk^+ allele, appears to be the result of the loss of a chromosome 14 or of a deletion from that chromosome. In groups 2 and 17, chromosome 14 monosomy is accompanied by the appearance of small marker chromosomes, and in group 5, there is a single copy of 14 and additional material on chromosomes 1 and 8. It is not known whether any of these abnormal chromosomes are derived from the missing chromosome 14.

Autosomal monosomy is rare in mammalian cells, although not unknown. Cox *et al.* (24) have reported that Chinese hamster cells monosomic for one or even two of the smallest autosomes are viable, although at a disadvantage in the presence of diploids or disomic tetraploids; by the 35th passage, these monosomic lines had either acquired a second copy of the chromosome or had become tetraploid. Monosomy could be maintained only by frequent subcloning. One of the CAK-B3 Adk^- mutants that segregated an Es-10 allele is composed of a mixture of cells with no consistent karyotype changes and cells in which the only evident karyotype change is the loss of a chromosome 14 (Toy^r-16, group 7). It is possible that this line is progressing toward a normal karyotype by duplication of the chromosome 14 carrying the Adk^- allele.

Chromosome loss and duplication may be a general mechanism by which cells containing two copies of chromosome 14 have become homozygous for Es-10 and Adk. In cells in which two normal homologues are present, this loss and duplication could have occurred by nondisjunction. In other cases, isochromosome formation, resulting from the failure of sister chromatids to disjoin at the centromeres, may have been the mechanism of duplication, or the isochromosome may have been formed as a result of the joining of two preexisting normal chromosomes at the centromere. It cannot be concluded on the basis of our results whether loss of the chromosome carrying the wild-type *Adk* allele usually occurred before or after duplication of the homologue carrying the mutant allele.

Whatever specific mechanisms or sequences of events are involved, it is clear that visible abnormalities of chromosome 14 are observed frequently in Adk^- mutants that have segregated an Es-10 allele. However, chromosome segregation events cannot account for the expression of all of the mutants in this line. More than half of the mutants remained heterozygous for Es-10 expression, and no abnormalities involving chromosome 14 were evident in their karyotypes. It is possible that, in these cases, secondary genetic events occurred that were not detectable by the methods used in this study. For example, deletion or inactivation of a small region of chromosome 14 that includes Adk but not Es-10 cannot be ruled out. There is evidence for regional hemizygosity at certain loci in CHO cells. In the case of emetine resistance (emt^r), this is clearly the result of a preexisting visible deletion (8). In other cases (25), the possibility of smaller deletions or regions of inactivation has to be invoked. It is unlikely that the degree of hemizygosity in CAK could be as extensive as has been hypothesized for CHO. CAK was established much more recently than CHO; it has a nearly normal karyotype, whereas CHO cells have numerous chromosomal rearrangements (26). The mutation frequency that we have observed for the Adk locus in CAK cells is several orders of magnitude lower than those reported for certain sublines of CHO (2, 3). It is also possible that there is a small subpopulation of CAK-B3 cells that is functionally hemizygous for the Adk locus but not for the Es-10 locus. In the V79/V6 Chinese hamster cell line, Siminovitch (27) has found evidence for a subpopulation that is apparently hemizygous at the emt locus and therefore produces emt^r mutants at

a much higher frequency than the cell line as a whole.

Mitotic recombination and gene conversion are other possible mechanisms for rendering an Adk^- mutation homozygous, although efforts to demonstrate mitotic recombination in mammalian cells have been unsuccessful so far (28). The occurrence of two independent Adk^- mutations, one in each allele, also cannot be ruled out.

Segregation of an allele on the same chromosome as a newly arising recessive mutation has been reported previously only in intraspecific hybrids. Chasin and Urlaub (29) found that in 50% of mutants at the X-linked Hprt locus in (CHO × CHO) hybrids that were heterozygous for X-linked glucose-6-phosphate dehydrogenase (G6PD), the expression of the $G6pd^+$ allele had been lost. (Loss of the $G6pd^-$ allele would not have been detectable in this system.) In this case, genetic segregation was probably the result of chromosome loss, because it was subsequently shown that segregation of the $Hprt^+$ allele from $Hprt^+/Hprt^-$(CHO × CHO) hybrids was nearly always associated with the loss of part or all of one X chromosome (30). However, cosegregation of two markers located on Chinese hamster chromosome 2 (emt^r and *chromate-resistance*) from (CHO × CHO) hybrids heterozygous at both loci was not associated with loss of that chromosome (31). Although these genes were autosomal, there were only two functional copies of each of them in the heterozygous hybrids, because the region of chromosome 2 in which they were presumably located has been deleted from one homologue.

Newly arising recessive mutants at the Adk locus in a mouse fibroblast line have been analyzed for the behavior of both a physically linked marker and the chromosome on which the two genes reside. Our results show that the segregation of such a marker, or of an entire chromosome, is frequently associated with the expression of an autosomal recessive mutation in karyotypically near-normal diploid mammalian cells. Similar processes may occur *in vivo*, resulting in homozygosity of new or preexisting recessive mutations. Such homozygosis of recessives in normal somatic cells could provide a mechanism for the expression of mutations leading to malignancy.

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