Development of a novel rapid assay to assess the fidelity of DNA double-strand-break repair in human tumour cells

S. J. Collis\textsuperscript{1,2}, V. K. Sangar\textsuperscript{1,5}, A. Tighe\textsuperscript{1,2}, S. A. Roberts\textsuperscript{3}, N. W. Clarke\textsuperscript{4,5}, J. H. Hendry\textsuperscript{1} and G. P. Margison\textsuperscript{2,*}

\textsuperscript{1}CRC Experimental Radiation Oncology Group, \textsuperscript{2}Carcinogenesis Group and \textsuperscript{3}Biostatistics Group, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Wilmslow Road, Manchester M20 4BX, UK, \textsuperscript{4}Department of Urology, Christie Hospital NHS Trust, Manchester M20 4BX, UK and \textsuperscript{5}Salford Royal Hospitals NHS Trust, Salford M6 8HD, UK

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ABSTRACT
Cellular survival following ionising radiation-mediated damage is primarily a function of the ability to successfully detect and repair DNA double-strand breaks (DSBs). Previous studies have demonstrated that radiosensitivity, determined as a reduction in colony forming ability in vitro, may be related to the incorrect repair (misrepair) of DSBs. The novel rapid dual fluorescence (RDF) assay is a plasmid-based reporter system that rapidly assesses the correct rejoining of a restriction-enzyme produced DSBs within transfected cells. We have utilised this novel assay to determine the fidelity of DSB repair in the prostate tumour cell line LNCaP, the bladder tumour cell line MGH-U1 and a radiosensitive subclone S40b. The two bladder cell lines have been shown in previous studies to differ in their ability to correctly repair plasmids containing a single DSB. Using the RDF assay we found that a substantial portion of LNCaP cells [80.4 ± 5.3(standard error)%] failed to reconstitute reporter gene expression; however, there was little difference in this measure of DSB repair fidelity between the two bladder cell lines (48.3 ± 3.5% for MGH-U1; 39.9 ± 8.2% for S40b). The RDF assay has potential to be developed to study the relationship between DSB repair fidelity and radiosensitivity as well as the mechanisms associated with this type of repair defect.

INTRODUCTION
The most critical lesion in terms of cell death following exposure to ionising radiation is considered to be the DNA double-strand break (DSB) (1), and cellular survival following radiation exposure is a function of the detection and successful repair of such lesions (2). Previous studies have attempted to relate the induction and rejoining of such lesions to the radiosensitivity of normal and tumour cells (3), but the conclusions from this body of work are conflicting. Whilst some have shown a correlation between induction/rejoining and radiosensitivity (4–7), others have not (8–10). Furthermore, the type of assay used to measure such parameters may affect whether or not a significant correlation is obtained (11).

An assay with the ability to predict the radiosensitivity of tumours would provide a useful tool for the further individualisation of radiotherapy of cancer patients (12). Some previous large studies have demonstrated that the fraction of tumour cells surviving after 2 Gy of radiation (SF\textsubscript{2}) in vitro is predictive of tumour control (13,14). However, the methods used to determine SF\textsubscript{2} can take up to 4 weeks and are thus not very practical in the clinical situation.

Whilst the majority of DNA damage can be successfully repaired by cellular mechanisms, it is the unrepaired or incorrectly repaired (misrepaired) DSB that may be the most critical types of damage in determining radiation-induced cell death. Indeed, it has been suggested that the misrepair of DNA DSBs influences cellular radiosensitivity (15–20). Also, it has been proposed that the misrepair of such lesions is an important underlying contributor to the high radiosensitivity of ataxia telangiectasia (A-T) cells (21).

The majority of existing in vitro assays for DSB misrepair are time consuming and labour intensive (16,22) and other more rapid repair fidelity assays involve cell-free systems (17,20,23). The aim of our study has been to design a rapid assay for the determination of DSB misrepair in whole tumour cells. In this paper we describe a novel rapid dual fluorescence (RDF) assay for measuring DSB misrepair and its application in three urological tumour cell lines. We also compare misrepair data obtained from two related bladder tumour cell lines using the RDF assay with previous results using an alternative plasmid-based assay.

\*To whom correspondence should be addressed. Tel: +44 161 446 3183; Fax: +44 161 446 3109; Email: gmargison@picr.man.ac.uk

Present addresses:
S. J. Collis, Johns Hopkins Oncology Center, Cancer Research Building, Department of Radiation Biology, Room 132, 1650 Orleans Street, Baltimore, MD 21218, USA
A. Tighe, The School of Biological Sciences, University of Manchester, 2.205 Stopford Building, Oxford Road, Manchester M13 9PT, UK
arised fragment was excised from the gel and the DNA purified. 0.8% low melting point agarose gel at 85 V for 2 h. The line-

**Figure 1.** (A) pREVY vector map and (B) site of linearisation. The unique Sgfl site is immediately 5' of the CMV promoter driving EGFP expression. The cleavage sites are indicated by triangles. Linear DNA will only express EYFP but not EGFP (as in pCGFP-1; Fig. 3A) unless correct repair of the DSB has taken place. Linearised pREVY was gel purified and quantified prior to transfection. Numbers show base-pair number within the pREVY sequence.

**MATERIALS AND METHODS**

**Cell culture**

The LNCaP prostate cancer cell line was obtained from the American Type Culture Collection. The MGH-U1 and S40b cells (courtesy of Prof. Trevor McMillan, University of Lancaster, UK, and Dr Simon Powell, Massachusetts General Hospital, Boston, USA) were derived from human bladder carcinoma. Cells were maintained as adherent monolayer cultures in RPMI (LNCaP) or HAMs F-12 (MGH-U1, S40b) culture medium (Gibco BRL), supplemented with 10% fetal calf serum (FCS; Biological Industries), 1 mM L-glutamine and 100 IU/ml penicillin and 0.1 mg/ml streptomycin (all obtained from Gibco BRL). Cells were grown at 37°C in a humidified atmosphere of 5% carbon dioxide, fed every 5 days with complete medium and sub-cultured when confluence was reached.

**Construction of pREVY**

The plasmid vector has been described previously (24). Briefly, the gene for enhanced yellow fluorescent protein (EYFP; obtained from pCGFP, Clontech) was inserted into a second cytomegalovirus (CMV) immediate–early (I.E.) promoter–enhancer-driven cloning site within the previously described vector pREV (24) to form pREVY (Fig. 1A). A control plasmid for CMV promoter–enhancer-driven EYFP expression (for FACS analysis) was constructed by inserting the EYFP gene into the plasmid pCneo (Promega) to produce pCYFP. A control plasmid (pCGFP-1) for enhancer-less CMV-driven EGFP expression was created by removing the ~650 bp CMV I.E enhancer sequence upstream of the EGFP gene within the plasmid pCGFP (25). Linearised pREVY was produced by incubating pREVY with the restriction enzyme Sgfl for 2 h at 37°C, which cuts between the CMV I.E. enhancer and promoter regions driving EGFP expression (Fig. 1B). The restriction digest was then electrophoresed on a 0.8% low melting point agarose gel at 85 V for 2 h. The linearised fragment was excised from the gel and the DNA purified using a Qiagen QIA quick gel extraction kit. The purified DNA was then quantified following gel electrophoresis using Bioline Hyper ladder I.

**Cell transfection**

A total of 1 × 10^5 cells were seeded into each well of a 6-well tissue culture plate (Falcon). The following day (when the cells were ~40–60% confluent) the culture medium was aspirated and the cell monolayer was washed with pre-warmed sterile phosphate-buffered saline (PBS). Cells were transfected using Transfast™ reagent (Promega) with 1 µg of pCGFP-1, pREV, pCYFP and circular pREVY or linearised pREVY in separate wells of a 6-well culture plate according to the manufacturer’s protocol. Green and yellow fluorescence was quantified 24 h later by FACS analysis.

**FACS analysis**

FACS analysis was carried out on transfected cells as previously described (25). Dual-colour FACS was performed as described by Lybager _et al._ (26), with the addition of a 480 nm ± 25 bandpass filter, which was added to overcome compensation difficulties and allow EGFP emission to be collected at 505 nm. Fluorescence was displayed as density plots using the program WinMidi 2.8, with EGFP and EYFP expression plotted on the x- and y-axes respectively, using the scales generated by the programme.

**Calculation of misrepair**

The data from the cells transfected with circular pREVY were used to obtain a linear regression line for green against yellow fluorescence. Cells with less than 10^2 fluorescence for both green and yellow i.e. cells having less than 100 times the fluorescence intensity of background levels (equivalent to channel 500 on the FACS plots) within the transfected cell population, were excluded from the calculation. To avoid the region of saturation of the green fluorescence very high fluorescence values (more than 3278 times the fluorescence background: channel 900 on FACS plots) were also excluded from the fit. In general, the fitted line was very close to the 1:1 relationship expected. The green fluorescence values for cells around this line were obtained, and the number of cells within two standard deviations of their distribution about the line was computed (~95% of cells). The fluorescence levels of linear pREVY-transfected cells falling within this range were taken as defining cells correctly repairing the introduced DSB. The fitted line and standard deviation obtained from the plots were then used to determine the proportion of cells misrepaired or not repaired (i.e. with fluorescence values more than two standard deviations from the fitted line) amongst those transfected with linear pREVY. Cells having less than 100 times the fluorescence intensity of background levels within the transfected cell population were again excluded from the fit. All calculations were performed using a purpose-written Fortran program. The RDF assay was repeated at least three times for each cell line, to give an average value for misrepair.

**Colony formation**

Cell survival was assessed using sub-confluent cell monolayers. The cells were trypsinised and diluted to form a single cell suspension of 1 × 10^5 cells/ml and then irradiated (1.12 Gy/min) on ice using a 137Caesium γ-ray source. After
irradiation the cells were diluted, so that the appropriate number of cells were seeded into 60-mm culture dishes, to give at least 50 colonies for counting. Ten (MGH-U1, S40b) to 21 (LNCaP) days after irradiation, colonies comprising of at least 50 cells were counted after staining with gentian violet. The data were fitted using the linear quadratic model, in which the value of the alpha parameter quantifies the initial slope of the survival curve, i.e. the radiosensitivity at low doses.

RESULTS

In vitro radiosensitivity

Colony-formation assays were carried out on each cell line and survival curves were produced (Fig. 2). The alpha values were 0.354 ± 0.01 Gy⁻¹ (LNCaP), 0.025 ± 0.03 Gy⁻¹ (MGH-U1) and 0.17 ± 0.02 Gy⁻¹ (S40b); SF₂ = 0.37 ± 0.05 (LNCaP), 0.94 ± 0.05 (MGH-U1) and 0.64 ± 0.04 (S40b).

Misrepair

Cells were transfected with pEGFP-1, pREV, pCYFP, circular pREVY or linearised pREVY and FACS analysed for EGFP and EYFP expression 24 h post-transfection. A typical set of data is shown in Figure 3. Our functional definition of misrepair is the inability to restore the complete function of the GFP expression cassette in the linearised plasmid, irrespective of the molecular basis of this. The percentage misrepair was assessed for each cell line by calculating the number of cells in the linear pREVY-transfected population lying outside the ± 2 SD range of the circular pREVY distribution. The use of ± 2 SD is arbitrary and actually implies that 95% of the circular pREVY-transfected cells express equivalent amounts of EYFP and EGFP. However, considering the distribution for linear vector-transfected cells shown in Figure 4D, increasing this to 100% would substantially reduce the numbers of cells estimated to have misrepaired the linear pREVY vector. Although there are cells that misrepair but do not exceed the ± 2 SD cutoff, ± 2 SD seems a reasonable compromise. Thus it is probably advisable to take the percentage misrepair as an index for comparative purposes, rather than an absolute figure. Other possible end points for the assay might be the magnitude of the shift in the fitted regression lines (the horizontal distance between the lines in Fig. 4B) or the variance of the linearised pREVY data about the fit of the circular data (compare Fig. 4C and D). It should also be noted that misrepair is assessed only in those cells in the population that were able to take up the plasmid.

The gpt/neo assay used by Powell et al. (22) is similar to the RDF assay in that it assesses the ability of a transfected cell to restore the function of the restriction endonuclease damaged gpt gene. However, in contrast to the RDF assay, this assay relies on the selection of stable clones that are resistant to the antibiotic neomycin and it might be that this in some way attenuates the ability to detect misrepair in the parent line.

Another possible explanation for the discrepancy between the two assays is based on the fact that in order to determine misrepair, a DSB was introduced in the pREVY plasmid between the CMV enhancer and promoter region driving the expression of EGFP. It was envisaged that incorrect repair such as deletions or insertions (28,29) would attenuate EGFP expression. Hence cells showing misrepair of the linearised plasmid would exhibit a ‘shift’ towards yellow fluorescence. However, insertion or deletion of a few base pairs between the CMV enhancer and promoter regions may not affect expression of EGFP. It is likely that there is a threshold of misrepair that can be tolerated within this region before transcription is

DISCUSSION

The present clonogenic survival data confirmed the differential radiosensitivity of the pair of bladder cell lines reported previously (22). The SF₂ values were ordered similarly but were both higher in the present study than in that of Powell et al. (22); 0.94 and 0.62 for MGH-U1 and S40b respectively, (present study) and 0.72 and 0.32 for MGH-U1 and S40b respectively (22). LNCaP cells exhibited radiation response similar to previously reported data (27).

Repeat experiments showed that the RDF assay was generally robust in terms of reproducibility (standard deviation of 10 percentage points among repeat experiments). There was a significant difference (approximately double) in the amount of misrepair between LNCaP cells and the two bladder cell lines, although there was not a significant difference in this measure of repair fidelity between MGH-U1 and S40b. This is in contrast to Powell et al. (22) who reported that MGH-U1 and its radiosensitive clone, S40b, had DSB misrepair values of ∼15 and ∼41% respectively. The corresponding values obtained using the RDF assay were ∼48 and ∼40%. Hence the present assay did not show the discrimination reported previously.

The assay end-point used is the number of linear pREVY-transfected cells, the green fluorescence of which lies outside the ± 2 SD range of the circular pREVY distribution. The use of ± 2 SD is arbitrary and actually implies that 95% of the circular pREVY-transfected cells express equivalent amounts of EYFP and EGFP. However, considering the distribution for linear vector-transfected cells shown in Figure 4D, increasing this to 100% would substantially reduce the numbers of cells estimated to have misrepaired the linear pREVY vector. Although there are cells that misrepair but do not exceed the ± 2 SD cutoff, ± 2 SD seems a reasonable compromise. Thus it is probably advisable to take the percentage misrepair as an index for comparative purposes, rather than an absolute figure. Other possible end points for the assay might be the magnitude of the shift in the fitted regression lines (the horizontal distance between the lines in Fig. 4B) or the variance of the linearised pREVY data about the fit of the circular data (compare Fig. 4C and D). It should also be noted that misrepair is assessed only in those cells in the population that were able to take up the plasmid.
reduced. If the repair of the engineered DSB involves a mechanism where bases are deleted either side of the lesion (28), then perhaps a loss of sequence within the upstream enhancer region may be less deleterious to EGFP expression than loss of the promoter region downstream of the DSB. It has been demonstrated that base deletion primarily occurs downstream of the DSB (17). Due to the close proximity of the DSB to the promoter driving EGFP expression the RDF assay should be very sensitive to such small unidirectional base loss downstream of the SgfI site. However, it not clear if this break

Figure 3. A typical set of FACS density plots produced following transfection with (A) pCEGFP-1, (B) pCEGFP, (C) pCEYFP, (D) circular pREVY and (E) linear pREVY. Green and yellow fluorescence is measured on the x- and y-axes respectively, both as log scales. Numbers in parentheses show wavelengths (nm) used to detect each protein. The percentages of the total population for each quadrant (set at 10² fluorescence for each protein) are shown. Data from circular and linear pREVY transfections were used to calculate the amount of misrepair in each cell line (Fig. 4). The slight bias towards green fluorescence is likely a consequence of the measurement of green fluorescence at its emission peak whereas for yellow fluorescence it was not optimal.
processing would affect the results of misrepair assays in one cell line rather than another.

The only other published misrepair assay that uses whole cells is that described by Löbrich et al. (16,21), who used a NotI restriction endonuclease fragmentation assay to demonstrate that the ability to correctly repair radiation-induced DSB is reduced in A-T homozygote fibroblasts. Whilst this assay allows the determination of the number of DSBs that are repaired correctly, it is limited by the resolution of the gel systems used. It may therefore not be sensitive to small amounts of misrepair

Figure 4. Computational analysis of FACS data for misrepair calculations. FACS data obtained following transfection of LNCaP cells with (A) circular pREVY and (B) linear pREVY were analysed using the misYG program developed within the Institute. The solid blue lines show the regression fits to the fluorescence data that falls between the two horizontal lines at 500 (cells having 100 times the fluorescence intensity of background levels) and 900 (cells having 3278 times the fluorescence background). The dashed blue line in (B) is the fitted line from (A). The histograms (C) and (D) show the residuals (in the horizontal ‘green’ direction) about the regression fit to the circular pREVY-transfected cell data, with the vertical blue lines indicating the ±2 SD limits of the circular data. The cells fitting outside these ±2 SD limits are those considered to have misrepaired the linear plasmid.
e.g. loss of a few hundred base-pairs, because very large chromosomal fragments are studied.

Other published misrepair studies have utilised cell-free assays to detect misrepair of restriction enzyme-induced DSBs (17,23), which use nuclear extracts from irradiated cells to repair a plasmid-based reporter gene in bacteria. Britten et al. (17) reported that the amount of misrepair calculated for several tumour cell lines correlated with SF2. However, using the same assay, North et al. (23) reported that A-T and normal transformed cell lines had similar amounts of misrepair, which is in contrast to the work of Löbrich et al. (21).

In conclusion, the RDAF assay detects DNA DSB misrepair in whole cell systems, may have the ability to detect misrepair with greater sensitivity than existing assays, is reproducible and lends itself to high throughput analyses, given that antibody selection is not required and analysis is by FACS. Therefore we feel that the RDAF assay has potential for exploitation and further development. Future studies will examine the molecular events associated with the misrepair process in a variety of cell lines.

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