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# EXPRESSION OF E. coli tag GENE ENCODING 3-METHYLADENINE GLYCOSYLASE I IN NIH-3T3 MURINE FIBROBLASTS

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NIH-3T3 fibroblasts were co-transfected with pSV2neo and pSG5tag by the calcium-phosphate precipitation method.The stable integration of the tag gene sequence and its transcription was verified by Southern and Northern blot analysis. 3-Methyladenine glycosylase activity in pSG5tag transfected 3T3 cells was approximately 400 times higher than in cells transfected with the control plasmid pSG5 or in the parental cells and was inhibited by 3-methyladenine. Bacterial tag gene can thus be expressed in mammalian cells and the encoded enzyme is functionally active. These transfected cells could serve as an important tool to investigate the importance of the repair of N3-adenine as a mechanism of protection against the mutagenicity and cytotoxicity of alkylating agents. 

3-Methyladenine (3-mAde) is one of the major DNA base modifications produced by alkylating agents or by the natural methyl donor S-adenosyl-methionine (1,2). Prokaryotic and eukaryotic cells contain various DNA repair enzymes to protect against DNA alkylation; DNA glycosylases that remove 3-mAde residues have been isolated from bacterial, yeast and mammalian sources (3-6). Two enzymes have been characterized in E. coli: 3-mAde glycosylase I (Gly I) is the constitutive enzyme encoded by the tag gene and removes only 3-mAde residues (7,8 ); 3-mAde glycosylase II (Gly II) is the inducible enzyme encoded by the alk A gene during an adaptive response (9-11). Gly II liberates 3-methylguanine, 7-methylguanine, O2-methylthymine and O2-methylcytosine in addition to 3-mAde residues. There is evidence that 3-mAde residues block DNA synthesis (12) and induce mutation in bacterial cells (13) and alkylation-induced death in yeast cells (14). However there is no evidence of the lethal or mutagenic effects of the 3-mAde lesion in the DNA of mammalian cells or of an in vivo protective role of mammalian 3-mAde-DNA-glycosylase.

A useful approach for evaluating the protective role of DNA repair enzymes involves the stable overexpression of DNA repair bacterial genes in mammalian cells (15). This study investigated whether the bacterial tag gene can be expressed in mammalian cells transfected with the gene and if the enzyme is functionally active in these cells.

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## MATERIALS AND METHODS

*Vector construction*
The E. coli tag gene cloned in pUC8 (the plasmid pCY5 was a generous gift from Dr. M. Sekiguchi; ref. 7) was recloned into pSG5 and thereby brought under the control of the early SV40 promoter. In brief, the tag gene was excised from plasmid pCY5 by Eco RI and Hind III, blunt ended and inserted into the previously Eco RI digested blunt ended pSG5 vector. The resulting plasmid was named pSG5tag.

*Cell culture*
NIH 3T3 cell line was grown in Dulbecco's Minimal Essential Medium (D-MEM) (GIBCO) supplemented with 10% fetal bovine serum, 5.3% sodium bicarbonate and 1% L-glutamine. The transfectant lines were selected in D-MEM containing 500 μg/ml G418 (GIBCO); the G418-resistant clones were maintained in the same medium supplemented with 200 μg/ml G418. All the cultures were maintained in incubators at 37℃ with 5% $CO_2$.

*Transfection and selection procedures*
For stable transfection, NIH 3T3 cells were plated at a density of $5x10^5$/100 mm dish; 24 hours later pSG5tag (20 μg/ dish) or pSG5 (20 μg/dish) and pSV2neo (5 μg/ dish) were introduced into the cells by the $CaPO_4$-DNA precipitation procedure (16); 24 hours after the addition of 1 ml of DNA co-precipitate to each 100 mm dish containing 10 ml of D-MEM, the medium was replaced and cells were allowed to grow for 48 hours. Then the cells were replated with a density of $5x10^4$/100 mm dish; 24 hours after plating, the medium on all dishes was replaced with selective medium containing 500 μg/ml G418. This medium was changed every three days for a total of four treatments. Resistant clones were picked using glass cloning cylinders for growth into mass culture.

*Southern and Northern blot hybridization*
Twenty μg of NIH 3T3 DNA were digested with EcoRI and subjected to 1% agarose gel electrophoresis. Ten μg of total RNA from NIH 3T3 cells were subjected to agarose gel electrophoresis in the presence of 6.7% formaldehyde. Nucleic acids were transferred to nylon membranes (Gene Screen Plus, New England Nuclear) using capillary transfer. RNA and DNA were hybridized with the random primed $^{32}$P-labeled EcoRI/Hind III 0.8kb fragment of the plasmid pCY5 (according to the manufacturer's protocol, Amersham). The membranes were hybridized to the probe for 16 hours, washed sequentially in 2xSSC at room temperature and in 2xSSC/1% SDS at 65℃ and then autoradiographed. RNA filters were rehybridized, after stripping, with the random primed $^{32}$P-labeled 1.8 Kb Pst I insert of the murine actin gene.

*Cell extract preparation*
Crude cell extracts were obtained by sonication (5x10 s) of the cellular pellets resuspended ($70x10^6$ cells/ml) in 50 mM TRIS-HCl buffer (pH 7.5 at 4℃) containing 1mM DTT, 0.1 mM EDTA, 0.1 mM MPSF. After centrifugation (1000xg/10 min) to remove cellular debris, the supernatants were collected and stored at -80℃ until enzymatic activity was determinated. Total protein concentration was determined by the Bradford dye-binding assay ( Bio-Rad).

*3-Methyladenine-DNA glycosylase (3mAG) assay*
Activity of 3mAG in the crude extracts was evaluated as previously reported (17). Briefly, aliquots of $^3$H-methylated DNA substrates, freshly preparated according to Wiestler et al. (18) containing about 1 pmol of 3-methyladenine, were incubated at 37℃ for variable intervals with crude extracts (0.1-0.2 mg protein) in a constant incubation volume of 140 μl. At stated times, reactions were stopped in ice and, after addition of the internal standard, DNA was precipitated by NaCl/Ethanol. Supernatants were then collected, dried, redissolved and the $^3$H-methyladenine content was analyzed by radiochromatographic HPLC. Similar samples were incubated without protein extracts in order to account for spontaneous depurination.

## RESULTS

The 800 base-pairs EcoRI-Hind III insert of pCY5 (7) was inserted into unique EcoRI cloning site of pSG5 (19) in the appropriate transcriptional orientation to yield pSG5tag.

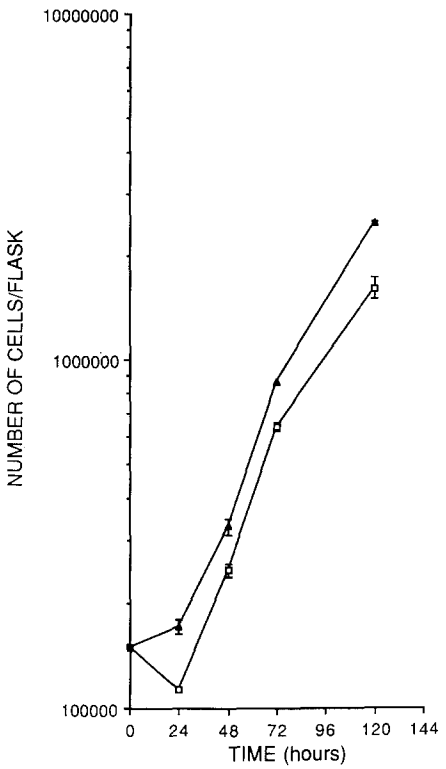


Fig.1. Growth of transfectant clones. Cells were counted at different times after plating at a density of $1.5x10^3$ cells/25 $cm^2$ flask. Each point represents the mean of three determinations ± standard error.
Symbols : □,clone 273 (pSG5) ; ▲, clone 394 (pSG5tag ).

Recipient NIH 3T3 cells ($5x10^5$/100 mm dish) were co-transfected with pSV2neo (5 μg) and pSG5tag (20 μg) or control plasmid pSG5 (20μg) using the calcium phosphate precipitation method. Cellular clones competent for the stable integration of exogenous DNA were obtained by growing the cells in the presence of 500 μg G418/ml. Two clones with similar growth rates (Fig.1) were selected for all the experiments: clone 394, transfected with pSG5tag, and clone 273, transfected with pSG5, were employed respectively as cells expressing the bacterial gene and control cells.

Southern blot analysis was carried out to verify the presence of the tag sequence in genomic DNA extracted from the clones. The Southern blots were prepared from high-molecular-weight DNA digested to completion with the EcoRI restriction enzyme and hybridized to the radiolabelled tag sequence. Panel A in Fig. 2 shows that the clone 394, transfected with pSG5tag, contains tag sequence in multiple sites, while neither clone 273, transfected with control plasmid pSG5, nor NIH 3T3 cells shows any detectable tag sequence. Expression of the tag gene in cloned cell lines was analyzed by Northern blot: total cellular RNA was prepared from clones and the NIH 3T3 cell line, after formaldehyde-agarose electrophoresis, transferred to a nylon membrane and hybridized with a 0.8 Kb tag probe. Panel B in Fig. 2 shows that the clone 394 expressed detectable amounts of tag-specific transcripts whereas clone 273 and NIH 3T3 cell line did not express any detectable tag transcripts.

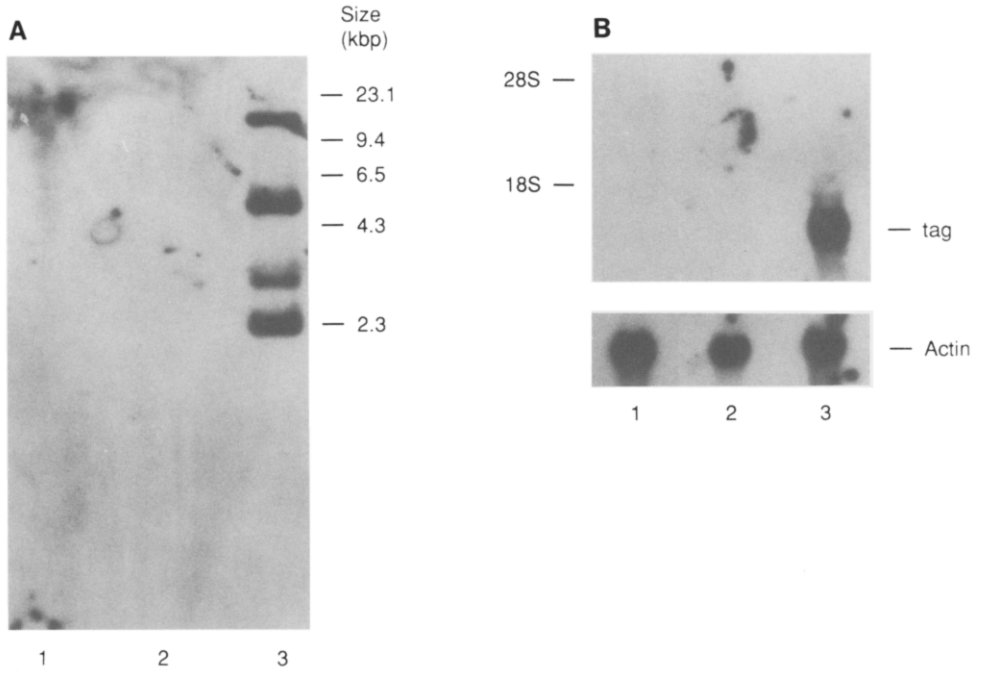


Fig. 2. Hybridization analysis of the NIH-3T3 genomic DNA and total RNA using the tag sequence as a probe. Panel A: Southern blot analysis of NIH-3T3 (lane 1), clone 273 pSG5 (lane 2) and clone 394 pSG5tag (lane 3) genomic DNA digested with Eco RI. Panel B: Northern blot analysis of NIH-3T3 (lane 1), clone 273 ( lane 2) and clone 394 (lane 3) total RNA.

The clones were expanded into massive culture, and protein extracts were prepared and assayed for 3-mAde glycosylase activity. Fig. 3 shows that 3-mAde glycosylase activity of clones 394 and 273 was respectively 1,501.2 fmol/min/mg prot. and 3.7 fmol/min/mg prot. In order to further verify that the increase in 3-mAde glycosylase activity of the clone 394 was due to the presence of the tag gene, we determined 3-mAde glycosylase activity of the clone 394 in the presence of 3-mAde as free base, because it is established that Gly I encoded by the tag gene is specifically inhibited by 3-mAde (3,4). Incubation for 5 minutes in the presence of different concentrations of 3-mAde caused dose-dependent inhibition of the 3-mAde glycosylase activity of clone 394 (at the dose of 5 mM 3-mAde as free base induced 65 % inhibition).

## DISCUSSION

Although it is recognized that N3-adenine is a major site of alkylation of many molecules which react with DNA, the repair mechanism of this lesion by specific enzymes and their relevance for protection from the carcinogenic and cytotoxic effects of alkylating agents have not been elucidated in mammalian cells. We have ample knowledge regarding bacteria where the structural and functional characterization of two 3-mAde glycosylases have been defined. In mutants of E. coli carrying mutations in tag and alk A, the cytotoxicity of methylating agents

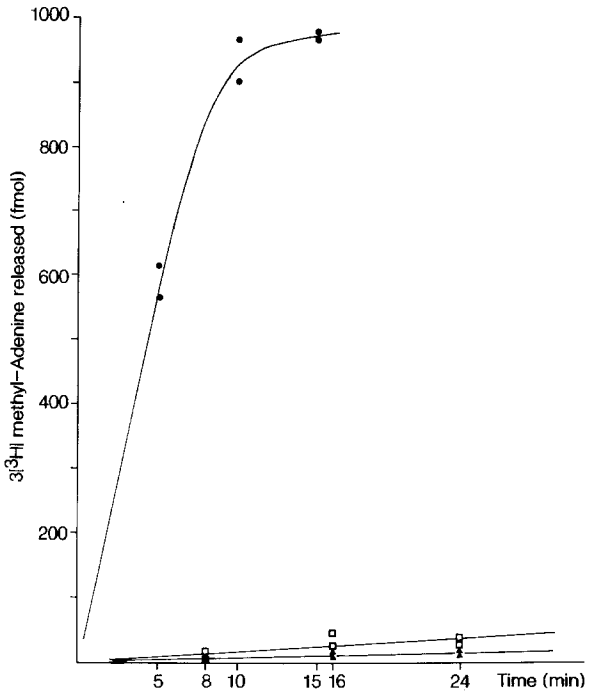


Fig. 3. 3-mAde glycosylase activity in crude cellular extracts. 3-mAde released during incubation at 37°C by 0.1-0.2 mg of cellular extract proteins from NIH-3T3 ( □ ), clone 273 pSG5 ( ▲ ) and clone 394 pSG5tag (●).

such as methyl-methane-sulphonate (MMS) and N-methyl-N-nitrosoguanidine (MNNG) was much higher than in the DNA repair competent wild type cells (9,10,12). The information available in eukaryotic cells is limited to a report in yeast in which deletion of the 3-mAde glycosylase gene produced a DNA alkylation repair mutant very sensitive to killing by MNNG and methylnitrosourea (MNU) but showing a lower mutation frequency than wild type cells when challenged with high doses of MNNG (14,20).

In the present study we show that the tag gene can be expressed at high levels in mammalian cells and that the encoded protein (Gly I) is functionally active. Since bacterial Gly I is specific for the repair of 3-mAde, the NIH-3T3 clone expressing the protein can be used to investigate whether and to what extent alkylation of N3-adenine is relevant for the cytotoxic effects of alkylating agents. Specific inhibition of the tag-encoded bacterial enzyme by 3-mAde (3,4) makes it possible to distinguish between the repair due to the bacterial enzyme and the repair of the mammalian glycosylase which should not be inhibited by 3-mAde.

Cells which over-express the bacterial tag gene can be useful as experimental models to assess the importance of N3-adenine alkylation for the mutagenicity and cytotoxicity of potentially carcinogenic alkylating agents. In addition they could help elucidate the importance of this lesion for the mechanisms of the cytotoxic and antitumoral action of alkylating antineoplastic drugs. This will be particularly important for some novel DNA minor groove binders such as CC-1065 (21) or the deformyl-benzoyl mustard derivative of dystamycin (FCE 24517) (22) which appear to be active as antineoplastic agents on account of their ability to determine DNA-sequence specific alkylation at N3-adenines.

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