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Direct Demonstration of Genetic Alterations at the Dihydrofolate Reductase Locus After Gamma Irradiation

LLOYD H. GRAF, JR.† AND LAWRENCE A. CHASIN*
Department of Biological Sciences, Columbia University, New York, New York 10027

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Gamma ray-induced mutants of Chinese hamster ovary cells lacking dihydrofolate reductase activity were screened for DNA sequence changes at the locus specifying this activity by using a cloned cDNA probe. Two of nine mutants screened displayed an altered restriction fragment pattern suggesting the occurrence of DNA deletions or rearrangements.

A major genetic effect of ionizing radiation on mammals is thought to be the induction of gross chromosomal changes such as breaks and deletions (3). Most of the evidence for deletions has come from specific locus tests in mice (1, 17, 19). More recently, several studies of cultured mammalian cells have shown that radiation-induced mutations at a given locus can be associated with specific cytogenetic aberrations (6) and can be multilocus in nature (11, 24). The precise definition of mutational lesions that are inducible in cultured mammalian cells requires both the ability to select specific-locus mutants and the ability to locate the lesion by fine-structure genetic mapping. A system that meets these two requirements is the locus specifying dihydrofolate reductase (DHFR) in Chinese hamster ovary (CHO) cells. Mutants of these cells lacking DHFR activity can be isolated by virtue of their resistance to tritiated deoxyuridine suicide (21). Physical mapping of DNA sequence changes at this locus should be possible by using nucleic acid hybridization to probes specific for this gene. In the work reported here, we used a cloned cDNA containing most of the sequence of mouse DHFR mRNA (pDHFR21) (5) as a probe for possible mutational changes in the Chinese hamster genomic *dhfr* sequences.

The mouse cDNA sequence contains sufficient homology to the Chinese hamster genes to allow its use as a probe. This finding was demonstrated in previous work characterizing amplification of the *dhfr* gene in a methotrexate-resistant CHO cell mutant (MK42) (14). Figure 1 shows the results of blot hybridization experiments displaying *Hin*dIII restriction fragments of total CHO cell DNA separated by agarose gel electrophoresis. Sequences with coding information for DHFR· mRNA were detected by using radioactively labeled plasmid DNA containing the cloned mouse cDNA sequence. In lane 1 of Fig. 1, four bands are readily demonstrable; here we used DNA from CHO mutant MK42, in which the *dhfr* gene has been amplified 150-fold relative to wild-type cells (14). The summed size of these four fragments was 36 kilobases (kb). Five other hexanucleotide-specific restriction enzymes (*Bgl*II, *Kpn*I, *Eco*RI, *Bam*HI, and *Xba*I) yielded similar results, with summed sizes ranging from 14 to 27 kb (data not shown). These results suggest the CHO cell *dhfr* gene, like the mouse gene (13), is very large relative to the structural information needed to specify the enzyme (ca. 500 to 600 base pairs).

Wild-type (nonamplified) hamster *dhfr* sequences could also be detected by using the mouse probe. The intensity of the bands (Fig. 1, lane 3) was considerably less than that obtained with homologous mouse wild-type DNA (Fig. 1, lane 2), presumably due to the inefficiency of cross-hybridization. In addition to the four bands of approximately 23, 8.5, 3.9, and 1.4 kb found in amplified DNA (Fig. 1, lane 1), there was a fifth band of 5.8 kb present in CHO-K1 DNA and all other nonamplified clones tested (see below). There are several possible explanations for this fifth band. It could represent a cross-reacting sequence from another locus, perhaps evolutionarily related. Alternatively, it could represent part of the *dhfr* gene that was not amplified in clone MK42. A third possibility is that the *dhfr* locus in CHO cells was both diploid and heterozygous with respect to size or *Hin*dIII cutting sites and that only one allele was amplified in clone MK42. We previously presented evidence for diploidy at this locus in CHO-K1 cells (21), although some contrary evidence exists in another CHO subline (10). There is also both cytogenetic (7, 14) and biochemical (2, 9, 12) evidence that only a single *dhfr* gene becomes amplified in any given clone. It is interesting that another independently isolated DHFR-overproducing CHO mutant (9) displays

† Present address: Laboratory of Cell Genetics, New York Hospital-Cornell Medical Center, New York, NY 10021.

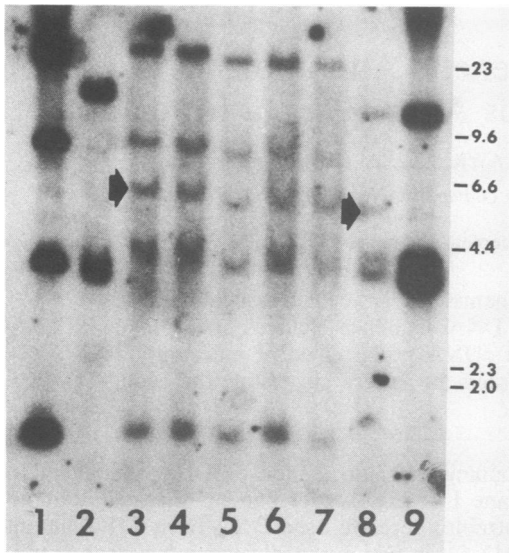

FIG. 1. Blot hybridization of genomic DNA restricted with *Hin*dIII (lanes 1 through 7) or *Bgl*II (lanes 8 and 9). DNA was purified (15) and subjected to restriction enzyme digestion as described by the supplier (Bethesda Research Laboratories or New England Biolabs). Either 10 μg (lanes 1, 2, and 9) or 30 to 40 μg (lanes 3 through 8 and 10) of the digests were applied directly to a 1-cm-thick 0.7% horizontal agarose gel. Electrophoresis (15) was carried out until the bromophenol blue marker reached the end of the gel. After the gel was stained with ethidium bromide and the fluorescent DNA was photographed, the gel was blotted onto a nitrocellulose filter membrane by the method of Southern (20) as modified by Wahl et al. (22) to include partial depurination and hydrolysis for improving transfer of large fragments. Filters were blocked, hybridized to ^{32}P-labeled plasmid pDHFR21 (5) DNA, and washed by a combination of previously described methods (18, 23, 26). Hybridization was carried out in sealed plastic bags with 10^6 cpm of probe per ml and 0.1 ml per cm^2 of filter material. The pDHFR21 plasmid DNA (kindly provided by R. Axel and S. Silverstein) was labeled by nick translation (16) to a specific activity of 200 to 500 cpm/pg. *Hin*dIII digestion: lane 1, MK42 (amplified mutant) DNA; lane 2, mouse L-cells; lane 3, CHO-K1 (wild type); lane 4, UKB25 (heterozygote); lanes 5 through 7, DUK22, DUK51, DXBA—three DHFR-deficient mutants. *Bgl*II digestion: lane 8, CHO-K1; lane 9, MK42. The arrows indicate restriction fragments found in wild-type DNA but not amplified in the methotrexate-resistant mutant MK42. *Hin*dIII fragments of lambda DNA were used as molecular weight (in kb) markers.

amplification of the same four *Hin*dIII fragments amplified in MK42 (data not shown) (25). All of these explanations predict the presence of the fifth band in single-copy amounts in the DNA from the amplified mutants. In fact, a faint 5.8-kb band was usually discernible (Fig. 1, lane 1). An extra band in nonamplified DNA was also found after digestion with *Bgl*II (Fig. 1, lanes 8 and 9), *Bam*HI, and *Eco*RI (data not shown).

Isolation of DHFR-negative mutants from CHO-K1 required the selection of a putative *dhfr* heterozygote in which one of the two genes had been rendered nonfunctional (21). This clone, designated UKB25, contains half of the DHFR activity of wild-type cells and can give rise to mutants completely devoid of DHFR activity at high frequency (21). Lane 4 of Fig. 1 shows that the mutation leading to the partial DHFR deficiency in the heterozygote UKB25 did not alter the gene structure detectable by comparing *Hin*dIII fragments. This finding is not surprising, since UKB25 was isolated after treatment with ethyl methanesulfonate (EMS), a base substitution mutagen, and since the probability of a single-base change occurring in a given restriction site is very small.

Presumed double mutants lacking all DHFR activity have been obtained, starting with the heterozygote UKB25 (21). The *Hin*dIII restriction patterns produced by two EMS-induced mutants are shown in lanes 5 and 6 of Fig. 1. Again, as expected, the mutations had no obvious effect on gene structure, as determined by this criterion. However, in two of nine mutants induced by gamma irradiation, alterations in the *dhfr* *Hin*dIII pattern were apparent. Mutant DXE11 displayed a new 7-kb *Hin*dIH fragment (Fig. 2, lanes 5 and 6), and mutant DXC11 displayed a new faint but reproducible band of 16 kb (Fig. 2, lanes 9 and 10). New restriction fragments were also observed for these two mutants upon digestion of DXE11 DNA by *Eco*RI and of DXC11 DNA by *Bam*HI (data not shown).

The appearance of a new band in *Hin*dIII digests of either DXE11 or DXC11 DNA was not accompanied by the disappearance of any of the original *dhfr* *Hin*dIII fragments. This finding supports the idea of diploidy at the *dhfr* locus. The gene copy which bears the first EMS-induced mutation remained present; it contributed an unaltered *Hin*dIII pattern. The gross alteration induced by gamma irradiation in the second allele gave rise to a new banding pattern that was superimposed on that of the first allele.

Although single-base changes cannot yet be ruled out in this preliminary study, the most likely explanation for the alteration in restriction pattern is deletion, insertion, or inversion at the *dhfr* locus. The precise definition of the mutational lesions will require homologous probes for the Chinese hamster *dhfr* gene. The construction of the probes is in progress.

The absence of detectable changes in 7 of 9 gamma ray-induced mutants analyzed (e.g., Fig. 1, lane 7 and Fig. 2, lanes 7 and 8) did not indicate that multinucleotide changes did not

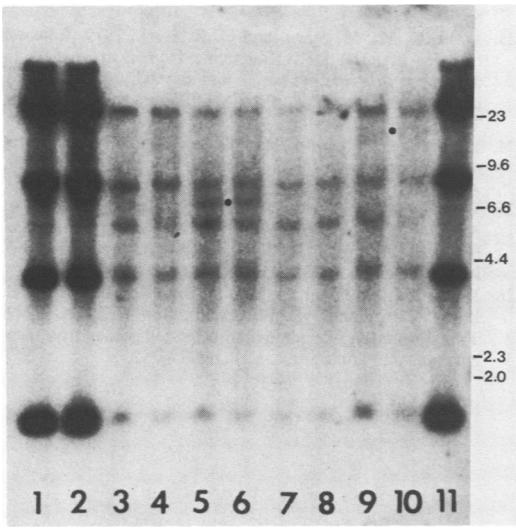

FIG. 2. Altered genomic *Hin*dIII restriction fragments in gamma ray-induced, DHFR-deficient mutants. Blot hybridization procedures are described in the legend to Fig. 1. Lanes 1 through 10 represent five pairs of duplicate DNA samples in which the second sample was digested with five times more enzyme than the first. Lanes 1, 2, and 11, MK42 (amplified mutant); lanes 3 and 4, UKB25 (heterozygote); lanes 5 and 6, DXE11; lanes 7 and 8, DXB11; lanes 9 and 10, DXC11 (three gamma ray-induced DHFR-deficient mutants). The dots indicate the new bands that appeared in two DHFR-deficient mutants.

occur. For example, the deletion of hundreds of base pairs from the 22-kb *Hin*dIII fragment would not have been detected in these experiments. Also, it is likely that there were restriction fragments from the *dhfr* gene which escaped detection because they either contained no exon sequences or contained exon sequences that failed to hybridize with the mouse cDNA probe.

Direct evidence for mammalian gene DNA sequence changes resulting from experimental mutagenesis has previously been reported only for immunoglobulin genes in immunocytes (8; S. L. Morrison and M. D. Scharff, CRC Crit. Rev. Immunol., in press). Deletion of genetic material at this locus in somatic cells is a regular developmental occurrence (4) that probably involves special mechanisms and hence may be a poor general model for mutational change. The changes described here represent direct evidence for induced mutations in perhaps a more typical mammalian gene. This system should be useful for the study of mutational mechanisms and gene structure-function relationships in these cells.

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