SOUTHERN ANALYSIS OF GENOMIC ALTERATIONS IN GAMMA-RAY-INDUCED APRT- HAMSTER CELL MUTANTS

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ABSTRACT

The role of genomic alterations in mutagenesis induced by ionizing radiation has been the subject of considerable speculation. By Southern blotting analysis we show here that 9 of 55 (approximately 16%) gamma-ray-induced mutants at the adenine phosphoribosyl transferase (aprt) locus of Chinese hamster ovary (CHO) cells have a detectable genomic rearrangement. These fall into two classes: intragenic deletions and chromosomal rearrangements. In contrast, no major genomic alterations were detected among 67 spontaneous mutants, although two restriction site loss events were observed. Three gamma-ray-induced mutants were found to be intragenic deletions; all may have identical breakpoints. The remaining six gamma-ray-induced mutants demonstrating a genomic alteration appear to be the result of chromosomal rearrangements, possibly translocation or inversion events. None of the remaining gamma-ray-induced mutants showed any observable alteration in blotting pattern indicating a substantial role for point mutation in gamma-ray-induced mutagenesis at the aprt locus.

Several lines of evidence suggest that chromosomal rearrangements may comprise a major element in the spectrum of mutations induced by ionizing radiation. These include the potent clastogenicity of ionizing radiation (BEIR Report 1980), the specific correlation of chromosomal rearrangements at the site of the HPRT locus with 6-thioguanine resistance in human fibroblasts (Cox and Masson 1978; Thacker and Cox 1984) and the inability of ionizing radiation to induce ouabain resistance (Arlett et al. 1975; Thacker, Stephens and Stretch 1978), a base substitution dependent mutation. Nevertheless, there has been no direct demonstration of the DNA sequence alterations in ionizing-radiation-induced mutants of mammalian cells.

Recently, several studies (Graf and Chasin 1982; Meuth and Arrand 1982; Nalbantoglu, Goncalves and Meuth 1983; Fuscoe et al. 1983; Goncalves, Drobetsky and Meuth 1984; King and Brookes 1984) have used nitrocellulose blotting techniques (Southern 1975) to analyze genomic alter-

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ations in mammalian cell mutants. This method enables the detection of deletsions or insertions of approximately 50 base pairs and larger, as well as the loss or gain of restriction enzyme cleavage sites. The latter events may reflect base substitutions or genomic alterations which would otherwise be below the limit of resolution. In the present report we have employed Southern analysis to study genomic alterations in gamma-ray-induced mutants at the adenine phosphoribosyl transferase (aprt) locus of Chinese hamster ovary (CHO) cells.

There are several selectable mammalian loci for which cloned probes for Southern analysis are available. These include aprt as well as hypoxanthine guanine phosphoribosyl transferase (hprt) and dihydrofolate reductase (dhfr). Although the aprt locus shares some features with the others, it offers advantages which are unique. Unlike hprt and dhfr, a detailed restriction map is known for aprt (LOWY et al. 1980; NALBANTOGLU, GONCALVES and MEUTH 1983), and pseudogenes do not complicate Southern analyses. Molecular analysis of the aprt locus is also facilitated by its small size (2.6 kb; DUSH et al. 1985; A. J. GROSOVSKY et al., unpublished results) as compared to dhfr (25 kb; MILBRANDT et al. 1983; CARROTHERS et al. 1983) and hprt (>33 kb in the mouse; MELTON et al. 1984).

As an autosomal locus, aprt is ordinarily present in diploid copy number. However, the CHO sub-clone, D422 (BRADLEY and LETANOVEC 1982), used in this study has been characterized as hemizygous at the aprt locus (NALBANTOGLU, GONCALVES and MEUTH 1983), thereby simplifying molecular analyses and enabling convenient isolation of APRT- mutants in a single step selection. APRT, like HPRT, is a nucleotide salvage pathway enzyme and, thus, is not essential for cell viability under ordinary culture conditions. Hence, all classes of mutational events can be expected among APRT- clones; the only restraint is the possible inclusion of essential genes within the region of hemizygosity. The results presented below demonstrate that any such restraints are not sufficient to prohibit the recovery of major genomic alterations among the APRT- clones induced by ionizing radiation.

Our results provide a quantitative determination of the prevalence of genomic alterations in a collection of 55 gamma-ray-induced and 67 spontaneous APRT- mutants. Observed genomic alterations have been characterized and localized on the restriction map of the aprt locus. Gamma-ray-induced genomic alterations were classified as either intragenic deletions or chromosomal rearrangements, and spontaneous alterations as restriction site loss.

MATERIALS AND METHODS

Maintenance and selection of wild-type and mutant strains: All 8-azaadenine (AA8) mutants used in this study were selected from a clonal isolate of D422, an aprt hemizygote that was originally derived from a diploid CHO line auxotrophic for proline. Cells were routinely propagated on 100-mm petri dishes (Nunc) in alpha minimal essential medium (Gibco) supplemented with 2.5% fetal calf serum and 2.5% heat-inactivated horse serum (Gibco).

Spontaneous APRT- mutants were obtained by growing initial inocula of 500 cells to a final cell number of approximately 2 \times 10^8. Selections were performed by seeding four replicates of 5 \times 10^3 cells per 100-mm petri dish in medium containing 0.4 mM
AA and 5% dialyzed serum. To ensure the independence of each mutant, just one AA^R colony was picked for each initial culture.

Gamma-ray-induced mutants were collected by irradiating independent cultures containing \( 4 \times 10^6 \) cells with a dose of 5.0 Gy using a \(^{137}\text{Cs} \) source (0.696 Gy/min.) After exposure, the medium was changed and a 5-day phenotypic expression period was allowed. Azaadenine selections were performed as for spontaneous mutants; one AA^R clone was picked for each independently irradiated culture.

Parallel unexposed and gamma-irradiated cultures were subcultured immediately after treatment and were tested for relative survival by seeding appropriate numbers of cells in 100-mm petri dishes. Mutation frequencies were also determined for these cultures by seeding four 75-cm² flasks with \( 4 \times 10^6 \) cells each for phenotypic expression and subsequent selection in AA.

All spontaneous and induced mutants were screened in AA after propagation in nonselective medium to ensure stability of the mutant phenotype, and up to \( 5 \times 10^5 \) cells of each mutant strain were grown in AAT medium (\( 10^{-5} \) m adenine, \( 10^{-6} \) m aminopterin, \( 10^{-5} \) m thymidine) to confirm the inability of AA^R cells to utilize adenine as the sole purine source.

**Southern analysis of wild-type and mutant DNA:** High molecular weight DNA was isolated from our strains by suspending approximately 150 \( \times 10^6 \) cells in 10 mm Tris-HCl, pH 7.5, 0.1 m EDTA, 0.5\% (w/v) SDS, and 25 \( \mu \)g/ml proteinase K (Bethesda Research Laboratories). After a 4-h incubation at 37°, NaCl was added (final concentration 0.1 m), the sample was extracted twice with an equal volume of phenol chloroform (1:1) and the nucleic acids were precipitated at \(-70^\circ\) for 1 hr with two volumes absolute ethanol. The precipitate was dried in a vacuum dessicator and resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (TE) and was incubated for 4 hr at 37° after addition of 50 \( \mu \)g/ml RNAse A (Sigma). As above, NaCl was added and the sample extracted with phenol chloroform. The DNA was precipitated with ethanol, spooled out, dried and resuspended in TE.

Approximately 10-\( \mu \)g mutant or wild-type DNA were digested with various restriction enzymes under conditions specified by the supplier (Bethesda Research Laboratories). Restricted samples were electrophoresed on 1% (w/v) agarose gels, transferred to nitrocellulose and hybridized to a nick-translated probe as described by SOUTHERN (1975), with some modifications. Filters were prehybridized for 3 hr at 65° in 3 \( \times \) SSC (1 \( \times \) SSC is 0.15 m NaCl, 0.015 m trisodium citrate), 10 \( \times \) Denhardt’s (0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll 400), and then overnight at 65° in 3 \( \times \) SSC, 10 \( \times \) Denhardt’s, 0.1% SDS, 50 \( \mu \)g/ml sheared salmon sperm DNA and 10 \( \mu \)g/ml polyA. Filters were then hybridized overnight at 65° in heat-sealable plastic bags containing prehybridization solution plus 10% (w/v) dextran sulfate and a nick-translated (RIGBY et al. 1977) 3.8-kb genomic probe prepared after digestion of the pHaprt plasmid (LOWY et al. 1980) with BamHI and HaeII. Filters were washed in three changes of 3 \( \times \) SSC, 0.1% SDS, followed by three changes of 0.1% SSC, 0.1% SDS, all for 30 min at 65°. Filters were then air dried and exposed to Kodak X-Omat AR film with Dupont Cronex Lightning Plus intensifying screens at \(-70^\circ\) for 1–3 days.

**RESULTS**

Spontaneous AA^R mutants were collected by inoculating independent suspension cultures with 500 cells. These were incubated until the cell number had reached \( 2 \times 10^8 \), at which time they were reseeded for AA selection. For collection of gamma-ray-induced mutants, independent cultures containing \( 4 \times 10^5 \) D422 cells were exposed to 5.0 Gy of \(^{137}\text{Cs} \) gamma rays. The resultant cytotoxicity and induced mutagenesis are shown in Table 1. The surviving fraction was 0.16. The frequency of AA^R in gamma-irradiated cultures was \( 23 \times 10^{-6} \) and \( 1.8 \times 10^{-6} \) in unirradiated controls. After accounting for cytotox-
TABLE 1
Gamma-ray-induced cytotoxicity and mutagenesis

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>Relative survival</th>
<th>Mutation frequency</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>$1.8 \times 10^{-6}$</td>
</tr>
<tr>
<td>5.0</td>
<td>0.16</td>
<td>$23 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

Figure 1.—Southern analysis of some spontaneous APRT$^-$ mutants following digestion with PstI. Strains are identified by numbers at the top of each lane. Fragment sizes (kb) are indicated by numbers along the side. A lost PstI cleavage site is the most likely explanation for the restriction polymorphism observed in strain 107.

icity and induced mutation frequency, there was an average of 14.3 gamma-ray-induced mutants and 1.1 spontaneous AA$^R$ mutants in each independent irradiated culture. For each mutant clone selected, the stability of the APRT$^-$ phenotype was checked by plating on AA and AAT after propagation in nonselective medium.

Figure 1 shows a Southern analysis of some spontaneous AA$^R$ mutants of D422. DNA isolated from these strains was digested with PstI and probed with a $^{32}$P-labeled BamHI fragment isolated from the plasmic pHaprt (Lowy et al. 1980) that contains the entire 2.6-kb hamster aprt gene plus 1.2 kb of 3' flanking sequence (see Figure 3). Only 2 of 67 spontaneous mutants analyzed have a detectable alteration. Both mutants, 104 and 107, have identical modifications of the PstI digestion pattern: The loss of the two wild-type bands (3.8 and 2.3 kb) and the appearance of a single, larger band (digestion of
strain 107 DNA is shown in Figure 1). Since this new fragment is about 6.1 kb, or the sum of the two wild-type fragments, it is likely that the mutational event in these strains involves the loss of the PstI site. This alteration can be adequately explained by a single base substitution, although the involvement of a small deletion or insertion event cannot be excluded by this type of analysis.

Three of the gamma-ray-induced mutants (X-15, 26 and 43) share much similarity and can be regarded as a group. Each was considerably altered from wild type and behaved in a similar fashion in seven restriction enzyme analyses. These analyses (two are shown in Figure 2) clearly indicate a substantial deletion of genomic sequences which hybridize to the aprt probe. When digested with PstI (Figure 2a) both wild-type bands are gone, replaced by a single band slightly smaller than the 3.8-kb wild-type fragment. Double digestion with BglIII and HindIII (Figure 2b) results in a single 1.6-kb fragment in place of the single 4.3-kb wild-type band.

A restriction map was constructed specifically from the data collected for X-15 (Figure 3); very similar maps can also be constructed for mutants X-26 and X-43. Bars above the wild-type restriction map show the unaltered fragments. The breakpoint, as indicated, has been localized just upstream of the PvuII restriction site in the 3' portion of the gene. All of the restriction enzyme cleavage sites on the 5' side of the breakpoint have been deleted. In summary, we have observed three gamma-ray-induced aprt deletion mutants, all of which may be identical.

The remaining six gamma-ray-induced genomic alterations (X-13, 29, 35, 41, 69 and 73) have been classified as chromosomal rearrangements since events such as translocations or inversions must be invoked to account for the complex blotting patterns observed. PstI digestion of the DNA from these six strains (Figure 2a) produces three fragments detected by the aprt probe, rather than by the two seen in the wild-type gene. In each case the cumulative sizes of the fragments are greater than the wild-type gene, suggesting the occurrence of an insertion. Furthermore, since each mutant in this group has an extra PstI fragment, the existence of a novel PstI site is also indicated. Extensive restriction analysis of this group of mutants with several additional restriction enzymes (Figure 2) generally confirms the PstI results. However, the tentative conclusion of an insertion with a single restriction enzyme site is rendered untenable by the observation that the size estimates for the "insertions" in each strain vary widely depending on the restriction enzyme used in the analysis.

The data for one mutant, X-69, have been tabulated and are presented in Table 2 as a model for the entire group. In the case of X-69, the estimated size of the "insertion" varies from 0.125 to 7.9 kb depending on the enzyme chosen. No two estimates are similar. For all of the restriction enzymes used, there appears to be a single new site; the one exception is TaqI digestion, which results in only 0.125 kb of additional material hybridizing to the aprt probe. In the case of digestion with two restriction enzymes, there is still a single new cleavage site as in the case of single digestions.
FIGURE 2.—Southern analyses of some gamma-ray-induced APRT<sup>−</sup> mutants with several restriction enzymes. Enzymes used for each individual analysis are as follows: a, PstI; b, double digestion with BglII and HindIII; c, BamHI; d, double digestion with BamHI and EcoRI. Strains are indicated by numbers at the top of each lane: D422 is wild type; 15, 26 and 43 have been classified as intragenic deletions; and 13, 29, 35, 41, 69 and 73 have been classified as chromosomal rearrangements. Numbers along the side indicate fragment size in kilobases. In (a) the wild-type fragments are 2.3 and 3.8 kb and in (b) the single wild-type fragment is 4.3 kb. Chromosomal rearrangement strains can be seen to have one additional fragment in each digestion as compared to the wild type. Strain X-13 has a small faint band following digestion with BamHI and with Bgl/Hind; the presence of each has been confirmed in repeat experiments and with longer autoradiographic exposures. A band that runs at a high molecular weight position is seen in each lane in (c) and (d). These bands have not been detected in repeat experiments.
FIGURE 3.—Restriction map of hamster aprt locus showing alterations detected in deletion mutant X-15. The wild-type restriction map shown confirms that of Lowy et al. (1980) and Nalbantoglu, Goncalves and Meuth (1983). An MspI site, identified from DNA sequencing data (A. J. Grosovsky et al., unpublished results), has been added at the 5' end of the gene immediately next to the BamHI site. The solid line indicates the 3.8-kb BamHI fragment that is used as the probe in our Southern analyses. Wavy lines represent the flanking hamster genomic DNA. Numbers indicate distance in kilobases. Bars above the wild-type restriction map indicate unaltered restriction fragments and restriction enzyme cleavage sites in X-15. The site of the breakpoint is as indicated; all of the locus on the 5' side of the breakpoint has been deleted.

TABLE 2
Analysis of genomic rearrangement in APRT mutant X-69

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>PvuII</td>
<td>3-kb insertion with one PvuII site</td>
</tr>
<tr>
<td>PstI</td>
<td>6-kb insertion with one PstI site</td>
</tr>
<tr>
<td>TaqI</td>
<td>0.125-kb insertion with no TaqI site</td>
</tr>
<tr>
<td>MspI</td>
<td>4.2-kb insertion with one MspI site</td>
</tr>
<tr>
<td>BamHI/XhoI</td>
<td>7.9-kb insertion with one site</td>
</tr>
<tr>
<td>BamHI</td>
<td>6.9-kb insertion with BamHI site</td>
</tr>
<tr>
<td>HindIII/BglII</td>
<td>0.9-kb insertion with one site</td>
</tr>
</tbody>
</table>

The restriction map for X-69 constructed from this data is shown in Figure 4. The genomic fragments which were found to be unaltered are indicated by bars above the wild-type restriction map. Both the 5' and 3' ends of the gene remain unaltered; the rearrangement involves the central portion of the gene.

The consequence of a chromosomal alteration involving one breakpoint within the aprt gene would be that each resulting fragment becomes joined to a novel DNA sequence. There would always be a restriction enzyme cleavage site in the new neighboring DNA regardless of which enzyme was utilized. The resultant restriction pattern would be an apparent insertion with one new cleavage site, although the size of the “insertion” would depend on the enzyme used and the location of the proximal cleavage site. This pattern would also be expected in double digestions for which the first site for either enzyme would represent the proximal cleavage site. Our analysis of this group of mutants (X-13, 29, 41, 69 and 73) has produced the pattern predicted for chromosomal rearrangements and is incompatible with a simple local event.
FIGURE 4.—Restriction map of chromosomal rearrangement mutant X-69. Bars above the wild-type restriction map indicate unaltered restriction fragments and restriction enzyme cleavage sites. The breakpoint for this chromosomal rearrangement has been localized to some position between the HincII and EcoRI restriction sites.

TABLE 3
Southern analysis of azaadenine-resistant (APRT⁺) mutants

<table>
<thead>
<tr>
<th></th>
<th>Total no. analyzed</th>
<th>No alterations detected</th>
<th>Classes of alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous mutants</td>
<td>67</td>
<td>65 (97%)</td>
<td>2 (3%)†</td>
</tr>
<tr>
<td>γ-Ray-induced mutants</td>
<td>55</td>
<td>46 (83.6%)</td>
<td>3 (5.5%)†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 (10.9%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9 (16.4%)</td>
</tr>
</tbody>
</table>

* Loss of restriction site (PstI).
† Deletions.
‡ Rearrangements.

A summary for all of the spontaneous and gamma-ray-induced mutants analyzed is presented in Table 3. No large genomic rearrangements were detected among 67 spontaneous mutants; the two observed cases of restriction site polymorphisms (3% of the total) were classified as restriction site losses. These findings are in contrast to an earlier report (NALBANTOGLU, GONCALVES and MEUTH 1983) in which deletions or insertions were detected in 13 of 127 APRT⁻ mutants. Among the 55 gamma-ray-induced mutations analyzed, nine (16.4%) had detectable genomic alterations; three of these (5.5%) were classified as intragenic deletions and six (10.9%) represented chromosomal rearrangements.

DISCUSSION

The results presented here show that approximately one in six gamma-ray-induced APRT⁺ mutants are attributable to a genomic alteration involving the aprt locus. Evidence is also presented that enables us to classify these alterations as either intragenic deletions or chromosomal rearrangements. One earlier report (GRAF and CHASIN 1982) found genomic alterations in two of nine gamma-ray-induced, dihydrofolate reductase mutants of mouse cells. Although the nature of these alterations was not well understood, the percentage of
mutants with rearrangements is in good agreement with the larger sample presented here.

Six genomic alterations were observed which were classified as chromosomal rearrangements since the restriction analysis data is incompatible with simple local modifications, such as insertions, but is consistent with the pattern predicted for such events as translocations or inversions. Ionizing radiation is well known as a potent clastogen (BEIR Report 1980), and one study (COX and MASSON 1978) has reported that up to 40% of X-ray-induced HPRT- human fibroblast mutants had a cytologically detectable chromosomal aberration consistent with the mapped position of the hprr locus.

The failure to observe complete deletions of the aprt locus or any deletion extending into the 3′ flanking sequence may be due to the presence of an essential gene in this hemizygous region. The three intragenic deletions which were observed appear to have identical breakpoints. This may suggest a sequence specific susceptibility to gamma-ray-induced DNA strand breakage and consequent deletions.

In view of the evidence presented here for sequence specificity in gamma-ray-induced deletions, sequence dependence in chromosomal rearrangements may also be considered. This possibility is consistent with cytological detection of X-ray-induced translocation hotspots in human fibroblasts (KANO and LITTLE 1985). The human cancer Burkitt’s lymphoma is characterized by a specific translocation involving the c-mye oncogene and the immunoglobulin gene complex, which has been extensively studied from the cytogenetic to the DNA sequence level (reviewed in VARMUS 1984). No evidence has been found for sequence specificity at the chromosomal breakpoints in these translocations. Similar studies have not, as yet, been reported for any mutagen-induced chromosomal rearrangements.

Mutants which were not found to contain genomic alterations may either be point mutations or may have deletions or insertions too small to be detected by Southern analysis. In view of the large number of mutants in this group (83.6% of the total), it is quite surprising that ionizing radiation is unable to induce ouabain resistance. This may reflect a very small target for ouabain resistance that, for sequence specificity reasons, is refractory to ionizing-radiation-induced, base substitution mutagenesis. Ionizing radiation is known to cause base substitution mutagenesis in E. coli (ISE, KATO and GLICKMAN 1984; GLICKMAN, RIETVELD and AARON 1980) and in the K-ras oncogene (GUERRERO et al. 1984). A DNA sequence analysis of our collections of spontaneous and gamma-ray-induced mutants is currently under way in our laboratory.

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