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Azacytidine-Induced Reactivation of a DNA Repair Gene in Chinese Hamster Ovary Cells

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Six X-ray-sensitive (*xrs*) strains of the CHO-K1 cell line were shown to revert at a very high frequency after treatment with 5-azacytidine. This suggested that there was a methylated *xrs*$^+$ gene in these strains which was structurally intact, but not expressed. The *xrs* strains did not complement one another, and the locus was autosomally located. In view of the frequency of their isolation and their somewhat different phenotypes, we propose that the *xrs* strains are mutants derived from an active wild-type gene. However, there is in addition a methylated silent gene present in the genome. Azacytidine treatment reactivated this gene. We present a model for the functional hemizygosity of mammalian cell lines, which is based on the inactivation of genes by de novo hypermethylation. In contrast to results with *xrs* strains, other repair-defective lines were found not to be reverted by azacytidine.

A large number of mammalian cell variants were isolated after mutagenic treatments, and there is convincing evidence that many of these are structural gene mutations, comparable to those isolated in various microorganisms (for reviews, see references 2, 7, and 36). Although the cells used were either diploids or originally derived from diploids, recessive autosomal mutants were often isolated at frequencies of 10^{-3} or 10^{-4}, suggesting that only a single gene copy is present. This has led to Siminovitch's (36) concept of "functional hemizygosity," in which a substantial part of the genome is effectively haploid. One way this could arise is by chromosome rearrangement and the loss of part of the diploid genome (36).

Although there is strong evidence for a genetic basis for stable cell variants, there remains the possibility that heritable variation might be caused by epigenetic alterations in gene activity (15). In support of this theory, it has recently been shown that several enzyme-deficient strains of rodent cells are reverted at very high frequency after treatment with 5-azacytidine (AC) (16–18, 27). This is only a weak mutagen, but is known to be highly effective in reducing DNA methylation, probably through the inhibition of a maintenance methylase (23, 40). The results make it likely that these enzyme-deficient strains are epigenetic variants, in which transcription has been shut off by methylation. Considerable independent evidence exists that transcription is associated with hypomethylation, and that certain silent genes, for instance those on the inactive X chromosome or in latent retroviruses, are hypermethylated and can be reactivated by AC (for reviews, see references 8, 11, 22, 32, and 33).

We previously described the isolation and partial characterization of six CHO strains which are sensitive to X rays (*xrs*) and defective in the repair of double strand breaks (21, 25). All of these are recessive traits and fall into one complementation group. We show here that all are reverted to X-ray resistance at very high frequency after AC treatment, indicating that the *xrs* strains have a copy of the *xrs* gene inactivated through methylation event(s). The strains were originally isolated after ethyl methanesulfonate (EMS) mutagenesis, at a frequency of approximately 10^{-3}. We propose that two copies of the gene exist in the parent line, but only one is active, the other being inactivated by DNA methylation. We discuss other published results which may be explained in the same way.

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MATERIALS AND METHODS

Cell culture conditions. The cells were routinely grown in GIBCO F15 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% (vol/vol) fetal calf serum (heat inactivated at 56°C for 60 min), penicillin-streptomycin (0.06 mg/ml and 0.1 mg/ml, respectively), glutamine (0.25 mg/ml), and pyruvate (0.11 mg/ml). For detaching cells, trypsin-versene (0.125% trypsin, 0.27 mM EDTA) was used. Cultures were incubated at 37°C in 5% CO_2.

Treatment with AC, EMS, X rays, and bleomycin. AC and EMS were purchased from Sigma Chemical Company, St. Louis, Mo., and bleomycin was a gift from Lundbeck Ltd. X-irradiation was from a Newton Victor X-ray machine operated at 120 kVp and 5.5 mA at a dose rate of 48 rads per min. To induce reversion by AC, cells were trypsinized and seeded at 1×10^5 cells per 25-cm^2 Falcon flask and were treated with AC 1 day later. AC is unstable in aqueous solution; therefore, it was prepared immediately before use (1 mg/ml). It was added at 1 to 3 μg/ml, and the medium was changed after 15 h. Cultures were routinely allowed 5 days of growth before plating under selective conditions and were passaged during this period if the cells became confluent. Selective conditions are described in Results.

For EMS mutagenesis, cells were seeded at 1×10^5 per 75-cm^2 Falcon flask on the day before exposure and treated with EMS for 24 h at 120 or 240 μg/ml. A total of 7 days of expression was allowed. During this period, the cultures were passaged when they became confluent, and then they were plated under selective conditions. The frequency of 6-thioguanine-resistant (TG^r) mutants was estimated by selection in TG (2 μg/ml).

Survival after X-irradiation was determined by plating the appropriate numbers of cells (10^2 to 10^5) into 60-mm tissue culture dishes and incubating for 4 h for attachment before irradiation. Cells were incubated for 7 days, and colonies were fixed in methanol-acetic acid (3:1) and stained with crystal violet (0.4 mg/ml).

TABLE 1. Reversion of *xrs* strains by AC

Strain (AC treatment [μg/ml])	Selective conditions	Frequency of reversion (%)
xrs 1 (1)	X rays	0.06
xrs 2 (1) (2×)[a]	Bleomycin	8
xrs 4 (1)	X rays	0.9
xrs 5 (0.5)	X rays	0.07
xrs 5 (1.0)	X rays	0.95–2.4 (4 expts)
xrs 5 (3)	X rays	0.63–9.6 (5 expts)
xrs 5 (1) (2×)	X rays	5
xrs 5 (1) (2×)	Bleomycin	8
xrs 6 (1)	X rays	3.3
xrs 7 (1)	X rays	1.5

[a] Two exposures to 1 μg of AC per ml given with an interval of 24 h.

Cell fusion and analysis of hybrid clones. Cells were fused by treatment with polyethylene glycol 1000, and hybrid clones were selected in hypoxanthine-aminopterin-thymidine (HAT) (hypoxanthine, 1×10^{-4} M; aminopterin, 4×10^{-7} M; thymidine, 1×10^{-5} M) and ouabain (1 mM) medium as described previously (20). Hybrid clones were shown to have the tetraploid number of chromosomes for metaphase preparations. After 2 weeks of growth in nonselective medium, TG^r segregants were selected by plating in TG (2 μg/ml). They were examined for X-ray resistance as described previously (20).

RESULTS

Selective conditions for *xrs* revertants. Initial experiments were done to optimize conditions for the selection of xrs^+ cells in the presence of a large number of xrs^- cells. When X- or γ-irradiation was given as a single dose, there was insufficient difference in sensitivity to allow the selection of wild-type cells in the presence of more than 10^3 mutant cells. Two selective conditions were chosen which overcame this problem: (i) (method A) exposure to 160 rads of X rays daily for 4 days (this reduced wild-type survival to 60% and the strain *xrs* 5 survival to less than 10^{-5}) and (ii) (method B) exposure to bleomycin (0.25 μg/ml). (Bleomycin was added on the day of plating and replaced with fresh medium containing bleomycin after 3 days. Under these conditions, wild-type survival was 67% and strain *xrs* 5 survival was less than 7×10^{-4}.)

Reversion of strain *xrs* 5 cells after azacytidine treatment. *xrs* 5 cells were treated with various concentrations of AC for 16 h as described in Materials and Methods and plated under selective conditions after allowing 5 days for expression. The reversion frequency was estimated by (number of X-ray-resistant clones)/(number of cells plated × cloning efficiency × survival CHO-K1), in which survival CHO-K1 is the survival of CHO-K1 cells under the same selective conditions. This takes into account the reduced survival of wild-type CHO-K1 cells under these conditions. This estimate of the reversion frequency assumes that revertant cells have the same survival as wild-type cells, and the frequency will be underestimated if this assumption is incorrect. After exposure to 1 to 3 μg of AC per ml, a reversion frequency of approximately 1% was usually observed. When sequential treatments with 1 μg of AC per ml were applied, reversion frequencies as high as 8% were obtained. Representative results are shown in Table 1.

A 1- to 3-μg amount of AC per ml caused considerable inhibition of cell growth, and the cloning efficiency at the time of plating under selective conditions was reduced from 35% for untreated cells to 19 and 9% for cultures treated with 1 and 3 μg/ml, respectively. The cloning efficiency of strain *xrs* 5 was itself low relative to the CHO-K1 parent (which is 60 to 90%), but *xrs* 5 cells did not appear to be more sensitive to AC than the CHO-K1 cells were. The reversion frequency was not significantly changed when cells were used after expression times varying from 2 to 7 days. During the course of these experiments, no spontaneous xrs^+ clones were observed out of $>5 \times 10^5$ *xrs* 5 cells that were examined. However, populations of *xrs* 5 cultures, grown continuously, have occasionally been overgrown by xrs^+ cells, indicating that reversion can occur spontaneously. These results show that AC can induce a 10^4 to 10^5 increase in the reversion of the *xrs* 5 strain to X-ray resistance (xrs^+).

Survival and stability of revertant clones. A total of 12 clones selected as xrs^+ by method A after AC treatment of strain *xrs* 5 were picked, and their survival after X-irradiation was examined. All were X-ray resistant. The survival of two of these clones after X-irradiation is shown in Fig. 1, together with that of xrs^+ and *xrs* 5 cells. These two clones showed the highest and lowest levels of survival; all the other clones had intermediate survival levels. Thus, all the clones showed reduced survival compared with the CHO-K1 parent, but were clearly much more resistant than the *xrs* 5 strain from which they were derived. In contrast to this, three clones obtained after AC treatment of strain *xrs* 2 and selected by method B (exposure to bleomycin) all showed wild-type levels of survival after treatment with X rays (data not shown). Two of the xrs^+ clones were main-

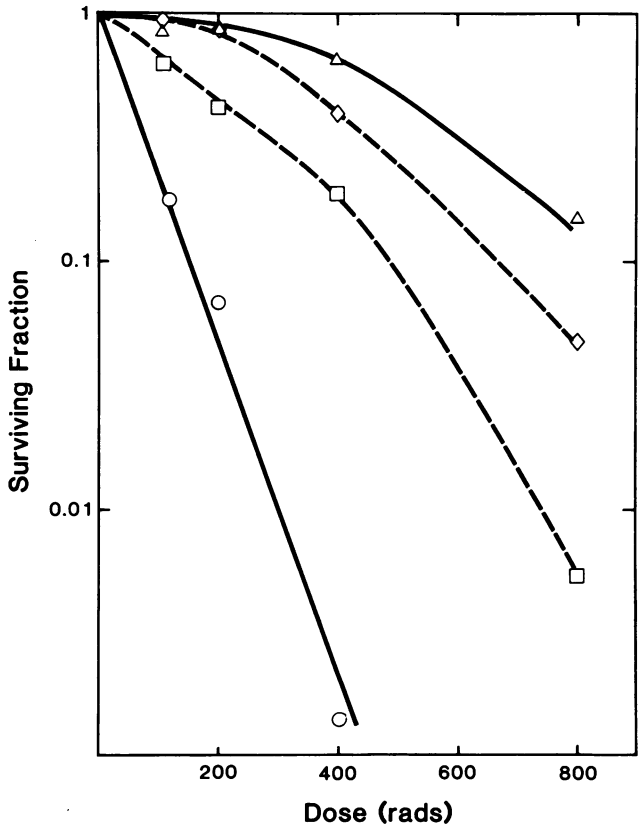

FIG. 1. X-ray survival of revertant clones. X-ray survival of CHO-K1 (△), *xrs* 5 (○), and two revertant clones (◇, □). The clones were selected from *xrs* 5 by method A (see text) after AC treatment.

TABLE 2. Reversion of *xrs* strains after treatment with EMS

Strain and treatment	Frequency (no. of colonies/no. of viable cells) of colonies resistant to:	
	X rays (*xrs*$^+$)	6-thioguanine (TG^r)
xrs 6		
Control	$<2.6 \times 10^{-4}$ ($0/2.6 \times 10^4$)	$<2.6 \times 10^{-4}$ ($0/2.6 \times 10^4$)
EMS (0.12 mg/ml)	5.8×10^{-4} ($5/0.85 \times 10^4$)	4.6×10^{-4} ($4/0.85 \times 10^4$)
AC (1 μg/ml)	0.6×10^{-2} ($16/0.28 \times 10^4$)	$<2.0 \times 10^{-4}$ ($0/2.8 \times 10^4$)
xrs 7		
Control	6.5×10^{-4} ($33/5 \times 10^4$)	$<10^{-5}$ ($0/10^5$)
EMS (0.24 mg/ml)	7.0×10^{-4} ($4/0.56 \times 10^4$)	3×10^{-3} ($21/0.56 \times 10^4$)
AC (1 μg/ml)	0.68×10^{-2} ($77/1.1 \times 10^4$)	ND[a]

[a] ND, Not done.

tained in culture for more than 2 months without exposure to X rays and retained their enhanced level of resistance.

Reversion after EMS treatment. To determine whether the strains were also revertible by mutagen treatment, we examined cells after treatment with EMS. However, the *xrs* strains were more sensitive to EMS than was the wild-type parent, and frequently poor results were obtained because of excessive killing by the mutagen. This was observed particularly with mutant *xrs* 5. The results of two experiments with strains *xrs* 6 and *xrs* 7 are shown in Table 2. Strain *xrs* 7 yielded a higher background frequency of X-ray treatment survivors, since it seemed more resistant to the repeated X-ray treatments, but no increase above this background frequency was observed after EMS treatment, although a high frequency of TG^r mutants was observed. With strain *xrs* 6 and a lower dose of EMS, the induction of X-ray-resistant and TG^r clones occurred at similar, although lower, frequencies. We conclude that EMS may have induced a low frequency of reversion to *xrs*$^+$, but certainly did not induce reversion at the high frequency observed after AC treatment.

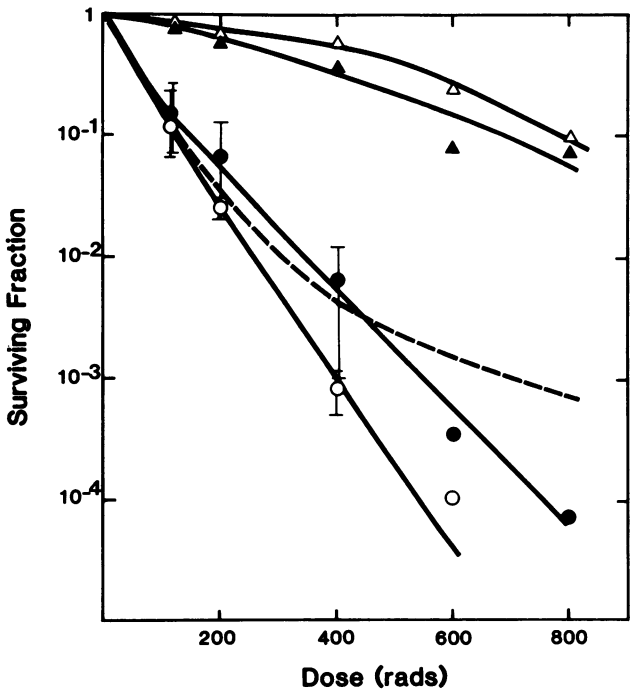

FIG. 2. X-ray survival of untreated and AC-treated populations of *xrs* 5 and *xrs*$^+$ cells. Cells were exposed to 3 μg of AC per ml for 16 h (closed symbols) or untreated (open symbols), and after 5 days of expression they were plated to estimate their survival after various doses of X rays (see Materials and Methods). *xrs*$^+$ (△, ▲), *xrs* 5 (○, ●). – – – – –, Theoretical estimate of 1% reversion frequency based on the survival of untreated *xrs* 5 and AC-treated CHO-K1 cells.

Resistance of *xrs* 5 and *xrs*$^+$ to X rays after AC treatment. The high frequency of reversion of strain *xrs* 5 to *xrs*$^+$ after AC treatment indicated that the survival of a population of AC-treated *xrs* 5 cells should appear as a biphasic curve. The survival curves of untreated and AC (3 μg/ml)-treated *xrs* 5 and *xrs*$^+$ cells are shown in Fig. 2. AC treatment slightly reduced the survival of *xrs*$^+$ cells. In contrast, the survival of AC-treated *xrs* 5 cells was greater than that of untreated cells, and this was more marked at higher doses. The shape of the survival curve did not follow that predicted for a population containing 1% wild-type cells, but the result was compatible with the observation (Fig. 1) that the *xrs*$^+$ revertants had a somewhat reduced survival compared with wild-type cells.

Examination of other repair-defective lines for reversion with AC. *xrs* 5 was one of six mutant strains isolated from a search for *xrs* mutants (21). The other five *xrs* mutant strains were examined for reversion by AC treatment, and all were found to be revertible at frequencies similar to that of *xrs* 5 (Table 1). To determine whether this was a feature characteristic of this gene or whether it was a property common to other repair-defective strains, we also examined certain other cell lines isolated on the basis of sensitivity to DNA-damaging agents for revertibility by AC (Table 3). UV-sensitive strains UV-5, UV-4, UV-24, UV-41, and UV-135 were isolated by Thompson et al. (42, 44) and are representatives of complementation groups 1, 2, 3, 4, and 5, respectively (41). Strains 43-3b and 27-1 were also isolated as UV sensitive (49). None of these lines appeared to be revertible

TABLE 3. Examination of repair-deficient strains for reversion by AC[a]

Strain (reference)	AC treatment (μg/ml)	Selective conditions
UV-4 (42)	1	10 nM mitomycin C
UV-5 (42)	1	5 J/m^2 UV (2×)[b]
UV-41 (42)	1	10 nM mitomycin C
UV-24 (42)	1	5 J/m^2 UV (3×)
UV-135 (44)	1	5 J/m^2 UV (3×)
43-3B (50)	1	20 nM mitomycin C
27-1 (50)	1	5 J/m^2 UV (2×)

[a] The reversion frequency for all strains was <0.01.

[b] Two or three exposures (as indicated) to 5 J of UV light per m^2 given at 24-h intervals.

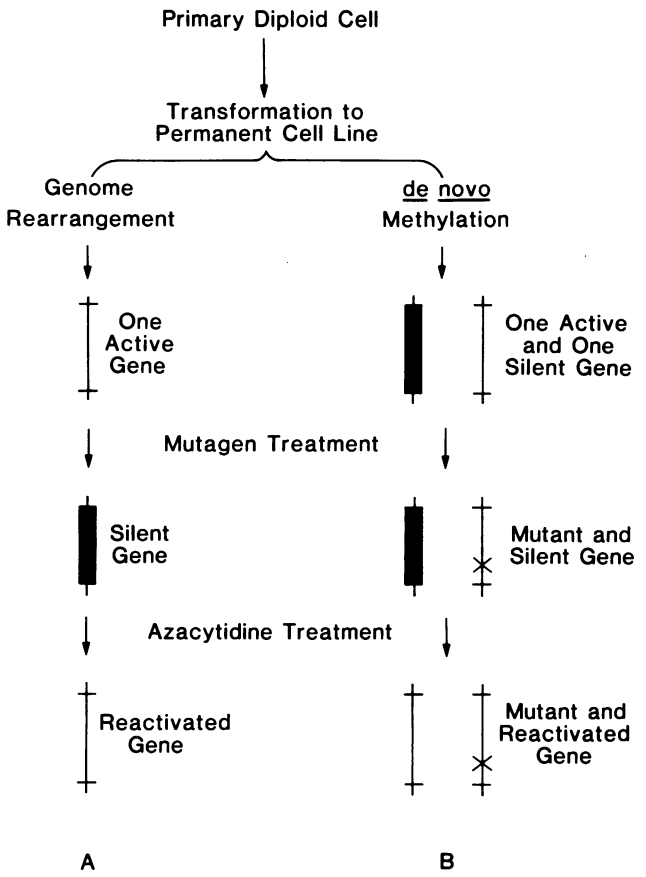

FIG. 3. Models for AC-induced gene activation. Possible mechanisms for the reactivation of a silent hypermethylated autosomal gene by AC, assuming that the genome contains one (A) or two (B) copies of the gene.

by AC under the conditions examined, although reconstruction experiments showed that wild-type cells could be selected in the presence of sensitive cells under the conditions used.

Autosomal locations of the *xrs* gene. To interpret the results of these experiments, it is important to know whether the *xrs* locus is on the X chromosome. For this purpose, hybrid clones were constructed from an *xrs* 5, TG^r, oua^R mutant and CHO-K1 by using selection in HAT and ouabain medium, as described in Materials and Methods. These hybrid clones have the wild-type level of resistance to X rays, showing the recessive nature of the *xrs* mutation (20). From a single hybrid clone TG^r segregants were selected and tested for X-ray sensitivity. TG^r segregants arose at a frequency of 6×10^{-4}, and of four segregants tested, all were HAT^s, TG^s, oua^R and showed a wild-type level of resistance to X rays (i.e., xrs^+). Since the frequency of spontaneous TG^r mutants is $<10^{-5}$, we infer that the TG^r clones are segregants that have lost the X chromosome and conclude that the *xrs* mutation does not segregate with hypoxanthine guanine phosphoribosyl transferase and that the gene is therefore not X-linked.

DISCUSSION

We showed that six X-ray-sensitive strains, originally isolated after treatment of CHO-K1 with EMS, reverted at high frequency to X-ray or bleomycin resistance after treatment with AC. The *xrs* strains were stable during normal growth and reverted at only very low frequency after treatment with EMS. The *xrs* gene is autosomal, but the frequency of *xrs* strains identified after mutagenesis was similar to the frequency of X-linked TG^r mutants. Since we previously showed that the *xrs* phenotype is recessive in hybrids (20), the results suggest that there is only one functional copy of the *xrs* wild-type gene in CHO-K1 cells.

There is considerable evidence that AC is a powerful agent in demethylating DNA, probably through its inhibition of DNA maintenance methylase after AC has been incorporated into DNA (23, 40). In contrast, this and other studies have shown that it is only weakly mutagenic (26). There are now many examples of the reactivation of nonexpressed genes by AC, including strains deficient in thymidine kinase (3, 16, 28, 30), glutamine synthetase (18), asparagine synthetase (39), ornithine decarboxylase (37), pyrroline-5-carboxylate synthetase (17), and metallothionein (4, 6). In some cases, reversion to the wild-type phenotype has been correlated with a loss of methylation (3, 4). A striking feature of the effect of AC is the very high frequency of revertants obtained, and in the case studied here up to 8% of the surviving clones were X-ray resistant. AC is also capable of reactivating silent genes on the inactive X chromosome (13, 29), which is known to be hypermethylated at the glucose 6-phosphate dehydrogenase and hypoxanthine guanine phosphoribosyl transferase loci (45, 48, 50). It also activates latent retroviruses, and these events have also been shown to be associated with the loss of 5-methylcytosine (5mC) (5, 14, 31). All these results support the hypothesis that hypermethylation of DNA sequences can shut off transcription and that AC reactivates genes by demethylating these sequences. Thus, there is an epigenetic control of gene activity in these instances. The results also show that the methylation which shuts off transcription is very stably inherited.

Our results indicate that the *xrs* gene is structurally intact in these radiation-sensitive strains and is regulated by cytosine methylation, but there are at least two possible ways that the results can be interpreted (Fig. 3). The first is that only one copy of the *xrs* gene exists in CHO-K1; in other words, it is hemizygous. The EMS treatment might then induce aberrant methylation, which shuts off the gene, at a frequency of about 10^{-3}. There is some evidence that DNA-damaging agents induce hypermethylation (9, 19); however, they have also been shown in several instances to reduce DNA methylation both in vitro (46) and in vivo (27), and repair tracts may be undermethylated (24). (Note that we cannot exclude the possibility that such hypermethylated variants arise spontaneously at a frequency of 10^{-3}, since only mutagenized isolates were tested in the search for *xrs* strains). If one methylated gene exists, then the AC treatment removes the methylation and reactivates the gene.

It was previously shown that the phenotypes of the six *xrs* strains are not identical; in particular, they vary in their cross-sensitivity to other DNA-damaging agents (21) and in their rate of repair of double strand breaks (25). These observations, taken together with the fact that all six strains are revertible by AC, leads us to favor a second interpretation of our results. We propose that there might be two copies of the *xrs* gene in CHO-K1, but that one has been inactivated at some stage during the origin or subsequent culturing of the line by de novo methylation. Thus, the CHO-K1 line is functionally hemizygous for the *xrs* gene because it has one silent copy inactivated by methylation. Therefore the *xrs* strains are indeed mutants resulting from

mutagenesis by EMS, and they have distinguishable phenotypes. However, when these mutants are treated with AC, the silent gene present in all the strains is reactivated at high frequency. This situation is outlined in Fig. 3B. We propose that epigenetic changes in gene activity based on DNA methylation should be referred to as epimutations to distinguish them from classical mutants, which are due to changes in DNA base sequences (R. Holliday, Heredity **55**:280, 1985). Thus we suggest that the wild-type CHO-K1 line contains a normal active gene and an epimutation at the *xrs* locus. Our CHO-K1 line is also *pro*$^-$ and cadmium sensitive (Cd^s), and these are further examples of epimutations, because AC can produce *pro*$^+$ and Cd^R phenotypes at high frequency. Verification of the model (Fig. 3B) requires the cloning of the *xrs* gene, and this is being undertaken.

It is possible that spontaneously arising variants (e.g., *pro*$^-$, Cd^s, and *glu*$^-$) in the CHO cell line have arisen because of de novo methylation during either the establishment of the line or routine subculture. In addition, certain cultured tumor cell lines have genes that are switched off by methylation (4, 28, 39). There is also other evidence that permanent cell lines are capable of de novo methylation. For example, genes reactivated by AC may become inactivated again after growth in the absence of the analog (3, 12). Moreover, the level of 5mC in 10T1/2 cells can be reduced by multiple treatments of 5-aza-deoxycytidine, but when these treatments are terminated, the level of 5mC gradually increases again (10). Teratocarcinoma cells are also capable of inactivating integrated retroviruses by de novo methylation (38). In contrast, primary diploid fibroblasts progressively lose 5mC during serial subculture, suggesting that they may have inefficient maintenance of DNA methylation and also may lack de novo methylase activity (47).

It may be that permanent lines with unstable karyotypes lose the usual controls of gene activity. One feature of this may be a given probability of inactivating structural genes by de novo methylation, if such inactivation confers no selective disadvantage. This provides one explanation of functional hemizygosity, but it does not rule out the possibility that chromosome rearrangements have produced physical hemizygosity for parts of the genome (1, 31). Moreover, we have shown here that other radiation-sensitive strains were not reverted by AC, and the frequency at which they were isolated suggests that a single gene copy is present (44). Preliminary mapping studies locate some of these genes to chromosomes which appear to be associated with structural rearrangements (43). Finally, there is evidence that large parts of the CHO-K1 genome remain diploid with two active alleles, since enzyme polymorphisms persist in these strains (34). It is possible, however, that in these cases the approximate halving of the amount of gene product by an epimutation may be selectively disadvantageous, so that such strains do not normally become established in laboratory populations.

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