Expression of a human O\textsuperscript{6}-alkylguanine-DNA-alkyltransferase cDNA in human cells and transgenic mice

C.Y. Fan, P.M. Potter, J. Rafferty, A.J. Watson, L. Cawkwell, P.F. Searle\textsuperscript{1}, P.J. O’Connor and G.P. Margison*

Cancer Research Campaign, Department of Chemical Carcinogenesis, Paterson Institute for Cancer Research, Manchester M20 9BX and \textsuperscript{1}Department of Cancer Studies, University of Birmingham Medical School, Birmingham B15 2TJ, UK

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ABSTRACT

A truncated human O\textsuperscript{6}-alkylguanine-DNA-alkyltransferase (ATase) cDNA was ligated into an expression vector under the control of the mouse metallothionein-1 gene promoter and upstream of part of the human growth hormone gene to provide splice and polyadenylation signals. Transfection of this construct into human cells resulted in very high levels of ATase expression (more than 300 fmoles/mg protein versus less than 2 fm/mg protein in parent vector transfected control cells). Microinjection of a 4.2 kb fragment of this vector into B6D2F2 mouse embryos and implantation of survivors into pseudopregnant females has so far generated 35 offspring. Southern analysis of tail tip DNA has shown that 11 of the offspring are transgenic for the human ATase gene, between 1 and at least 30 copies of the gene being detected. Human ATase transcripts were detected in total RNA extracted from liver obtained from two male transgenic mice by partial hepatectomy. Cell free extracts of liver samples from five transgenic mice showed up to 4 times higher ATase levels than control livers.

INTRODUCTION

Alkylating agents exert a wide range of biological effects in both pro and eukaryotes and there is ever increasing evidence that these are mediated via interactions with DNA. The majority of evidence, much of it correlative, indicates that O\textsuperscript{6}-alkylguanine is a mutagenic, toxic and probably carcinogenic lesion (1–4). Repair of O\textsuperscript{6}-alkylguanine in DNA can be mediated by ATase and cells or tissues that express low levels of ATase are generally more susceptible to the toxic and mutagenic effects of alkylating agents than high level expressors (3,4). More compelling evidence for the importance of this lesion in DNA has come from experiments in which mammalian cells sensitive to the toxic effects of alkylating agents have attained increased resistance following transfection and expression of the cloned E.coli ATase genes ada (4), and more recently ogt (5).

To further examine the possible role of O\textsuperscript{6}-alkylguanine in carcinogenesis by alkylating agents, we have produced ada (6) and ogt (5) transgenic mice in attempts to generate a strain with greatly increased repair capacity. However, we have so far found little or no evidence of expression of the bacterial ATases (5,6) although more recently, ada-expressing transgenic mice have been described (7,8).

The availability of the human ATase cDNA nucleotide sequence (9) has allowed us to produce a human ATase cDNA inducible expression vector which we have shown to achieve high levels of expression in human cells. We also report the generation of human ATase transgenic mice, the presence of human ATase transcripts and up to 4 times higher levels of ATase activity in liver tissue.

MATERIALS AND METHODS

Isolation of human alkyltransferase cDNA

Oligonucleotides 1075, 207 and 238 (Figure 1a) were synthesised on a Dupont Coder 3000 using standard phosphoramidite chemistry and purified by preparative polyacrylamide gel electrophoresis (10). Oligo-dT selected RNA (10/tg) prepared from human CEM cells was converted to cDNA by specific priming of the first strand using an oligonucleotide (1075, Figure 1a) complementary to the 3' region of the ATase sequence (9). Second strand synthesis was performed according to Gubler and Hoffman (11) and the cDNA then amplified by the polymerase chain reaction using human ATase specific oligonucleotides 207 and 238 (Figure 1a) (9). These create Bam HI restriction endonuclease recognition sites 4bp upstream of the initiation and 12bp downstream of the termination codons respectively. Approximately 100ng of dscDNA was amplified in a total volume of 50\mu l containing 10mM Tris-HCl pH8.3, 1.5mM MgCl\textsubscript{2}, 1 unit of Taq polymerase (Cetus), 100ng of each oligonucleotide and 400 \mu M of each dNTP. After overlaying with 75 \mu l of paraffin oil, 35 cycles of amplification were performed (93°C-20 seconds, 60°C-1 minute, 72°C-1 minute). Aliquots (5 \mu l) were then subjected to agarose gel electrophoresis to determine the extent of amplification.
4 days between washes.

65 °C and exposure was with an intensifying screen for up to 

Filters were washed sequentially in 6x, 1 x and 0.1 x SSC at 

^P-labelled nick-translated human ATase 648 bp probe (16); 

described (10).

(16) as previously 

electrophoresed in 1 % agarose gels and transferred to nylon filters 

DNA was isolated (15) and 5 /tg samples were 

digested, 

BamHl 

DNA isolation and analysis 

Production of human ATase transgenic mice

B6D2F2 mouse embryos were microinjected using standard procedures (15). Drop cultures were incubated at 37°C in an atmosphere of 95% air 5% CO₂ overnight and surviving embryos were implanted into pseudopregnant females. Offspring were weaned at 28 days and tail tips were removed for DNA 

tissue for DNA and total RNA extraction, and for ATase assay.

Eukaryotic expression vector construction

The 464 bp Bam HI ATase fragment was isolated from phAT1 and ligated into the vector p(RG48)MT-hGH which consists of the mouse metallothionein-1 gene promoter and most of the 

transcribed region of the human growth hormone gene containing a number of introns and a polyadenylation signal. This vector is basically similar to that described in (12) but it contains a unique 

BamHI site. A 4.2 kb EcoRI-HindIII fragment of this construct (Figure 1b) was isolated from 2 sequential low melting point agarose gels and made up at 2 ng/ul in buffer for microinjection.

Expression of ATase construct in mammalian cells

In order to confirm that pMThAThGH was capable of expressing the human ATase the intact plasmid (or p(RG48)MT-hGH in control experiments) was transferred by lipofection (Gibco BRL) into SV40 transformed Xeroderma pigmentosum fibroblasts (XP12ROSV ref 13): 18 ug of plasmid DNA was cotransfected with 2/ig of pZipneoSV(X)l (14) and cells were selected in MEM 

containing 10% foetal calf serum and 0.5 mg/ml G418. Resistant clones were isolated after 23 days, expanded and extracts prepared for ATase assay.

RNA isolation and analysis

Fresh liver samples were homogenised using the guanidinium method as recommended (10). The samples were then repeatedly (3 x) phenol:chloroform extracted before the RNA was recovered by sequential isopropanol precipitations.

Denatured RNA samples were separated on 1 % agarose gels 

containing 2.2M formaldehyde and 1 X MOPS running buffer 

as described (10), except 2/ul of 0.5 /tg/ml EtBr was added to 

each sample before loading. This allows visualisation of the major 

RNA species without the usual background fluorescence associated with this technique. Following electrophoresis (40V, 

6 hrs) the gels were washed in water prior to capillary transfer of RNA to Hybond-N in the usual manner (10). An RNA ladder (BRL) was used as a size marker. All hybridisations were performed in 40% formamide, 6xSSC, 1 % SDS, 1 x Denhardt's solution and 250 µg/ml denatured salmon sperm DNA at 42°C.

Alkyltransferase assay

Extracts of cells or tissues were prepared and assayed for ATase 

activity as described (17) except that in some experiments incubation volumes were 500µl and the amount of 4M perchloric acid added following incubation (2h at 37°C) was reduced to 

200µl. Specific activities were calculated from the linear portion of the graph of fmolues [3H]methyl transferred to protein versus amount of protein assayed.

The amplified 671 bp fragment was digested with BamHI and 
ligated into BamHI restricted pUC 8.1 to generate phAT1.

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isolation and analysis. Partial hepatectomy (1/3) provided liver 

tissue for DNA and total RNA extraction, and for ATase assay.

DNA isolation and analysis

DNA was isolated (15) and 5 µg samples were BamHI digested, 

electrophoresed in 1 % agarose gels and transferred to nylon filters 

(Hybond N, Amersham International) essentially as previously described (10).

Prehybridisation and hybridisation were as described using a 

32P-labelled nick-translation human ATase 648 bp probe (16): 

Filters were washed sequentially in 6x, 1 x and 0.1 x SSC at 

65°C and exposure was with an intensifying screen for up to 

4 days between washes.

Figure 2. Alkyltransferase activity in extracts of G418 resistant clones isolated after transfection of Xeroderma pigmentosum cells with the human ATase cDNA containing vector pMThAThGH (•—•, clone 5b; O—O, clone 6B) or control 

vector (□—□, clone 1b) see text for further details.
Table 1. Status of mice surviving to weaning age

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**Fluorography**

SDS-PAGE and fluorography was carried out as previously described (23) except that 16% gels were used.

**RESULTS**

**Expression of transgenic vector in human cells**

The XP cell line used has previously been reported to express very low levels of ATase (less than 2 fm/mg protein, 12). Following lipofection of the ATase vector (pMThAThGH) together with the selectable vector pZipneoSV(x)1, 10 G418 resistant clones were isolated and 3 of the 8 assayed showed marked increases in ATase activity. Fig 2 shows the results of the ATase assay of clones 5b and 6b which had specific activities of 350 and 158 fmoles/mg protein respectively. In contrast, three G418 resistance clones isolated after lipofection of the parent vector together with pZipneoSV(X)1 expressed very low levels of ATase (also 2fm/mg protein).

**Production of transgenic mice**

Following microinjection of 700 eggs, 240 survivors were implanted into 12 pseudopregnant D6B1 females (10 per oviduct). DNA was isolated from tail tips (removed after weaning) of the

**Figure 3. Southern analysis of tail-tip DNA.** 5 µg of DNA was digested with Bam HI, electrophoresed in 1% agarose and transferred following denaturation to Hybond-N. The filter was prehybridised and hybridised with a 32P-labelled human ATase cDNA probe as described in the text. The position of the 648 bp fragment is shown.

**Figure 4. Northern analyses of total liver RNA from a normal mouse (lane N) and from transgenic mice 7.1 (lane 2) and 7.3 (lane 3).** Exactly 10 µg of total liver RNA (as determined by E260 measurements) were run in each lane. Sizes of markers in kb are shown.
two male transgenic mice contained ATase activity close to 148 fmoles ATase/mg total protein. Extracts of the liver of hepatectomy of six normal male control animals contained 96 and to assess whether this protects against these biological effects. However, the evidence is correlative and in Fig. 5.

As shown in Fig 5, extracts of liver obtained by partial hepatectomy of six normal male control animals contained 96 and 148 fmoles ATase/mg total protein. Extracts of the liver of two male transgenic mice contained ATase activity close to control levels (mice 6.0 and 6.2) two contained twice as much (mice 6.4 and 7.1) and one more than 4 times as much (mouse 7.3). Fluorography of liver extracts of control and transgenic (7.1 and 7.3) mice (Fig. 6) showed a band of around 22 kDa, the intensity of which corresponded to the ATase activities shown in Fig. 5.

**DISCUSSION**

The carcinogenic and other biological effects of alkylating agents in mice are well established (18). Of the various lesions produced in DNA, O\(^6\)-alkylation of guanine in has been implicated in the carcinogenic effects. However, the evidence is correlative and not entirely consistent (19). One approach to providing more conclusive evidence would be to generate transgenic mice that express very high levels of an O\(^6\)-alkylguanine repair function and to assess whether this protects against these biological effects.

This approach has been used very successfully in a variety of mammalian cells, and more recently in yeast (20) by a number of different groups (reviewed in 4).

Although our attempts to produce *E. coli* ada gene transgenic mice that express high levels of the bacterial ATase have been unsuccessful (6), other groups using different expression vectors have been able to show low levels of ada expression (7,8). The inherent disadvantage of using the ada gene is that the 39 kDa protein encoded contains two ATase active sites that act on alkylphosphotriesters and on O\(^6\)-alkylguanine and O\(^4\)-alkylthymine (21). Thus it would be difficult to exclude a possible contribution from any of these products if changes in the responses of such mice to the biological effects of alkylating agents are observed.

The ATase encoded by the *E. coli* ogt gene (22,23) more closely resembles the mammalian equivalent in as much as it lacks activity on alkylphosphotriesters (22). However, though ogt transgenic mice have been produced (5) no indication of expression has been found.

The cloning and sequencing of the human ATase cDNA (9) has enabled us to generate a transgenic vector containing this gene. In the vector used, ATase expression is controlled by the heavy metal inducible mouse metallothionein-1 gene promoter: the presence of the human growth hormone sequences downstream provide introns which have been reported to increase expression in transgenic mice (24). The ability of the construct to express at high levels in human cells (Fig. 1) and to increase expression following addition of Zn\(^{2+}\) to the growth medium of *E. coli* (data not shown) confirmed its integrity before proceeding with transgenic mice generation.

In the present experiment approx 30 percent of the offspring surviving to weaning age were shown to be transgenic by the presence of a 648 bp BamHI fragment seen on Southern analysis of tail-tip DNA: ATase gene copy numbers ranged from 1 to, in one mouse, greater than 30 per genome. A more comprehensive molecular analysis of these mice will be presented elsewhere. In two of the male transgenic mice subjected to partial hepatectomy, human ATase message-containing transcripts were

![Graph showing Alkyltransferase specific activities](https://academic.oup.com/nar/article-abstract/18/19/5723/1104927/5726Nucleic-Acids-Research-Vol.18-No.19?refid=1E65241C-3F3C-3C5E-9E3D-0D062F61D338)
readily detectable in total RNA isolated from excised liver: transcripts were very much more abundant in mouse 7.3 than in 7.1.

Alkyltransferase activity in normal mouse liver extract was in the range of % to 148 fmoles/mg protein, close to that reported for other strains used in such studies (6-8). Two of the five transgenic mice assayed for hepatic ATase activity showed similar levels of activity whereas in the other 3 activity was considerably higher, particularly in mouse 7.3 which contained more than 4 times the mean control level. Fluorography further confirmed the considerably higher hepatic ATase activity in mouse 7.3 in comparison with 7.1 and a control mouse. However, in the gel system used, no resolution of the transgene and the endogenous mouse ATases was obtained, probably because of the amounts of protein loaded (200 µg/lane) to detect the ATases.

There was a very approximate correlation between the hAT cDNA copy number and the hepatic ATase activity and this correlation was also evident with mRNA abundance, mouse 7.3 appearing to produce considerably more transcripts than mouse 7.1. Large numbers of analyses will be required before any consistent relationships can be established: in the present work we have addressed the question only of whether it is possible to express the human gene in transgenic mice.

In E.coli ada gene transgenic mice (7,8) the ATase expressed acts on O^-alkylguanine and alkylphosphotriesters (21,23) and half of the total additional ATase activity is due to the latter function. After correction for this, O^-alkylguanine ATase activity in hAT transgenic mice is approx 3 times higher than in the ada mice. Thus, if O^-alkylguanine is important in the adverse biological effects of alkylating agents, protection against these effects may be expected to be more extensive in the human ATase expressing mice. The tissue-dependence of ATase expression, the production of a homozygous transgenic mouse line and the assessment of the response of these animals to specific biological effects of alkylating agents are in hand.

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