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Mutation Research 405 (1998) 209–220

Fundamental and Molecular Mechanisms of Mutagenesis

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Accepted 15 May 1998

Abstract

The restriction site mutation (RSM) assay (see Steingrimsdottir et al. [H. Steingrimsdottir, D. Beare, J. Cole, J.F.M. Leal, T. Kostic, J. Lopez-Barea, G. Dorado, A.R. Lehmann, Development of new molecular procedures for the detection of genetic alteration in man, Mutat. Res. 353 (1996) pp. 109–121] for a review) has been developed as a genotypic mutation detection system capable of identifying mutations occurring in restriction enzyme sites of genomic DNA. Here we will report the steps taken to overcome some of the initial problems of the assay, namely the lack of quantitative data and limited sensitivity, the aim being to achieve a methodology suitable for the study of low dose chemical exposures. Quantitative data was achieved in the RSM assay by the inclusion of an internal standard molecule in the PCR amplification stage, thus allowing the calculation of both spontaneous and induced mutation frequencies. The sensitivity of the assay was increased through the discovery that intron sequences of genomic DNA accumulated more mutations in vivo compared to the exons, presumably due to differential selective pressure within genes [G.J.S. Jenkins, I.deG. Mitchell, J.M. Parry, Enhanced restriction site mutation (RSM) analysis of 1,2-dimethylhydrazine-induced mutations, using endogenous *p53* intron sequences, Mutagenesis 12 (1997) pp. 117–123]. This increased sensitivity was examined by applying the RSM assay to analyse the persistence of *N*-ethyl-*N*-nitrosourea (ENU)-induced mutations in mice testes. Germ line mutations were sought in testes DNA 3, 10 and 100 days after ENU treatment. Mutations were detected in exons and especially intron regions, the intron mutations were more persistent, still being detected 100 days post-chemical treatment. Assignment of these mutations as ENU induced was complicated in some cases where the spontaneous mutation level was high. This theme of mutation persistence was further investigated by studying the presence of 4-nitroquinoline-1-oxide (4-NQO)-induced DNA mutations in vitro. This study also analysed the relationship between DNA adduct formation and DNA mutation induction by the concurrent RSM analysis and ^{32}P post-labelling analysis of 4-NQO treated human fibroblasts. The results demonstrated that early DNA mutations detected 4 days post-treatment by the RSM assay were probably ex vivo mutations induced by Taq polymerase misincorporation of 4-NQO adducted DNA, due to the maximum levels of 4-NQO adducts being present at this time point. A later mutational peak, after the adduct level had declined, was assumed to be due to DNA sequence changes produced in the fibroblasts by the in vivo processing of DNA adducts.

Keywords: RSM; Mutation; DNA lesion; Intron; 4-NQO; ENU

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0027-5107/98/$19.00
PII: S0027-5107(98)00138-9

1. Introduction

We describe here the development of the restriction site mutation (RSM) assay to the stage where it may be employed as a sensitive mutation detection system suitable for use at low dose exposures to genotoxins. We also highlight some improvements made in the assay, which clarify the relationship between DNA sequence changes produced in experimental animals and cells and those produced during subsequent PCR amplification. These developments were achieved through the RSM analysis of mutations induced by two potent genotoxins *N*-ethyl-*N*-nitrosourea (ENU) and 4-nitroquinoline-1-oxide (4-NQO).

The restriction site mutation (RSM) assay [1–8] is a genotypic mutation system capable of detecting DNA mutations induced in restriction enzyme sites in target DNA regions (see Fig. 1 for the basic procedures involved in the methodology). In the procedure, undigested (mutated) DNA is selectively amplified by PCR, with any wild type DNA being removed by the restriction step. The resulting enzyme resistant PCR product can be sequenced to ascertain the nature of the mutation, which rendered the enzyme site indigestible. In vivo mutational data has been produced by the RSM assay in a variety of studies involving *Xenopus* sp. [9], the mouse [10] and the rat [11], albeit with relatively low sensitivity (10^{-4}–10^{-5}) and without accurate quantitative data.

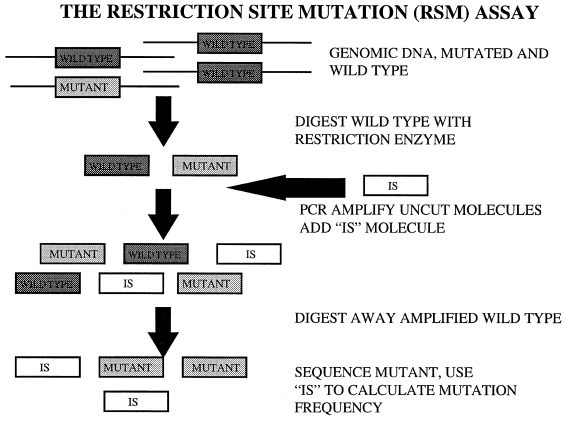

Fig. 1. The basis of the restriction site mutation (RSM) assay. Restriction enzymes fail to cut mutated recognition sites and uncut (mutated) copies may be selectively amplified by PCR.

In order to employ the RSM assay as a research tool in mutational studies, it is necessary for the assay to provide sensitive quantitative data on induced mutagenesis.

The challenge involved in developing the RSM assay revolved around increasing the sensitivity, such that rare mutations could be characterised and quantified [2]. In order to provide quantitative mutational data, internal standard (IS) molecules were synthesised and employed in competitive PCR [12]. These IS molecules were designed to be amplified with the same primer pair as the target region but were synthesised to be shorter in length by deleting the RSM enzyme sites, thus ensuring the IS molecules were resistant to digestion and easily identified in the RSM analysis.The IS molecules were cloned into plasmid vectors in order to increase their molecular size to prevent their preferential PCR amplification. Due to the fact that the IS molecules are included in the PCR step of the RSM assay at a known copy number (approximately 50), it was possible to quantitate the amplification efficiency of the PCR step. Therefore, because the target and IS molecule share common PCR primers it can be assumed that their amplification characteristics are almost identical [13]. Hence, it is possible to calculate the copy number of the mutant population in the DNA before amplification, by comparison to the post-amplification copy number, i.e., the mutation frequency can be calculated.

In order to increase the sensitivity of the assay, two approaches were taken. Firstly, the digestion and amplification steps were optimised for each restriction enzyme/PCR primer pair, such that any mutations present were readily detectable. This allowed multiple analyses to be performed with a high probability of mutations being detected. This lead to an increase in the sensitivity from 10^{-5} (single RSM analysis) to 10^{-6}–10^{-7} (10–100 replicates). Secondly, several target regions of the *p53* gene were analysed by the RSM assay and it was discovered that the non-coding intron 6 region contained approximately 10-fold more mutations than the coding regions in dimethylhydrazine (DMH) treated mice [2]. This increase in mutation induction in intron 6 of mouse *p53* was also evident in two further RSM studies involving two different genotoxic chemicals [14]. A second intron region (intron 8) was also

analysed and a similar pattern of increased intron mutagenesis compared to the exon regions was identified [14], hence the effect was not confined to a single intron region. One explanation for our data is that introns mutate more frequently than exons due to the reduced selective pressure acting on non-coding regions.

The increase in RSM sensitivity, due to the targeting of intron regions was assessed here by evaluating the RSM assay as a germ cell mutation detection system. At present, there is limited information on germ cell mutagenicity, partly due to the fact that the current tests, such as the dominant lethal test and the specific locus test have low sensitivity. These current tests also fail to provide quantitative data on mutation induction or any information on base specificity of test mutagens. Germ cells are known to be less sensitive to mutation induction than somatic cells, this has been demonstrated by results showing that the ENU induced adduct level in testes was 4-fold lower than that of the liver [15]. The reasons for this lower mutability include more stringent repair, higher fidelity replication and the protective effect of the Sertoli cell barrier [16]. The application of the RSM assay may provide germ line mutational data currently not available. Here, we present the results of RSM analyses on ENU-induced mutations in the testes of the mouse 3, 10 and 100 days post-exposure in order to determine their persistence.

In addition to analysing the persistence of induced mutations using the RSM assay, we also addressed the question of the role of DNA lesions in the detection of DNA mutations. It is possible that at early time points post-exposure, mutational analyses can confuse the presence of DNA lesions with DNA mutations and these lesions could well be converted into mutations ex vivo through Taq polymerase misincorporation. This problem is also encountered in transgenic systems, where mammalian DNA adducts are processed in the host bacteria and ex vivo mutations are induced [17]. This can have a profound effect on the analyses of true DNA mutations and ambiguous results may be produced. The analysis of DNA lesions and their conversion to DNA mutations ex vivo can also shed light on the process of DNA mutagenesis, as misreplication of damaged DNA is known to be a major source of mutations in vivo. In order to assess the role of DNA lesions in induced mutagenesis, human fibroblasts were treated with the known genotoxin 4-nitroquinoline 1-oxide (4-NQO). After 4-NQO treatment, the cells were cultured through successive passages for 80 days and the presence of DNA mutations and DNA lesions were assessed by the concurrent application of the RSM assay and the ^{32}P post-labelling assay [18,19].

2. Materials and methods

2.1. In vivo treatments

Male CD-1 mice (Charles River UK, Manston, Kent) were orally treated with ENU (Sigma–Aldrich Poole, UK) (CAS No. 759-73-9) at a dose level of 275 mg kg^{-1} day^{-1} in 1% sodium methylcarboxycellulose (SCMC). The animals were dosed on days 1 and 2 and groups of animals [4] were sacrificed on days 3, 10 and 100. Control animals [5] were dosed similarly, with the vehicle only and sampled at days 3, 10 and 100. The testes were removed from the cadavers and were homogenised in Tris–HCl (pH 7.4) before DNA extraction.

2.2. Cell lines and 4-NQO treatment

Human foreskin fibroblasts (HF12) were cultured in 800-ml tissue culture flasks (Nunc, Roskilde, Denmark) using 30-ml Dulbeco's modified Eagle medium (DMEM) supplemented with 15% (v/v) foetal calf serum (FCS) (Gibco, Paisley, Scotland) in a humidified atmosphere of 5% CO_2/95% air at 37°C. The cells were regularly subcultured once confluence occurred (every 2–4 days). A single cell suspension was obtained by removing the culture medium from the confluent monolayer, washing the monolayer twice with 20 ml of Hanks solution, then with 10 ml of Trypsin EDTA (0.25% Trypsin, 0.02% EDTA in Hanks solution), after detachment had begun, the Trypsin EDTA was removed and the flask gently shaken. A total of 5 ml of the cell suspension was then subcultured into 30 ml of fresh DMEM + FCS medium in a fresh 800-ml flask and further incubation at 37°C continued.

The cells were treated with 5 μM 4-NQO (CAS No. 56-57-5) dissolved in dimethylsulphoxide (DMSO) in 10 separate flasks, 10 flasks were also

set up as negative controls receiving DMSO only. The dose of 4-NQO was chosen as the concentration, which produced 50% survival, obtained from a cytotoxicity study (results not shown). After 4 h, the media was removed, the cells washed with warm Hanks solution and fresh media added. Two of the 10 4-NQO treatment flasks were used to extract DNA 24 h post-treatment. The second DNA extractions occurred after 4 days using two other flasks. The remaining six flasks were grown until confluency (3–4 days after 4-NQO treatment) and were subcultured into two fresh flasks each (giving 12 in total). Two fresh flasks were sampled 24 h and 4 days after subculturing as detailed above and grown until confluency. The process was then repeated until day 80 (passage number 26). The control flasks containing DMSO were processed similarly.

2.3. DNA extraction

2.3.1. Mouse testes samples

The DNA was extracted from the homogenised testes by the high salt method [20] using the kit of the same name (Stratagene, Cambridge, UK). The concentration and purity of the DNA was analysed by spectrophotometry at 260/280 nm. The concentration was adjusted to 0.1 μg/μl and the DNA was stored at −20°C.

2.3.2. Human fibroblasts

The media was removed from the cultured cells and the monolayer was washed twice with ice cold phosphate buffered saline (PBS). A total of 10 ml of ice cold PBS was added to the flasks and the cells were scraped off the flask using a cell scraper (Falcon, Oxford, UK)). The cells were pelleted by centrifugation at $2000 \times g$ for 15 min in a Beckman J-6 centrifuge. The cell pellet was then subject to the high salt DNA extraction method of Miller et al. [20] and its concentration and purity were checked as above.

2.4. Construction of the internal standard for exons 4 and 5 and intron 6 of mouse p53

This is detailed elsewhere [13,14] but essentially followed the same method as for human *p53* exon 7 (below).

2.5. Construction of internal standard molecule for human p53 exon 7

An IS molecule (69 bases) was synthesised on a PCR MATE DNA synthesiser (ABI, Warrington, UK) following the manufacturers recommendations. The 69 base molecule was designed to possess the same primer sites as the 79-bp *p53* exon 7 sequence, but had 10 bases deleted including the *Hae*III and *Msp*I restriction sites. The 69mer was amplified by PCR using the exon 7 specific primers and cloned into the pCR II plasmid using the TA cloning kit (Invitrogen, Leck, Netherlands), in order to prevent the preferential amplification associated with small synthetic DNA fragments. The IS was included in the PCR stage of the RSM assay at a copy number of approximately 50 copies per assay. The mutation frequency was calculated from the initial copy number of mutant genomic DNA, which was calculated as follows:

$$n\text{Target} = \{N\text{Target}/N\text{Internal Standard}\} \times n\text{Internal Standard}$$

where n denotes initial vales and N denotes final values.

The calculated initial mutant copy number is divided by the copy number of the *p53* gene in 1 μg of DNA (3×10^5) to give the mutation frequency.

2.6. Restriction site mutation analysis

2.6.1. Mouse p53 exons 4 and 5 and intron 6

RSM analyses were performed in the two exons and intron 6 as previously described [2]. Aliquots of DNA (1 μg) were subject to digestion with 20 units of restriction enzyme overnight at 37°C in Taq polymerase buffer (Promega, Madison, WI, USA). The restriction enzymes employed were *Nco*I, *Ban*II (exon 4); *Nco*I, *Hae*III, *Nla*IV (exon 5); *Ban*II and *Hae*III (intron 6). The digested DNA was then subjected to PCR amplification. Amplification was performed in a DNA engine (MJ Research, Watertown, MA, USA) with hot lid capability. The amplification reaction contained 10 pmol of each primer, 400-μM dNTPs, 1.5-mM $MgCl_2$, 1.25 units Taq DNA polymerase (Promega) in 1× Taq polymerase buffer. The primers, cycling conditions employed and other details of the methodology are as Jenkins et al. [2].

The amplified DNA was subsequently re-restricted with 20 units of enzyme in the manufacturer's recommended buffer, electrophoresed on 6% polyacrylamide gels and stained with silver to identify any resistant products. Resistant RSM products were isolated from polyacrylamide gels using Spin-X columns (Costar, Cambridge, MA, USA), re-amplified by PCR and sequenced using an ALF DNA sequencer (Pharmacia, Upsala, Sweden). In order to quantitate the mutation frequencies, the resistant products were applied to an ALF DNA sequencer (Pharmacia) and the fluorescent intensities were compared between the IS band and the target band.

2.6.2. Human p53 exon 7

RSM analyses were performed in the human *p53* gene encompassing exon 7. PCR primers were designed to amplify a 79-bp region around the hot spot codon 248 (forward primer 5′-ATGTGTAACAGTTCCTGCATGG-3′ and reverse primer 5′-CTGACCTGGAGTCTTCCAGTG-3′). Cycling conditions were as follows (34 cycles of: 94°C 1 min, 60°C 30 s, 72°C 30 s, followed by 72°C for 10 min). The forward primer was fluoroscein labelled to allow fluorescent intensity quantitation and the reverse primer was biotinylated to allow immobilisation for DNA sequencing. This hot spot region contains the *Hae*III (GGCC) and *Msp*I (CCGG) restriction sites. The RSM method was otherwise identical to that of the mouse testes (above).

2.7. ^{32}P post-labelling method

The method employed was as described by Jones et al. [21]. Calf thymus DNA was run as a negative control and 4-NQO treated *Mytilus* sp. DNA was used as a positive control. The method consisted of hydrolysing 10 μg aliquots of DNA with micrococcal nuclease (0.2 U/μl) and calf spleen phosphodiesterase (Sigma/Aldrich, Poole, UK) (2 mU/μl) for 6 h. The hydrolysed nucleotides were then enhanced by P1 nuclease (Sigma/Aldrich) (2 μg/μl) digestion to remove the normal nucleotides [22]. The remaining nucleotides were labelled with T4 polynucleotide kinase (Boehringer-Mannheim, Lewes, UK). The labelled nucleotides were then spotted onto TLC plates (BDH Merck, Poole, UK) and run in four directions with four different solutions (D1: 1 M sodium phosphate, pH 6.8; D3: 3.5 M lithium formate, pH 3.5, 8.5 M urea; D4: 0.8 M lithium chloride, 0.5 M Tris–HCl, pH 8.0, 8.5 M urea; and D5: 1.7 M sodium phosphate, pH 6.0). The plates were then exposed to hyperfilm (Amersham, Bucks, UK) for 72 h. The levels of radioactive adducts were determined using an AMBIS radioanalytical scanning system (Lablogic, Sheffield, UK). The relative adduct levels (RAL) were calculated using the equation:

$$\mathrm{RAL} = \mathrm{Adduct\ (cpm)} / \mathrm{total\ nucleotides\ (cpm)},$$

cpm: counts per minute (the total nucleotide cpm is calculated from normal nucleotides without enhancement, run alongside the enhanced nucleotides).

3. Results

3.1. RSM analysis of ENU treated mouse testes

The results from the RSM analysis of ENU treated mouse testes are shown in Table 1. These results demonstrate that the RSM assay can detect ENU induced and spontaneous testes mutations, even at 100 days post-treatment, the presence of spontaneous mutations, especially in intron 6 at 100 days, leads to a possible classification problem, these 100-day

Table 1
N-Ethyl-*N*-nitrosourea (ENU) induced mouse testes mutations in the *p53* gene

Gene region	3 days		10 days		100 days	
	Treated[a]	Untreated[a]	Treated[a]	Untreated[a]	Treated[a]	Untreated[a]
Exon 4	0/12 (0%)	0/15 (0%)	2/22 (9%)	0/17 (0%)	0/20 (0%)	0/20 (0%)
Exon 5	0/12 (0%)	0/15 (0%)	1/16 (6%)	1/16 (6%)	0/16 (0%)	0/16 (0%)
Intron 6	3/24 (13%)	0/24 (0%)	2/18 (11%)	0/16 (0%)	2/25 (8%)	2/20 (10%)

[a] Number of resistant RSM products/total number of analyses (percentage of analyses resistant).

ENU-induced mutations may well be spontaneous in origin. The ENU-induced mutations in exons 4 and 5 were only detectable in day 10 samples and were undetectable after 100 days (the exon 5 mutation may also be spontaneous in origin, given the level of spontaneous mutations detected after 10 days). Despite the possible contribution of spontaneous events, the mutations detected in the ENU treated testes can be assumed to be predominantly due to the effect of ENU, this is evident from the 3-fold increase in total mutations in ENU treated testes compared to untreated testes (10:3). The high level of spontaneous mutations in intron 6 after 100 days compared to 3 or 10 days echoes unpublished results from our laboratory on spontaneous bone marrow mutations, which were also highest after 100 days. This may reflect an increase in the spontaneous mutation rate as these animals mature.

3.2. Mutation frequency of ENU induced testes mutations

Table 2 shows the ENU-induced and spontaneous mutation frequency data obtained by the inclusion of an IS molecule in the RSM assay. The results demonstrate a low background mutation frequency of approximately 10^{-6} in the exon regions at days 3 and 100. The mutation frequency detected after 10 days in the ENU treated exon 4 region was approximately 100-fold higher than background values, but the exon 5 values were almost identical between ENU treated and untreated testes, perhaps reflecting that they were both spontaneous in origin. The highest intron mutation frequency was obtained in the ENU treated testes taken 100 days post-treatment. This mutation frequency is approximately 10-fold greater than the exon mutation frequencies and 10-fold greater than the background mutation levels detected, even though similar numbers of mutations were detected between treated and untreated testes. The spontaneous mutation frequencies detected in intron 6 were similar to the values for the ENU induced exon mutation frequencies, further emphasising the greater mutability of the intron region.

3.3. Sequence specificity of ENU induced testes mutations

The 10 ENU-induced resistant RSM products (seven in intron 6, two in exon 4 and one in exon 5) yielded 14 mutations upon sequencing (nine in intron 6, three in exon 5, and two in exon 4), with several resistant products containing multiple mutations. The sequencing demonstrated that the most prevalent mutation induced was the GC → AT transition (7/14), followed by the GC → TA transversion (4/14), there were also two AT → CG transition and one GC → CG transversion. Twelve of the 14 mutations detected in ENU treated testes were at GC bases with two mutations detected at AT bases. This implicates the O^6-ethylguanine adduct as the main cause of ENU induced testes mutation. Given that guanine is the major target for ENU-induced mutation and given also that, of the 14 mutations induced, 10 mutations were at guanine bases on the non-transcribed strand, this implicates the removal of transcribed strand mutations/lesions by the transcription coupled DNA repair process [23]. The three spontaneous mutations detected contained a total of four mutational events, three GC → AT transitions and one GC → TA transversion. Fig. 2 displays the location of the ENU induced and spontaneous mutations.

3.4. RSM analysis of 4-NQO induced DNA mutations in human fibroblasts in vitro

The results of the RSM analysis of exon 7 of the human *p53* gene after 4-NQO treatment are shown

Table 2
ENU induced *p53* gene mutation frequencies in mouse testes samples

Gene region	3 days		10 days		100 days	
	Treated	Untreated	Treated	Untreated	Treated	Untreated
Exon 4	$< 1.4 \times 10^{-6}$	$< 1.4 \times 10^{-6}$	1.4×10^{-4}	$< 2.5 \times 10^{-6}$	$< 2.3 \times 10^{-6}$	$< 2.3 \times 10^{-6}$
Exon 5	$< 1.4 \times 10^{-6}$	$< 1.4 \times 10^{-6}$	1.68×10^{-4}	1.6×10^{-4}	$< 2.8 \times 10^{-6}$	$< 2.8 \times 10^{-6}$
Intron 6	1.2×10^{-4}	$< 0.9 \times 10^{-6}$	1.7×10^{-4}	$< 2.8 \times 10^{-6}$	1.1×10^{-3}	4.0×10^{-4}

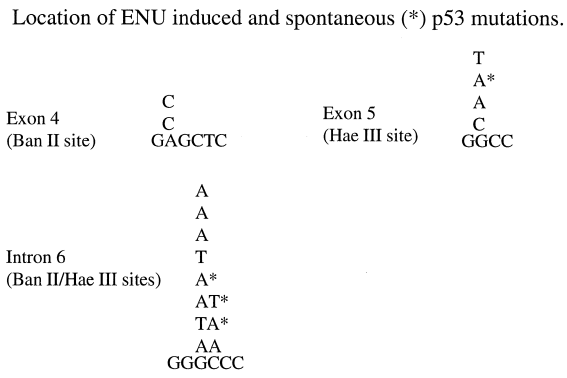

Fig. 2. The location of ENU induced and spontaneous testes mutations as detected by the RSM assay. * = Spontaneous mutations.

in Table 3 and are graphically represented in Fig. 3. The results demonstrate that two peaks of DNA mutations appear at day 4 and day 46 post-exposure. There were relatively few mutations detected between the two peaks and the mutations apparently declined after 46 days, with no mutations detected in the 64- to 80-day period. No spontaneous mutations were detected in any control samples exposed to DMSO alone.

3.5. Sequence specificity of 4-NQO-induced mutations in human fibroblasts

The mutations characterised demonstrated that all of the base substitution mutations detected by RSM were GC → AT transitions located at the second position of codon 248 (C*G*G), as shown in Table 3. Therefore, all mutations were detected in the *Msp*I site that encompassed codon 248, with no mutations detected in the *Hae*III site encompassing codon 249. Two insertional events were detected 62 days post-treatment. These samples contained insertions of 211 and 220 base pairs. Furthermore, given that 4NQO targets guanine bases and that all guanine bases targeted here were on the non-transcribed strand, this is consistent with preferential repair of the transcribed strand [23].

3.6. Calculation of mutation frequency in human fibroblasts

The mutation frequency was calculated by the inclusion of the IS molecule in the RSM assay. The calculated mutation frequencies are given in Table 4. The data parallels the number of resistant RSM products (mutations) shown in Table 3, in that there

Table 3
Number of resistant RSM products detected in exon 7 of the human *p53* gene by the RSM assay after 4-NQO treatment of human fibroblasts

Days post-exposure	Passage no.	Resistant products[a]	Mutation	*p53* codon
1	11	3/35	GC → AT	248/2
4	11	14/32	GC → AT	248/2
10	11	6/16	GC → AT	248/2
18	12	0/12		248/2
20	13	0/20		248/2
24	14	3/18	GC → AT	248/2
31	15	2/32	GC → AT	248/2
40	16	2/46	GC → AT	248/2
44	17	10/44	GC → AT	248/2
46	18	48/56	GC → AT	248/2
50	19	7/38	GC → AT	248/2
54	20	0/41		248/2
58	21	0/49		248/2
62	22	2/48	insertions	
62	22	3/48	GC → AT	248/2
64–80	23–26	0/155		248/2

[a] Number of resistant RSM products/total number of analyses (percentage of analyses resistants).

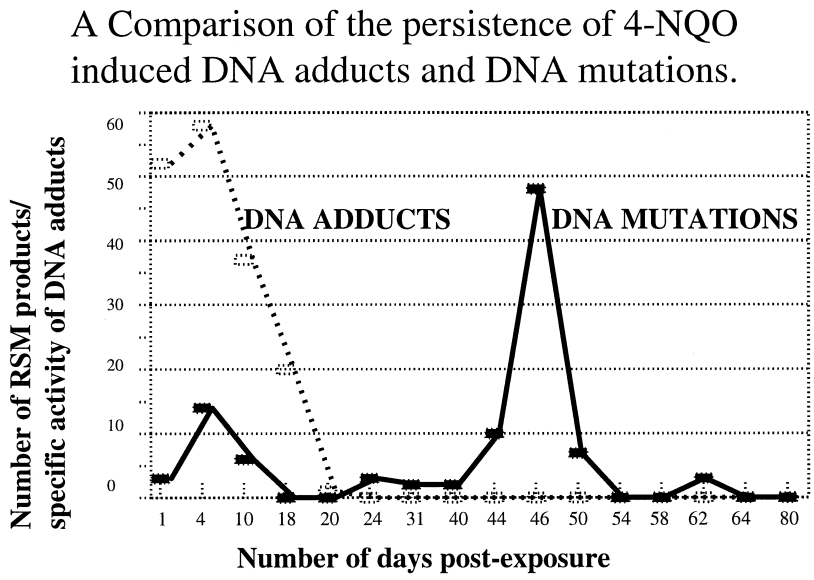

Fig. 3. Graphical representation of the persistence of 4-NQO induced DNA mutations and DNA adducts. The DNA adducts are measured in units of specific activity and these are compared with the numbers of RSM products detected.

was a high mutation frequency at days 1–10, which drops to undetectable levels until day 24, when it peaks again briefly, the mutation frequency then drops until day 46 when it reaches its highest value and then tails off becoming undetectable after 64–80 days. The spontaneous mutation frequency was estimated from the number of untreated RSM analyses, which were negative, as previously described [2].

Table 4
^{32}P post-labelling and mutation frequency data for 4-NQO treated human fibroblasts

Days post-exposure	Passage number	Mutation frequency ($\times 10^{-6}$)	Relative adduct level	Specific activity
1	11	21	16 ± 2.3	51.84 ± 7.4
4	11	15	18 ± 5.19	58.32 ± 16.8
10	11	15.3	11.4 ± 5.17	36.93 ± 16.7
18	12	ND	6.3 ± 5.95	20 ± 19.2
20	13	ND	1.75 ± 1.75	0.56 ± 0.56
24	14	14.6	ND	ND
31	15	0.3	ND	ND
40	16	1.27	ND	ND
44	17	7.1	ND	ND
46	18	510	ND	ND
50	19	ND	ND	ND
54	20	ND	ND	ND
58	21	2.43	ND	ND
62	22	6.5	ND	ND
64–80	23–26	ND	ND	ND
Post-labelling negative control			ND	ND
Post-labelling positive control			326 ± 36	1056 ± 116

The spontaneous mutation frequency was estimated to be $< 2 \times 10^{-6}$ for each passage.
(ND: Not detectable).

Given that at least 12 analyses were performed on untreated samples at each passage, the spontaneous mutation frequency was estimated to be $< 2 \times 10^{-6}$.

3.7. ^{32}P post-labelling of 4-NQO induced DNA lesions in human fibroblasts

The ^{32}P post-labelling data is also shown in Table 4 and demonstrates high adduct levels at days 1–10, which tail off by day 24, being undetectable thereafter. The negative control contained no detectable adducts and the positive control (4-NQO-treated *Mytilus* sp. DNA) had a value 20-fold higher than the highest 4-NQO treated fibroblast sample. The post-labelling results showed that the day 1, 4 and 10 samples contained two distinct adducts adjacent on the TLC plates (results not shown). These were assumed to be the two 4-NQO induced adducts of guanine (N2-dG and C8-dG). These two adducts were barely distinguishable and hence common quantitative data obtained by use of the AMBIS analyser is presented for the two adducts.

3.8. Comparison of the persistence of DNA adducts relative to DNA mutations in human fibroblasts

The comparison of the persistence of DNA adducts and DNA mutations is displayed in Fig. 3. The results show that the DNA adducts were detected in days 1–18, being at a maximum at day 4. However, their presence decreased to undetectable levels after day 20. Fig. 3 also shows that the levels of DNA mutations attain two peaks, at day 4 (similarly to the DNA adducts) and at day 46. A low level of DNA mutation was detected between these two peaks and after the second peak, no mutations were detectable after day 62.

4. Discussion

The results presented here highlight the steps undertaken to enhance the application of the RSM assay. The results demonstrate the use of internal standard (IS) molecules to produce quantitative data on mutations induced by *N*-ethyl-*N*-nitrosourea (ENU) in mouse testes and in human fibroblasts treated with 4-nitroquinoline-1-oxide (4-NQO) in vitro. The results also demonstrate the persistence of ENU testes mutations in vivo and 4-NQO-induced mutations in vitro. Furthermore, by the concurrent analysis of DNA mutations and DNA adducts in the 4-NQO treated fibroblasts, the role of DNA adducts in mutagenesis at early time points post-treatment could be elucidated.

The detection of germ line mutations by the RSM assay was made possible by the demonstration that intron regions were more sensitive to mutagenesis than exon regions [2]. The application of the RSM assay to study germ line mutagenesis was indicative of the increase in sensitivity achieved and represents the first report of in vivo germ line mutations detected by the RSM assay. The RSM assay has now been developed such that it is sufficiently sensitive to detect spontaneous germ line mutations and hence may be employed as a germ cell mutagenicity test, applicable to low dose exposures to genotoxins. The RSM assay offers significant advantages over current tests. These advantages include the quantitative analysis of any gene, the production of base specificity information and the investigation of the role of sequence context.

The ENU induced mouse testes mutations were mainly at GC bases, consistent with the formation of O^6-ethylguanine. This GC base specificity directly contradicts reports of ENU induced transgenic mutations, which were shown to be mainly at AT bases [16,25]. The resistant RSM products detected were mainly present in the intron 6 region (7/10), with all the 100-day mutations being identified in this region. This supports previous data, showing the *p53* intron 6 region to be hypermutable compared to exons 4 and 5 [2]. The third base of the *Ban*II site in intron 6 was shown to be a hotspot for induced mutation as reported previously [2]. Of the 14 mutations detected in testes samples, six were identified at the same base in the *Ban*II site in question (GG*G*CCC). The next most prevalent base for mutagenesis was the second base of the *Hae*III site in exon 5 (G*G*CC), where four of the 14 mutations were detected. All 14 mutations were detected at bases preceded by a 5′G base as predicted previously [26]. The RSM assay was also able to detect four spontaneous germ line mutations in exon 5 [1] and intron 6 [3]. The prevalence of intron 6 mutations is consistent with the notion that intron mutations, being non-coding per-

sist in the genome, whereas coding region mutations are selected against. This was further supported by the presence of ENU induced intron 6 mutations in the testes, 100 days post-treatment, whereas the exon testes mutations were only detected at day 10. The fact that the exon mutations were only detected after 10 days, but not at 3 days supports transgenic data showing that ENU induced testes mutations rise after treatment and reach a maximum after 35 days [15,24]. This is a consequence of spermatogenesis where the expression time (10 days) involves the clonal expansion of mutated spermatogonial stem cells to form post-spermatogonial sperm cells. However, it is important to bear in mind the contribution of spontaneous mutations to those labelled as ENU induced, given the high level of spontaneous mutation at certain time points.

It is possible that the 3-day and 10-day ENU-induced mouse testes mutations are the result of the misreplication of ENU lesions ex vivo by Taq polymerase. However, the half life of the main mutagenic lesion (O^6-ethylguanine) is reported to be 38 h in the testes [16], therefore only 25% of these lesions would be present after 3 days and less than 2% after 10 days, thus their contribution, particularly in the 10 day samples can be assumed to be minimal.

The concurrent analysis of DNA adducts and DNA mutations in human fibroblasts treated in vitro with 4-NQO appears to implicate DNA adducts in producing ex vivo DNA mutations at early time points. The adducts were shown to peak at day 4 and decline to undetectable levels after day 20, whereas two mutational peaks at day 4 and day 46 were detected. The first peak of mutation induction at day 4 is probably due to the miscoding of the 4-NQO lesions by Taq DNA polymerase in the RSM assay, due to the fact that this corresponds to the days where DNA adducts were at a maximum. However, the second mutational peak, at day 46, is presumably due to genuine fixed mutations, induced in the cells by the in vivo miscoding of 4-NQO adducts during cell division. Hence, it has been possible to estimate the expression time of 4-NQO induced mutagenesis in treated human fibroblasts (about 20 days). This expression time represents the time taken for all 4-NQO induced DNA adducts to be converted into DNA mutations during successive rounds of cell division. This information may provide a better understanding of the role of DNA adduct processing within cells and hence could lead to better dosing and sampling regimes in future mutation studies. These results also indicate the potential use of the RSM assay at early expression times to detect DNA adducts as an alternative to the use of the post-labelling assay.

The subsequent reduction in DNA mutations after 46 days suggests that the human fibroblasts are actively selecting against DNA mutations in the hotspot regions of exon 7 of the *p53* gene (unlike the intron mutations in the testes which persisted for 100 days). This experiment is currently being extended to include both intron and exon targets in order to determine if the selection against exon 7 mutations described in this paper is evident for the intron regions, or whether the intron mutations, being non-coding, are tolerated.

The 4-NQO-induced mutations identified by the RSM assay are consistent with those expected from the published data. 4-NQO's major DNA base target has been shown to be guanine [27]. The predominant mutation detected was shown to be the GC → AT transition, supporting earlier data [28,29]. The sequence specificity of the 4-NQO-induced mutations (at the second base of codon 248) may reflect the sequence context of this codon. This hotspot codon CGG contains the highly mutable CpG dinucleotide [30] which has been shown to spontaneously deaminate after cytosine methylation [31]. Deamination at the CpG dinucleotide on the transcribed strand would yield a GC → AT transition at the second base of codon 248. Hence, it is conceivable that increased deamination of methyl-cytosine when it is paired opposite 4-NQO adducted guanine could provide an alternative mechanism to misreplication of adducted guanine residues resulting in the sequence specific mutations detected in this study. The deamination of methyl-cytosine has been shown to be increased when paired opposite alkylated guanine [32]. The contribution of spontaneous events to the 4-NQO-induced mutation detected here, is negligible, as no mutations were detectable in the untreated cells.

The fact that all the 4-NQO induced base substitution mutations were located on the non-transcribed strand suggests that transcription coupled DNA repair [23] occurred, removing the DNA damage from the transcribed strand. In a study of ENU induced

mutagenesis in human *p53* exon 7, GC → AT transitions were reported at the second base of codon 248 on the non-transcribed strand [33], identical to that of 4-NQO mutations detected here. Thus, a similar mechanism involving the preferential deamination of methyl cytosine when placed opposite adducted guanine residues, may be responsible for these results and these reports together may explain why codon 248 is frequently mutated in many human cancers.

The spectrum of 4-NQO-induced mutations in human fibroblasts did not alter significantly with expression time, although two complex insertional events were induced at day 62, which were not apparent at earlier time points. The 211-bp and 220-bp insertional events at 62 days post-exposure represented DNA sequences, which were unidentifiable by comparison with the Genbank/EMBL databases and hence their origin is unknown. These later insertions may reflect the chronic DNA damage induced as a result of *p53* inactivation. It is known that the *p53* gene product plays a major role in protecting the genome from DNA damage [34], hence if the *p53* gene becomes inactivated, it is possible that gross structural changes may occur unchecked. The *p53* gene is known to be inactivated by point mutations in codon 248 and this represents the most frequent *p53* mutation associated with human cancer [35]. Therefore, due to the fact that all the mutations induced by 4-NQO were in codon 248, it is conceivable that the *p53* gene was inactivated in these cells and hence they may have become genetically unstable, which would account for the insertional events detected.

Therefore, in summary, the RSM assay has been shown to detect 4-NQO-induced mutations in vitro and provide information on the role of DNA adducts and also, more significantly, it has been possible to detect both ENU induced and spontaneous germ line mutations. Hence the development of the RSM assay has progressed such that it may now be applied to detect rare DNA mutations and provide quantitative mutational data at low dose exposures to genotoxins.

Acknowledgements

The work described here was funded in part by grants from the European Union Environmental Research Program. We are grateful to Dr. Munira Kadhim for the human fibroblasts cell line used in this study. We are also grateful to Dr. Jim Harvey and Dr. Selwyn Page for the 4-NQO treated *Mytilus* DNA, to Brett Lyons for assistance with the ^{32}P post-labelling technique and also to Mrs. Diane Elwell for her skilled technical support.

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