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DNA-mediated gene transfer as an indicator of DNA damage and its repair by recipient cells

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Preparations of plasmid containing the thymidine kinase gene (pHSV106) were treated with the alkylating agents methyl methanesulphonate or *N*-methyl-*N*-nitrosourea prior to transfection into thymidine kinase-deficient mouse L-cells using the DNA-calcium phosphate co-precipitation technique. Relative to transfection with unmodified plasmid, a reduced transformation efficiency was observed using alkylation-damaged plasmid, *N*-methyl-*N*-nitrosourea causing the greatest inhibition. Treatment of recipient cells with arabinosyl cytosine or dideoxythymidine during the expression period following transfection by the 'damaged' plasmid reduced transformation efficiency, suggesting that DNA repair 4–6 h post-transfection was required for gene expression.

DNA repair; Transfection; Thymidine kinase gene; Methylmethanesulfonate; *N*-Methyl-*N*-nitrosourea

1. INTRODUCTION

Foreign genes may be integrated stably into mammalian cells and expressed for several generations using calcium phosphate-DNA-mediated gene transfer [1]. This technique has been used by several groups to examine the effects of carcinogen damage to the transfecting gene and its repair by recipient cells [2–4]. Reports of the transforming efficiencies of damaged plasmids differ, however, with ultraviolet radiation causing a dose-dependent increase in transformants [2] and reaction with benzo[*a*]pyrene diol-epoxide effectively reducing the transforming ability of plasmid preparations [3]. That transfected cells are capable of repairing plasmid lesions introduced in vitro has been demonstrated following both ultraviolet damage [4] and double-stranded breaks caused by restriction endonucleases [5,6]. We have sought to describe the effect of the alkylating agents MMS and MNU on the biological activity of a recombinant plasmid containing the viral thymidine kinase gene (pHSV106), following transfection into TK-deficient mouse L-cells. In order to assess the potential of transfection-based assays to characterize DNA damage and repair preliminary examination has been made of the effect of inhibitors of DNA polymerases at various stages throughout the expression period.

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Abbreviations: ara-C, 1-β-arabinofuranosyl cytosine; ddT, dideoxythymidine; MMS, methyl methanesulphonate; MNU, *N*-methyl-*N*-nitrosourea; TK, thymidine kinase

2. MATERIALS AND METHODS

For plasmid modification, ^{14}C-labelled MMS or MNU (Amersham, Bucks, England) in DMSO solution at the indicated concentrations were reacted with 2 μg of plasmid pHSV106 DNA [7,8]

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(generous gift of Dr M. Sleigh, CSIRO, North Ryde, NSW) in 10 mM Tris-HCl/1 mM EDTA (TE buffer, pH 7.5) or citrate buffer (pH 6.0) respectively prior to ethanol precipitation of the DNA and redissolving in TE buffer (200 μl). The percentage modification by MMS and MNU was determined by calculating the molar ratio of DNA to chemical using A_{260nm} values and the specific activity of the labelled compound.

Mouse LTK$^-$/aprt$^-$ cells [9], maintained as monolayers in Dulbecco's modified Eagle's medium (Gibco) with 10% calf serum and antibiotics, were routinely passaged 1:3 once per week. 4×10^5 mouse LTK$^-$ cells were seeded 24 h before transformation and transfections were carried out using the calcium phosphate-DNA coprecipitation technique [1]. Briefly, cells were exposed to the precipitate for 24 h, and following removal of the transfecting DNA, were allowed 24 h in fresh medium before selection. TK$^+$ colonies were then selected in HAT medium and were fixed, stained and counted 14 days following transfection. For studies involving inhibitors, medium was removed from the cells for 2 h periods during the 24 h post-transfection interval and, where appropriate, replaced with fresh medium or medium containing ara-C (20 μM) or ddT (1 mM). After the 2 h pulse period, fresh medium was provided for the remainder of the 24 h growth period. Cells were then selected in the usual manner.

3. RESULTS

The effect of alkylation on the biological activity of the TK gene was assessed by determining the transformation efficiency. Upon selection of stable transformants using HAT medium, numbers of colonies were invariably reduced after transfection with modified DNAs compared to untreated plasmid (table 1). Transfection with either MMS- or MNU-damaged plasmid yielded a consistent relationship between increasing levels of modification and inactivation of the gene. Loss of gene expression occurred at much lower levels of modification with MNU than with MMS.

The effects of ara-C and ddT on the expression of the exogenously added TK gene were determined by addition of the inhibitors to the medium for 2 h periods throughout the 24 h following exposure to the DNA-calcium phosphate coprecipitate. Addition of either ara-C or ddT to the medium decreased transformation efficiency when the inhibitors were present immediately following transfection, ara-C reducing the yield to 22% of control value and ddT to 65% (table 2). Addition of ara-C at other times also reduced thymidine kinase expression, the most marked effect occurring when the compound was present 22–24 h post-transfection. In contrast, ddT did not reduce transformation efficiency when present 4–6 h and 22–24 h after deposition of DNA. Addition of ara-C or ddT during the 0–2 h or 22–24 h periods following transfection with alkylated DNA yielded similar results to those obtained with undamaged plasmid. However, addition of the inhibitors during the 4–6 h period markedly decreased the transformation efficiency observed with DNA damaged by MNU (table 3). The presence of ara-C during this period decreased, by a further 50%, the transformation efficiency observed with undamaged plasmid. More significantly, ddT, which caused no decrease in transformation efficiency at this pulse time following transfection of undamag-

Table 1

Effects of modification on expression of the TK gene following transfer to LTK$^-$ cells

Carcinogen/ DNA	Concentration (μg/μg)	% modification	TK$^+$ clones/ 10 ng DNA
−/pHSV106	−	−	204.0 ± 19.1
MMS/pHSV106	1	33	75.1 ± 8.5
	0.1	11	91.4 ± 4.3
	0.01	0.3	167.7 ± 18.9
	0.001	−	143.0 ± 19.3
MNU/pHSV106	1	6.3	11.7 ± 2.0
	0.1	1.2	55.9 ± 15.4
	0.01	0.12	88.9 ± 20.5
	0.001	−	121.8 ± 9.9

Plasmid pHSV106 was incubated with various concentrations of MMS or MNU. A 10 or 25 ng aliquot of modified DNA was used for transfection, which was performed as described in section 2. 48 h total expression time was allowed before selection in HAT medium. TK$^+$ colonies were scored 14 days after transfection. The numbers of TK$^+$ colonies indicate the mean ± SD of several experiments

Table 2

Effects of DNA replication/repair inhibitors on the expression of the TK gene

DNA	Time of medium change (h)	Additions	TK^+ clones/ 10 ng DNA	% control[a]
pHSV106	24	–	162 ± 3.6	100
pHSV106	0– 2	ara-C	40 ± 3.2	22
	4– 6	ara-C	77 ± 13.5	43
	22–24	ara-C	10 ± 2.0	8
pHSV106	0– 2	ddT	119 ± 25.6	65
	4– 6	ddT	220 ± 26.0	122
	22–24	ddT	132 ± 7.5	97

[a] The percentage control values were obtained by comparison with the transformation efficiencies obtained in experiments in which only the medium was changed for the stated 2-h periods

Cells were exposed to 20 μM ara-C or 1 mM ddT in fresh medium for 2-h periods within the 24 h following removal of the DNA-calcium phosphate co-precipitate

Table 3

Effects of inhibitors on the transformation efficiency of DNA damaged by MNU

DNA	Time of medium change (h)	Additions	% control trans-formation efficiency[a]
–/pHSV106	4–6	ara-C	43
	4–6	ddT	122
MNU/pHSV106	4–6	ara-C	20
	4–6	ddT	58

[a] % control transformation efficiency is expressed by comparison with identical transfection plates in which only the medium is changed during the stated periods

Experiments were performed as described in table 2 except that DNA modified by reaction with MNU (1 μg/μg DNA) was used for the transfection

ed plasmid, decreased the transformation efficiency by 50% when damaged plasmid was transfected.

4. DISCUSSION

Although expression of pHSV106 in mouse LTK^- cells was decreased following damage to the plasmid by alkylating agents, transformation efficiency subsequent to damage was sufficiently high to allow further reduction by manipulation of cellular DNA repair processes. In analogous experiments, dose-response relationships between carcinogen-induced damage to DNA and variation in transformation efficiency have been described [2,3]. These, and the present observations have been made despite indications [10] that the transfection process per se results in DNA damage. The effect of such 'background' damage on repair processes of recipient cells has yet to be clarified. In terms of gene inactivation MNU caused a greater effect at lower levels of modification than MMS, suggesting either that removal of MNU damage is less efficient or that generation of a critical lesion occurs more frequently with MNU.

Reduction of the transformation efficiency (table 2) by addition of either ara-C or ddT to the medium immediately following removal of undamaged transfecting DNA suggested a possible toxic effect of inhibitors on cells recovering from the transfection procedure. The decrease in transformation efficiency observed when ara-C is present 22–24 h post-transfection, implying that such inhibition of DNA polymerase α prevents ex-

pression, is consistent with relatively late replication of the cellular DNA with incorporated viral TK gene [1,11].

Evidence for the role of DNA repair in transfection has been previously obtained using repair-deficient xeroderma pigmentosum cells as recipients in transfection assays with transient expression of psV2-cat as an end point [12,13]. In both cases, reduced expression of ultraviolet-damaged plasmid was observed in xeroderma pigmentosum cells compared to normal recipients. In our studies, transformation efficiency was markedly decreased by addition of repair inhibitors during the 4–6 h post-transfection period when the plasmid was damaged with MNU (table 3). The effect of ddT is conspicuous because, with intact plasmid, this agent did not reduce the number of colonies. The susceptibility of expression to inhibitors of DNA polymerases α and β [14] suggests that repair processes requiring both enzymes are involved during this period. Similar inferences regarding rapid repair arise from the observations of Mooibroek et al. [4] who examined transfection of ultraviolet-damaged DNA into fibroblasts and demonstrated no repairable damage persisting in the plasmid DNA 2–3 h following transfection. Our observations suggest that modification of the processes of DNA replication and repair throughout the expression period following plasmid transfection can yield useful information on factors influencing the expression of exogenous DNA in mammalian cells.

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