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DNA-Mediated Transfer and Expression of a Human DNA Repair Gene That Demethylates O^6-Methylguanine

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Human liver DNA was transfected into CHO cells (*mex*$^-$) along with pSV2gpt and colonies were selected first for resistance to mycophenolic acid and then to chloroethylnitrosourea. Transformants were obtained that contained approximately 10,000 molecules of O^6-alkylguanine alkyltransferase (*mex*$^+$) per cell. Their genome contained at least three copies of the human *Alu* sequence.

Alkylation of DNA at the O^6 position of guanine represents a potent mutagenic and carcinogenic lesion. In *Escherichia coli*, O^6-methylguanine is repaired by an 18-kilodalton protein, O^6-alkylguanine alkyltransferase, that directly transfers the methyl group in stoichiometric manner to a cysteine residue on the protein, with resultant suicide inactivation (12). A similar alkyltransferase mechanism has been identified in mouse (1), rat (11), and human livers (13). Cells that express this repair activity have been defined as *mex*$^+$ (16). It has been proposed that in *E. coli* the alkylated protein acts to induce an adaptive response that is manifested as enhanced resistance to mutation resulting from increased alkyltransferase and enhanced resistance to toxicity (8). The latter resistance has been attributed to enhanced production of another DNA repair enzyme, 3-methyladenine glycosylase(II), which removes both 3-methyladenine and 3-methylguanine; it is 3-methylguanine that has been postulated as the critical toxic lesion (8). It has recently been demonstrated with *E. coli* that the alkyltransferase is a fragment of the 37-kilodalton *ada* gene protein that regulates the adaptive response (18).

An adaptive response in mammalian cells was first reported for CHO cells (15). Pretreatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) resulted in enhanced survival when the cells were subsequently challenged with the same agent. Resistance to mutation was not studied, but these cells, which do not possess any alkyltransferase, i.e., *mex*$^-$ (5), would not be expected to alter their mutation frequency. A subsequent study with rat hepatoma cells demonstrated enhanced survival and resistance to mutagenicity (7). To understand the regulation of this adaptive response, we started to isolate the genes involved. We took advantage of the fact that cellular resistance to 1-(2-chloroethyl)-1-nitrosourea (CNU) is mediated by the *mex* protein, which repairs monofunctional alkylation products at the O^6 position of guanine before they can rearrange to produce DNA interstrand crosslinks (3, 19, 23). We transfected DNA from human liver (*mex*$^+$) (13) into the *mex*$^-$ CHO cells (5) and selected for CNU resistance. We report here the successful strategy used to obtain expression of the human alkyltransferase gene in CHO cells.

CHO cells, apart from being *mex*$^-$, are also excellent recipients for introduction of foreign DNA, having previously been used in similar studies of the repair genes for UV light-induced DNA damage (9, 14, 20). The strategy for the isolation of the human repair gene is shown in Fig. 1. At 24 h before transfection, 5×10^5 CHO-D422 cells were plated into each of 60 100-mm culture dishes containing 10 ml of α minimal essential medium (α-MEM) supplemented with penicillin, streptomycin, 2.5% dialyzed horse serum, and 2.5% dialyzed fetal bovine serum. High-molecular-weight DNA was purified from human liver and sheared to an average fragment size of 40 to 50 kilobase pairs by four passages through a 20-gauge needle, and 20 μg of DNA per culture dish was transfected into CHO cells as a calcium phosphate precipitate (21). A marker gene, bacterial xanthine-guanine phosphoribosyltransferase (*gpt*), in the form of the plasmid pSV2gpt (5 μg), was simultaneously cotransfected. This cotransfection protocol derives from the observation that selection for a marker gene present in excess enriches for a subpopulation that is competent for the stable integration of exogenous DNA (22). This approach also reduces the possibility of selection of an alkyltransferase repair-proficient CHO revertant. The CHO cells were incubated with the calcium phosphate precipitate for 24 h, after which the medium was removed and the cells were treated with 15% glycerol for 2 min. Fresh growth medium was added, and 1 day later the cells were trypsinized and replated in T-150 flasks containing α-MEM plus 25 μg of mycophenolic acid per ml, 25 μg of adenine per ml, and 250 μg of xanthine per ml. The medium was replaced 3 days later. When *gpt*$^+$ colonies contained about 50 to 100 cells, they were trypsinized, replated, and incubated for 1 h with 60 μM CNU (Developmental Therapeutics Program, National Cancer Institute). This leads to 1% survival of the cells. The surviving cells were grown to 50% confluence, replated, and incubated with 90 μM CNU. After the survivors reached 50% confluence they were again replated and incubated with 120 μM CNU. A total of six colonies were detected in one culture dish. Of these colonies, one was selected and grown for subsequent study.

Based on the number of cells initially seeded before transfection, the frequency of *gpt*$^+$ transformation was calculated as approximately 10^{-4}. As a test for genetic stability, CHO *gpt*$^+$ cells were grown for 8 weeks in the absence of selection pressure and then were reselected in mycophenolic acid-containing medium. The transformants retained about 50% plating efficiency. A similar rescue rate was observed when 500 CHO *gpt*$^+$ cells were seeded together with 5×10^5 wild-type CHO cells.

The ability of the selected cell to survive in CNU was compared with that of wild-type CHO cells. One day before drug treatment, 500 to 50,000 cells were plated in 60-mm culture dishes. Appropriate dilutions of CNU (dissolved at

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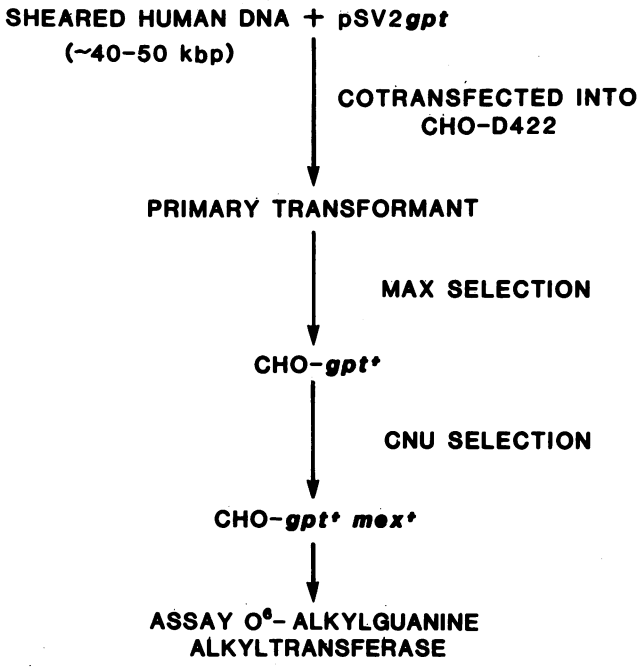

FIG. 1. Strategy for isolation of the human O^6-alkylguanine alkyltransferase gene.

10 mM in 95% ethanol immediately before use) were added to cultures containing serum-free α-MEM and incubated for 1 h at 37°C. After replacement with fresh medium, the cells were incubated at 37°C until colonies were large enough to be stained and counted. Almost complete survival of the resistant cells was observed at concentrations of CNU that kill most of the wild-type cells (Fig. 2A). To verify that this resistance was due to expression of alkyltransferase, the cells were incubated with 2 μM MNNG (1 mM stock in 95% ethanol) for 1 h, a procedure known to saturate the alkyltransferase (23). Survival after a subsequent incubation in CNU demonstrated that the pretreatment had caused reversion of the resistant phenotype (Fig. 2A).

To confirm the expression of alkyltransferase, a crude cell extract was prepared from the transformants as follows. A total of 3×10^8 cells were suspended in 1.5 ml of TEDG (50 mM Tris hydrochloride [pH 7.8], 1 mM disodium EDTA, 5 mM dithiothreitol, 5% glycerol, 500 μM phenylmethylsulfonyl fluoride) and were sonicated. NaCl to 0.5 M was added to dissociate the protein from the DNA, and the sample was centrifuged at 15,000 × *g* for 10 min. The supernatant was dialyzed against TEDG, and activity was assayed with [^{3}H]CH_3-DNA containing 1 pmol of O^6-methylguanine as described previously (1). No detectable alkyltransferase activity was observed in the wild-type CHO cells. The extract from the CNU-resistant cells, however, removed 63 fmol of O^6-methylguanine per ng of extracted protein. This represents approximately 6,000 molecules of alkyltransferase per cell, assuming that each molecule demethylates only one O^6-methylguanine molecule. In parallel experiments with similar preparations from rat liver, generally about 60% of the activity present in tissue homogenates was recovered by this simple extraction procedure. Correcting for this recovery, we calculated that the resistant cells would have about 10,000 molecules per cell. The equivalent values for HeLa and NRK cells are 100,000 and 20,000 molecules per cell, respectively (4). Human lymphoid cell lines exhibit 10,000 to 25,000 molecules per cell (6). Based upon this evidence of expression of alkyltransferase, the transformed cells were designated CHO *mex*$^+$.

Human cells have previously been designated *mer*$^+$ or *mer*$^-$, depending on whether they are able to reactivate virus that has been damaged by MNNG (2). All *mer*$^+$ cells were resistant to MNNG-induced toxicity and were proficient for repair of O^6-methylguanine. This lesion was therefore considered lethal if unrepaired. We therefore tested our CHO

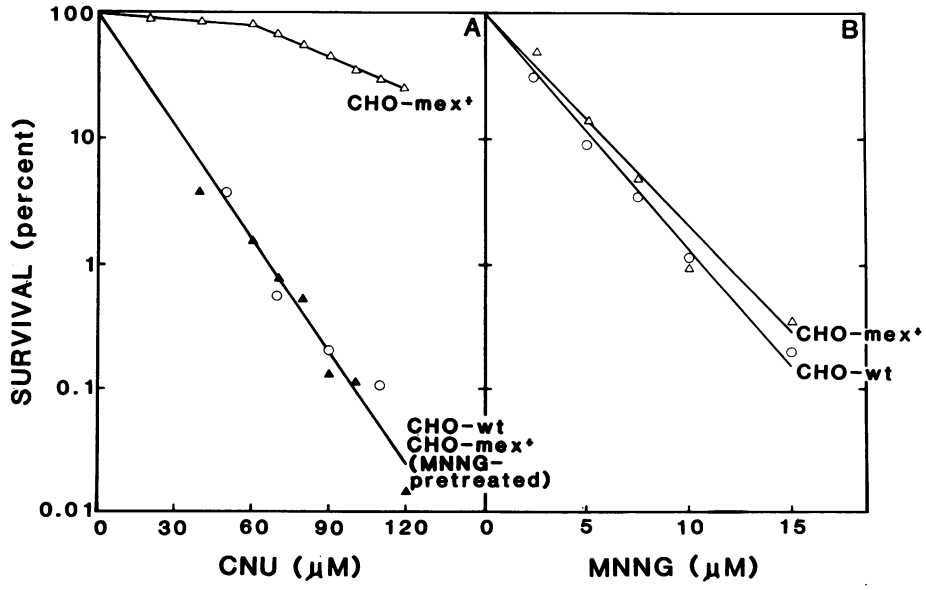

FIG. 2. Survival of CHO wild-type (○) and CHO *mex*$^+$ cells (△) after treatment with CNU (A) and MNNG (B). CHO *mex*$^+$ cells were also pretreated with 2 μM MNNG before examining survival in CNU (▲).

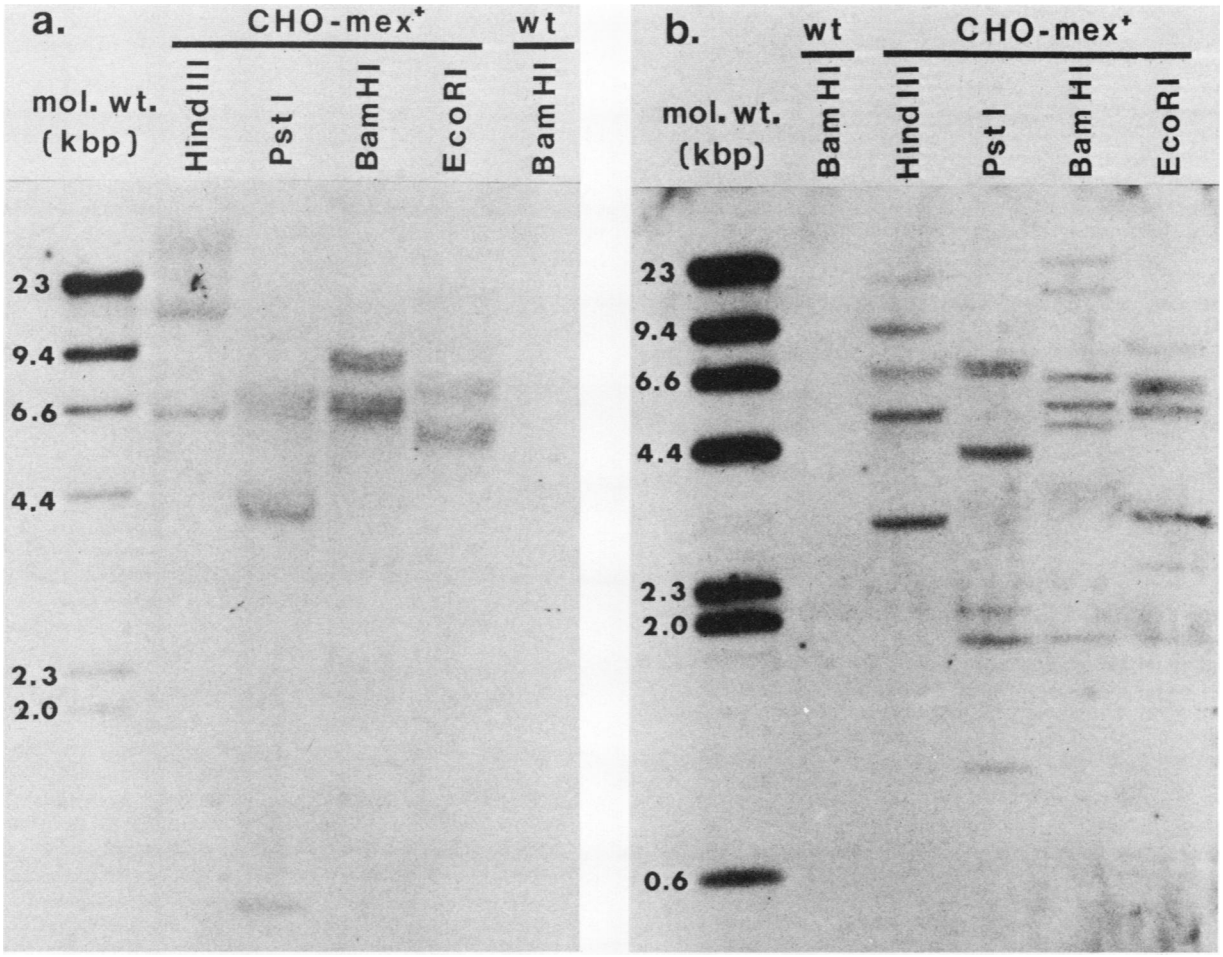

FIG. 3. Gel electrophoreses of CHO *mex*$^+$ DNA hybridized to ^{32}P-nick-translated human repetitive *Alu* sequence (a) or ^{32}P-nick-translated pSV2gpt DNA (b). CHO wild-type DNA (wt) was also electrophoresed to demonstrate lack of hybridization to this DNA.

mex$^+$ transformant for resistance to MNNG. The introduction of the *mex* gene did not affect the response of the cells to MNNG (Fig. 2B). This suggests that O^6-methylguanine is not a toxic lesion in mammalian cells, a situation analogous to that in *E. coli* (8). In addition, *mer*$^+$ human cells are also resistant to the toxicity induced by methyl methanesulfonate, an alkylating agent that produces only very low levels of O^6-methylguanine (2). The terms *mer* and *mex* have often been used interchangeably. The results here, however, show that they describe different phenotypes, thereby posing the question as to which alkylation derived from methylating agents represents the toxic lesion(s), if not O^6-methylguanine. In *E. coli*, either 3-methylguanine (8) or methylphosphotriesters (10) may be toxic. The enzymes for repair of both of these lesions are also induced as part of the adaptive response. Repair of methylphosphotriesters is, in fact, a property of the 37-kilodalton *ada* gene product (18).

In addition to the biological evidence presented above, molecular evidence is required to establish that the *mex*$^+$ phenotype is due to the integration of human DNA. High-molecular-weight DNA was purified from the CHO *mex*$^+$ cells, 20 μg was digested with several restriction endonucleases for 3 h at 4 U/μg of DNA and for a further 3 h with an additional 2 U/μg of DNA. The fragments were separated by electrophoresis overnight on a 0.8% agarose gel. After Southern transfer to nitrocellulose (17), the DNA was hybridized to a ^{32}P-nick-translated pBlur 8 probe containing the human *Alu* sequence. Upon autoradiography, up to 3 restriction fragments were observed that specifically hybridized to the human DNA probe (Fig. 3). Parallel restriction endonuclease digestions of CHO *mex*$^+$ DNA were hybridized to ^{32}P-nick-translated pSV2gpt DNA. Up to 6 restriction fragments hybridized to this probe (Fig. 3). As each of the restriction endonucleases cuts within the plasmid DNA, this suggests that CHO *mex*$^+$ cells contain a minimum of 3 copies of the *gpt* gene. After a single round of DNA-mediated transfection as performed here, it is common to see many more fragments that hybridize to *Alu* DNA. We can use this phenomenon to advantage since the presence of so few positive fragments should obviate the need for a second round of transfection for further enrichment of the *mex* gene. The gene can probably be isolated directly from these cells.

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