Non-phenotypic selection of N-methyl-N-nitrosourea-induced mutations in human cells

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ABSTRACT

The distribution of mutations in a particular gene as detected by a selective mutation assay could be affected by the structural properties of the target protein. To investigate this, we have analysed N-methyl-N-nitrosourea (MNU)-induced mutations in two restriction recognition sequences of a target gene for mutation analysis and compared these data with what previously observed in a phenotypic mutation assay. DNA base changes in the NciI and EcoRV sites of the *gpt* gene maintained in human cells by a shuttle vector system were measured by restriction fragment length polymorphism/polymerase chain reaction (RFLP/PCR) technique. After MNU-treatment of human cells, mutations were detected in the NciI recognition sequence but not in the EcoRV site. DNA sequencing analysis revealed that all NciI-resistant mutations were GC to AT transitions located over four bases of the NciI recognition sequence. Only one of these mutations drastically affected the functionality of the GPT protein. The NciI-resistant mutations were randomly distributed in both DNA strands of the *gpt* gene and were preferentially targeted at guanine residues flanked 5' by a guanine. Our results indicate that the structure of the GPT protein is the main contributor to the strand-specificity of MNU-induced mutations previously reported by using a phenotypic mutation assay. The potential use of the RFLP/PCR technique as a general tool for mutation detection is also discussed.

INTRODUCTION

In the last decade considerable efforts have been directed towards the molecular analysis of mutations after exposure to physical and chemical agents. The non-randomness of mutation distribution has been extensively described (1, 2), but only recently have factors such as adduct distribution, nearest neighbour effects and sequence-specific repair been identified as likely contributors to mutation distribution. For instance, the strong site-specificity of S_N1-type alkylating agent-induced mutations has been associated with the reaction mechanism of

this class of mutagens (3, 4, 5). More recently, the preferential fixation of mutations in the non transcribed DNA strand of the hypoxanthine-guanine-phosphoribosyltransferase gene in rodent cells (6) has been associated with the preferential repair of UV-photodimers from the transcribed DNA strand of the same gene (7). Similarly, we have shown (5) that N-methyl-N-nitrosourea (MNU)-induced mutations in human cells are preferentially located in the non transcribed DNA strand of the xanthine-guanine-phosphoribosyltransferase (*gpt*) gene stably maintained and expressed in the host human cells by a shuttle vector system (8). However, in that case, the strand specificity of MNU-induced mutations was shown to be independent of the transcriptional activity of the target gene. Our data strongly indicated the sequence-specificity of MNU mutagenesis together with the structural properties of the GPT protein as contributing factors to mutation strand bias. The analysis of mutation distribution is in fact always based on the 'window' of observation of the selective mutation system used (often based on resistance to the toxic effects of drugs), in which an alteration in a cell phenotype indicates that a mutation has occurred in a particular gene. The drawback of the phenotypic mutation assays is that mutation analysis is confined to those genetic alterations which produce a functionally altered gene product.

In order to evaluate the effect of the selection system on mutational distribution we explored the feasibility of using a non-selective method to discriminate mutated sequences from wild-type DNA (for a review see 9). The restriction fragment length polymorphism (RFLP)/polymerase chain reaction (PCR) approach (10) was chosen and used for the first time to detect and analyse gene mutations arising in a human cell line after treatment with a carcinogen. The RFLP/PCR technique is based on the acquisition of resistance to digestion by a restriction endonuclease due to mutation at the recognition sequence of the enzyme. The mutant DNA molecules are detected after several cycles of digestion with the restriction endonuclease followed by PCR amplification of the sequence of interest. In this study, the type and location of MNU-induced mutations were determined in two restriction recognition sequences, NciI and EcoRV, located within the coding sequence of the *gpt* gene stably expressed in human cells by the shuttle vector pF1-EBV (8). By using the

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same shuttle vector system, we have previously shown that MNU-induced *gpt* mutations were almost exclusively GC to AT transitions, preferentially targeted at guanine residues flanked 5' by a purine (5). Therefore, on this basis, the NciI site (5'-ACCGGGT-3') is expected to be a mutational 'hot-spot' for MNU, while the EcoRV site (5'-TGATATCC-3') should be a less favourable target.

MATERIALS AND METHODS

Chemicals

MNU (Sigma) was dissolved in Dulbecco's modified Eagle's medium (DMEM) without serum at pH 6 shortly before use and quickly diluted in growth medium at the required concentration.

Cell culture and mutagenesis

6NT human *mer*⁺ cell line (8, 5) was cultured in DMEM supplemented with 10% foetal calf serum and grown in 10% CO₂ at 37°C. This cell line was obtained from human embryonic kidney 293 cells (11) by transformation with the EBV-derived shuttle vector pF1-EBV (8). This shuttle vector contains as target gene for mutation analysis the bacterial *gpt* gene under the control of the mouse metallothionein I (MT-I) promoter. Mutagenesis experiments were carried out as previously described (8). Briefly, cells at approximately 30% confluence were exposed to MNU and cultured until they reached confluence. Shuttle vector DNA was isolated from 6NT cells by the alkaline extraction method (12) and further purified by drop dialysis (13). The recovery of plasmid DNA was quantified by Southern blotting analysis (14). After electrophoresis on a 1% agarose gel, the DNAs were denatured, neutralized and blotted onto nitrocellulose filters. They were then hybridized with ³²P-nick-translated pF1-EBV DNA. The amounts of plasmid DNA isolated from 6NT cells were estimated by comparison to known amounts of pF1-EBV DNA.

Selection for mutations in restriction recognition sequences of the *gpt* gene by RFLP/PCR

Restriction endonuclease digestion and PCR amplification. 1 ng of pF1-EBV DNA or aliquots of the shuttle vector DNA isolated from 6NT cells were extensively digested with the selected restriction endonuclease. Three rounds of 1 hr digestion with 16 U of NciI (Biolabs) or 20 U of EcoRV (Biolabs) were performed. Both restriction endonucleases have a single recognition sequence within the promoter/coding region of the *gpt* gene. The restricted DNA was purified by drop dialysis for 1 hr at 4°C. The *gpt* DNA sequences resistant to cleavage were then amplified by PCR. The sequences of the primers were *gpt/EcoRI*: 5'-GGGAATTCTG-TACCTTCTCCTCACTTACTCCG-3' which hybridizes to the MT-I promoter sequences, and *gpt/BamHI*: 5'-CCGGATCCG-CAGTGCCAGGCGTTGAAAAGA-3' which hybridizes to the SV40 termination sequences of the *gpt* gene. Both PCR primers present a 8-nucleotide tail containing the recognition sequence of EcoRI or BamHI. The resulting PCR product contained the promoter and the coding sequence of the bacterial *gpt* gene. The oligonucleotides were synthesized by the phosphoramidite method with a Beckman automated DNA synthesizer and 5' end-labeled with gamma-³²P-ATP and T4 polynucleotide kinase (BioRad) according to standard conditions. PCR was performed by adding 2.5 U of Taq polymerase (Bio-Rad) in the amplification buffer (10 mM Tris.HCl pH 8.4, 50 mM KCl, 2.5 mM MgCl₂, 200

μM each dNTP) with the following cycle profile: 1) denaturation step at 94°C for 1 min; 2) primer: template annealing at 50°C for 2 min; 3) extension step at 70°C for 3 min.

Purification of amplified DNA and cloning into pUC8 vector. After 30 cycles of amplification, the PCR products were purified by drop dialysis and resuspended in TE buffer. The samples were then treated with EcoRI and BamHI in order to free the ends of the amplified fragments and digested with EcoRV or NciI to select for mutations at these recognition sequences. The PCR products resistant to cleavage by the selected restriction endonuclease were isolated from the gel by glass-milk (Geneclean). DNA aliquots were ligated to EcoRI/ BamHI restricted pUC8 plasmid molecules and the ligation products were transformed into the *Escherichia coli* DT2 strain, a *gpt*⁻ derivative of HB101.

Colony hybridization and DNA sequencing analysis. A specific probe for the promoter/coding region of the *gpt* gene corresponding to the EcoRI-BamHI PCR product (Fig. 1) was used to screen for bacterial colonies containing *gpt* sequences. The probe was prepared by adding to the amplification buffer 1 μl of alpha-³²P dCTP (3000 Ci/mmol, 10 mCi/ml, Amersham). The colony screens were prehybridized for 1 hr at 65°C in 14% sodium dodecyl sulfate, 1 M Na H₂PO₄ and 1M Na₂HPO₄. The discs were then hybridized for 3 hr with the radioactive probe at 65°C in the prehybridization buffer. Three 10 min washings were performed at 65°C in 5×SSC followed by 2×SSC and 1×SSC (0.15 M NaCl, 17 mM sodium citrate, pH 7.0).

DNA sequencing analysis was performed by the method of Sanger et al. (15) using a 24-nucleotide primer starting at the position -115 of the *gpt* gene.

Fig. 1. Detection of mutation at the NciI and EcoRV recognition sequences by RFLP/PCR assay. The regions of the mouse MT-I gene promoter and the termination sequences of SV40 are indicated by a stippled and an open box, respectively. The black-filled box represents the promoter and coding sequences of the *gpt* gene.

Enzymatic activity of the restriction endonuclease-resistant *gpt* molecules. pUC8-*gpt* plasmid molecules containing specific mutations at the NciI site were transformed into the *gpt*⁻ *E. coli* DT2 strain and plated in medium containing 6-thioguanine (6-TG) to determine their phenotype. *Gpt* mutants are able to grow in these selective conditions, while DT2 cells containing wild-type *gpt* genes are unable to grow in the presence of 6-TG.

The *gpt* enzymatic assays were performed as follows. DT2 cells transformed with the mutant plasmids were grown overnight in 20 ml of LB broth. Bacteria were harvested by centrifugation, washed once and sonicated in a lysing buffer containing 10 mM Tris.HCl pH 8, 1 mM dithiothreitol, 10% glycerol and 50 mM NaCl. Cell extracts aliquots (0.1 to 5 μ g of protein) were added to a 100 μ l reaction mixture containing 5 mM MgCl₂, 30 mM Tris.HCl, 1.25 mM 5-phosphoribose-1-pyrophosphate (Sigma), 900 μ M cold xanthine and 30 μ M ¹⁴C-xanthine (50 μ Ci/ μ mole, Amersham). After 10 min incubation at 37°C, the reaction was stopped by adding EDTA to a final concentration of 20 mM. 10 μ l aliquots were spotted onto DE81 paper (Whatman), washed three times with 4 mM Tris.HCl pH 8, dried and counted in Filter Counter scintillation liquid (SupelChem).

Selection for *gpt* mutant plasmids

Phenotypic selection of *gpt* mutants was performed as previously described (8) with minor modifications. Briefly, shuttle vector DNA was transformed into the *gpt*⁻ *E. coli* DT2 strain by electroporation (16) (Bio-Rad electroporation system). Transformation mixtures were plated on minimal salt plates supplemented with ampicillin (50 μ g/ml) and 6-TG (54 μ M final concentration, Sigma) (MATG) to select for colonies containing *gpt* mutant plasmids. To confirm their phenotype, mutant colonies were streaked on MATG plates.

RESULTS

Development of a mutation assay at a restriction endonuclease recognition sequence

The experimental strategy is depicted in Fig. 1. pF1-EBV DNA isolated from MNU-treated and untreated human cells was extensively digested with NciI or EcoRV. The DNA molecules which are resistant to cleavage were selectively amplified by PCR. The PCR products were then digested again with the selected restriction endonuclease and analysed by agarose gel electrophoresis. The mutant molecules were discriminated from wild-type DNA on the basis of their size. In the case of NciI digestion, wild-type *gpt* molecules are expected to be restricted to two fragments of 369 and 361 bp, whereas mutations at the NciI site are expected to produce a full size (730 bp) *gpt* fragment. In the case of EcoRV digestion, wild-type *gpt* molecules are expected to give as digestion products two fragments of 627 and 103 bp, while EcoRV-resistant mutant molecules will remain 730 bp fragments. The band corresponding to the mutant molecules was extracted from the gel and subcloned into pUC8 vector. The last steps of the procedure included DNA sequencing analysis and characterization of the *gpt* gene product.

In order to calibrate single bp mutations by the RFLP/PCR method, a pF1-EBV plasmid containing a single bp substitution (a kind gift of E. Kohfeldt) was used as a model. This mutant

Fig. 2. Quantitative detection of mutations at the NciI site by RFLP/PCR assay. **a)** Autoradiographs of agarose gels after RFLP/PCR selection for mutations at the NciI site. 1 ng of pF1-EBV DNA was mixed with different quantities of model mutant DNA (point mutation at the NciI site). Lane 1: 1000 fg of model mutant DNA; lane 2: 100 fg; lane 3: 10 fg; lane 4: 1 fg; lane 5: wild-type DNA only. **b)** Calibration curve based on densitometric scanning of wild-type and mutant bands.

Fig. 3. Induction by MNU of mutations at the NciI and EcoRV recognition sequences in 6NT human cells. Lane 1: shuttle vector DNA from 6 NT untreated cells selected for resistance to EcoRV cleavage; lane 2: from MNU-treated 6NT cells selected for resistance to EcoRV cleavage; lane 3: from 6 NT untreated cells selected for resistance to NciI cleavage; lane 4: from MNU-treated 6NT cells selected for resistance to NciI cleavage.

has a GC to AT transition at nucleotide position 128 of the *gpt* coding region within the NciI recognition sequence. Increasing amounts of mutant DNA (from 1 to 1000 fg) were added to 1 ng of wild-type plasmid. Mutant molecules are resistant to NciI digestion while wild-type molecules are cleaved once in the coding region of the *gpt* gene at nucleotide position 123. The wild-type/mutant mixtures were subjected to the RFLP/PCR procedure by using end-labeled primers. The PCR products were digested with NciI and wild-type and mutant *gpt* molecules were separated by 2% agarose gel electrophoresis. As shown in Fig. 2a, an increase in the intensity of the NciI-resistant band was observed as function of the initial levels of model mutant DNA present in the mixture. The relative amounts of mutant and wild type DNA in each lane were determined by densitometric scanning (Fig. 2b). The ratio mutant:wild-type DNA after RFLP/PCR plotted against mutant DNA (initially present in the mixture) showed an hyperbolic relationship. This curve is expected to approach an asymptote when one species (in this case the mutant DNA) is present in vast excess. The DNA sample with a ratio mutant:wild-type of 10^{-6} , is undistinguishable from the control pF1-EBV DNA. In conclusion, under our experimental conditions, this methodology is able to identify single bp changes, small deletions and insertions which result in the elimination of the NciI restriction site occurring with a frequency above 10^{-5} .

MNU-induced mutations: a phenotypic versus a genotypic mutation assay

6NT human cells harbouring pF1-EBV shuttle vector molecules were treated with 150 μ g/ml of MNU. Plasmid DNA was isolated from MNU-treated and untreated cells. A portion of this DNA was transformed into *gpt*⁻ *E. coli* DT2 cells. Transformants were plated on minimal salt medium to determine the transformation efficiency and, in parallel, on the same medium added with 6-TG to select for colonies containing *gpt* mutant plasmids (8). The spontaneous mutation frequency in untreated cells was 2.5×10^{-5} . A 40 fold increase over background was observed for the plasmid molecules recovered from MNU-treated human cells (1×10^{-3}). DNA aliquots from the same shuttle vector DNA isolate, corresponding to approximately 1 ng (as estimated by Southern blotting analysis), were subjected to RFLP/PCR technique. Selection for mutations at two restriction

endonuclease sites, NciI and EcoRV, was performed. As shown in Fig. 3, a significant fraction of *gpt* sequences isolated from MNU-treated 6NT cells was resistant to cleavage by NciI (lane 4). In contrast, no mutants at the EcoRV site were observed (lane 2). The control samples derived from untreated 6NT human cells were completely digested after both EcoRV (lane 1) and NciI (lane 3) digestion. The densitometric scanning of lane 4 indicated a mutation frequency at the NciI site in the order of 10^{-4} as calculated from the calibration curve (Fig. 2b).

The presence of NciI-resistant *gpt* sequences after MNU exposure of 6NT cells was confirmed by performing RFLP/PCR amplification of plasmid molecules isolated from a set of independently treated cells (data not shown). The RFLP/PCR procedure was repeated at least twice on the same DNA isolate obtaining similar results.

DNA sequencing analysis of NciI-resistant *gpt* sequences

The NciI-resistant sequences were subcloned into pUC8 plasmid and the recombinant plasmids containing the *gpt* sequences were transformed into *gpt*⁻ DT2 *E. coli* cells. Characterization of the mutants was performed by restriction endonuclease digestions and DNA sequencing analysis.

The recognition sequence of KpnI (5'-GGTACC-3') overlaps the recognition sequence of NciI (5'-CCGGG-3') in the *gpt* gene. Therefore, the resistance to cleavage by KpnI identifies mutations targeted at the two cytosine residues of the NciI site located at nucleotide positions 124 and 125 (Cyt₁₂₄, Cyt₁₂₅). 9/30 NciI-resistant mutants were also KpnI-resistant showing that 30% of MNU-induced mutations is localized at Cyt₁₂₄ and Cyt₁₂₅. It is interesting to recall that the analysis of 85 *gpt* mutants detected in the same cell system after treatment with MNU by using the *gpt* phenotypic selection system did not show mutations at these two cytosine residues (5).

DNA sequencing analysis was performed for 21 NciI-resistant *gpt* sequences. As shown in Fig. 4, all mutations detected at the NciI site were GC to AT transitions as expected from the miscoding properties of O⁶-methylguanine (O⁶-meGua). The majority of the mutations (10/21) were targeted at nucleotide position 128; 5/21 mutations were located at position 124 and 127 respectively, and only 1/21 was detected at nucleotide position 125. Only mutations at nucleotide position 128 were previously detected in the phenotypic mutation assay (5). One additional mutation outside the NciI site (within the 130 nucleotides analysed per gene) was observed in 2 cases out of 21. Because of their nature (one AT to GC transition and one AT to TA transversion), these mutations are likely due to miscoding errors of Taq polymerase (17, 18).

Fig. 4. Distribution of MNU-induced GC to AT transitions in the NciI site of the *gpt* gene.

Table 1. Xanthine-phosphoribosyltransferase activity of NciI-resistant mutants detected by RFLP/PCR assay

Strain	Amino acid change	growth in MATG	U/mg of protein
M124 ^a	Pro-Ser	—	13.2
M125 ^a	Pro-Leu	—	16.3
M127 ^a	Gly-Ser	—	17.0
M128 ^a	Gly-Asp	+	N.D. ^b
wild-type		—	13.8

a: M=mutant; the number indicates the mutation site. The first base of *gpt* coding sequence is considered as position number 1.

b: Not detectable

Effects of the NciI-resistant mutations on the functionality of the GPT protein

Gpt⁻DT2 *E. coli* cells containing the NciI-resistant *gpt* molecules were plated in medium containing 6-TG in order to test the activity of the GPT protein. As shown in Table I, mutations located at nucleotide position 128 gave rise to a mutant *gpt* phenotype, while mutations at nucleotide positions 124, 125 and 127 were highly tolerated by the GPT protein causing bacterial cell death in the presence of 6-TG. The properties of these mutants was further investigated by xanthine-phosphoribosyltransferase assays on cell extracts. Mutants at position 128 were confirmed to be defective in their ability to convert xanthine to its respective ribonucleotide, while mutants at positions 124, 125 and 127 were able to catalyse this synthesis (Table I).

In conclusion, by using RFLP/PCR technique we were able to detect MNU-induced mutations leading to a GPT protein which is still functional. These mutations would be undetectable by a phenotypic mutation assay.

DISCUSSION

We describe in this study the first application of the RFLP/PCR technique to detect and analyse mutations in specific gene sequences after treatment of human cells with a carcinogen. We were able to detect MNU-induced mutations occurring at a frequency of 10^{-4} in a restriction endonuclease site of the *gpt* gene carried by an EBV-derived shuttle vector episomically maintained in human cells. The analysis of the mutations at the NciI site by RFLP/PCR technique revealed that 4 out of 5 potential target sites for MNU mutagenesis were mutated by a GC to AT transition. However, only one of these mutations (i.e. mutation at nucleotide position 128 which leads to Gly-Asp amino acid change) affected the functionality of the GPT protein. This mutation was the only one previously detected by using the selective mutation assay based on resistance to cell killing by 6-TG (5). On the basis of this phenotypic mutation assay two main conclusions were drawn: 1) MNU-induced mutations are preferentially located at guanine residues which are flanked 5' by a purine, and 2) these mutations are predominantly generated in the non transcribed DNA strand of the *gpt* gene. The sequence-specificity of MNU mutagenesis was confirmed by the relative frequency of mutation at the different guanine residues of the NciI site. Gua₁₂₈ which is the favourite target for MNU mutagenesis (10/21) is in fact preceded by a guanine residue, while the less favourable site, Gua₁₂₅, (1/21) is flanked 5' by a pyrimidine. In contrast, no strand bias in mutation distribution was observed in the NciI site. Mutations were detected in both DNA strands: at Cyt₁₂₄ and Cyt₁₂₅ as expected from miscoding of O⁶-meGua adducts located in the non transcribed DNA strand as well as at Gua₁₂₇ and Gua₁₂₈ due to miscoding of O⁶-meGua located in the transcribed DNA strand. Moreover it is interesting to notice that Cyt₁₂₄ and Cyt₁₂₅ both lie in the codon sequence of Pro. No mutations at Pro codons were recovered by the *gpt* phenotypic mutation assay after treatment with MNU. The abundance of mutations at Gly and Trp codon sequences led us to advance the hypothesis that the functional constraints of the GPT protein might play a key role in the DNA strand asymmetry of MNU-induced mutations. The results of this study showing that GC to AT transitions at nucleotide positions 124 (Pro to Ser) and 125 (Pro to Leu) leave a protein which is still functional strongly support this hypothesis. The mutation at nucleotide

position 127, which changes a Gly into a Ser, is also highly tolerated by the GPT protein.

The estimation of the mutation frequency by RFLP/PCR assay deserves some comments. Besides the mutagenic potential of the DNA-damaging agent, two factors drive the final relative amount of mutant DNA molecules detected in a specific DNA sample: the completeness of the digestion of wild-type DNA by the selected restriction endonuclease and the error frequency of Taq polymerase (strictly dependent on the number of amplification cycles). After treatment with MNU, we detected a mutation frequency at the NciI site of 10^{-4} and a *gpt* mutation frequency of 10^{-3} . In a previous study (5), the analysis of the MNU-induced mutational spectrum revealed that 6% of MNU-induced *gpt* mutants (5/85) were localized within the NciI site (at position 128). Therefore, on the basis of the phenotypic assay, the expected mutation frequency at the NciI site would be 6×10^{-5} . Considering that almost 50% of the NciI-resistant mutations are phenotypically silent, the mutation frequency estimated by RFLP/PCR (10^{-4}) is in full agreement with what predicted by the phenotypic assay. These results would suggest that in our case no major differences in digestion efficiency and number of amplification cycles occurred among the various DNA samples.

We were unable to find EcoRV-resistant in the shuttle vector DNA isolated from MNU-treated human cells. By using the *gpt* phenotypic assay, no mutations at this site were detected in either mammalian cells (5) or *E. coli* cells exposed to a variety of alkylating agents (19, 20). The inability of the genotypic assay to detect mutations at this site confirms that EcoRV recognition sequence is a cold spot for MNU-mutagenesis even if we cannot exclude that the mutation frequency at this site is below the detection limit of our assay.

In conclusion, the analysis of MNU-induced mutations by RFLP/PCR assay shed light on the factors responsible for the strong strand specificity of mutation distribution previously reported for the *gpt* gene in both bacterial (19, 20) and mammalian (5) cells. Moreover, the application of this technique to detect carcinogen-induced mutations seems very promising. In a previous study a similar approach was applied to detect oncogene mutations after treatment with a carcinogen (21). In this case, Ha-ras mutations occurring with a frequency of 10^{-6} were detected by selection for appearance of a new restriction site. However, this experimental strategy allows to detect only the specific mutation which generates the new restriction endonuclease site. The approach that we have used has a larger window of detection which is extended to every type of mutation altering the recognition sequence of the selected restriction endonuclease. We have shown the feasibility of this approach to detect and analyse carcinogen-induced mutations in human cells. This protocol has been recently improved to detect mutation frequencies in the order of $10^{-7}/10^{-8}$ (22). Several potential fields of application of this technique are emerging. This technique will allow the characterization of the molecular nature of mutations arising in any relevant genes of any tissue and organism and, because of its sensitivity, its use in biomonitoring studies is foreseen.

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