Kinetics and mechanism of DNA repair: an automated programmable apparatus for fast time-resolved studies of the repair of mammalian DNA after u.v. irradiation

Christopher W. WHARTON,*†, Rosalind A. MELDRUM,* Christopher REASON,† John BOONE† and William LESTER†

*School of Biochemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K. and †Laser Support Facility, Rutherford–Appleton Laboratory, Chilton, Didcot, Oxon OX11 0QX, U.K.

An automated apparatus, designed and constructed for use in fast time-resolved studies of mammalian DNA repair after u.v. irradiation, is described. The methodology makes use of *caged* DNA-break-trapping reagents, e.g. [α - 32 P]dideoxyadenosine triphosphate [see Meldrum, Shall, Trentham and Wharton (1990) *Biochem J.* 266, 885–890] and the apparatus incorporates excimer lasers both for the delivery of u.v. damage and for the photo-activation of the *caged* reagents. The design is based on a sample-changing turntable which permits the sequential irradiation and quenching of samples. A maximum of eight

samples can be processed in a single experiment, the sequence of events being programmed on a microcomputer, which permits a very generalized experimental sequence. Pipettes for addition of cells, nucleotides and quenching agent are driven pneumatically. A pair of pneumatically operated platinum electrodes allows electroporation of cells for loading of negatively charged reagents prior to irradiation. The time resolution of the apparatus is dependent upon the experimental scheme used and can be very short (e.g. 1 ms) when *caged* reagents are used; a more usual period is 1 s for the shortest incubation.

INTRODUCTION

The consequences of environmentally and human-induced DNA damage in mammalian cells has been widely studied in relation to the risk of mutagenesis and the parameters of cell death [1,2]. The spectacular increase in public awareness has lagged behind scientific interest, but has now given increased impetus to this area of research.

Enormous efforts have been expended in very many laboratories in attempts to gain a rigorous understanding of the process by which mammalian cellular DNA is repaired after damage [2]. Despite this, our understanding of the details of molecular involvement in the repair of both chemical and radiation damage is sufficiently incomplete for much further work to be required [3].

The overall process of repair of u.v.-induced lesions in mammalian cellular DNA by the excision repair process takes many hours [4]. Replication is attenuated until such time that repair is complete, so it has seemed that repair can be leisurely, since replication occurs only every 12–24 h. Despite this seemingly simple and superficially convincing analysis, it is intuitively compelling that there must be segments of the genome that require immediate attention to damage. Such segments may represent genes which require to be constantly transcribed, owing perhaps to an unstable mRNA or protein. There is some evidence that an induced gene is repaired more rapidly than quiescent genes in *Escherichia coli* [5] and in mammalian cells [6]. At relatively high doses and high intensities of u.v. damage it seems likely that there will be some direct strand breaks induced in the DNA as well as pyrimidine dimer and other adduct damage and this may have to be repaired rapidly [7]. It may be necessary for moderately or seriously damaged cells to effect some immediate damage-limitation repairs in order, for example, to maintain

DNA strand continuity. If strand continuity is lost in both strands then this may be lethal and the cell can be expected to do all it can to avoid this outcome. Such a response may have features in common with the stress response which occurs when cultured cells are exposed to excess oxygen or ethanol and might be termed a ‘panic’ response [8].

Very little work has been done on the analysis of the repair response to u.v.-induced damage in terms of the formalism of classical enzyme kinetics; most workers have made observations over an extended time period with a view to monitoring the performance of polymerase inhibitors or mutant cell lines [9,10 (and references cited therein)]. To study the events that occur shortly after u.v. damage it is necessary to adopt the concepts and methods of classical enzyme kinetics and combine them with those of fast reaction techniques [11,12].

We have previously described some early work directed towards the assessment of the feasibility of making such measurements of the repair of pyrimidine dimers [13,14]. The method relies upon the use of a break-trapping agent, in our case α - 32 P-labelled dideoxyadenosine triphosphate (ddATP), which will label sites which are ‘open’ during the polymerization phase of the excision-repair process by incorporation of the trapping agent, which is a chain-termination inhibitor [15]. In order to implement effective fast reaction studies of the early events in DNA repair, it is essential to have a clean but fast method of triggering both the source of damage (i.e. a u.v. laser pulse) and the monitoring of the repair process. Lasers represent an obvious choice both for the damaging event and for initiation of the monitoring process, since they are capable of providing powerful pulses of light in a very short time. In order that lasers might be used for the initiation of the repair monitoring process we have adopted ‘caging’ technology which allows photorelease of active break-trapping reagent at variable times after the damaging laser

Scheme 1 The experimental protocol used with the automated apparatus

The time intervals shown are approximate, but represent the shortest times that can be used reliably for these operations without the risk of interference between sequential operations.

pulse [16]. Scheme 1 shows the overall experimental design. The caging method involves the synthesis of an *o*-nitrobenzyl ester of [α - ^{32}P]ddATP (or [^3H]TTP), which is photolabile (to yield ddATP on photolysis) at a wavelength distinct from that which is used to cause the DNA damage [13].

An important problem in the use of nucleoside triphosphates in studies of (intact) cellular metabolism is the permeability barrier of the cell membrane to negatively charged species. In order to circumvent this problem we have adopted the method of pulse electroporation, and this has been incorporated into the automated apparatus which is described in the present paper [17].

DESIGN CRITERIA FOR THE APPARATUS

Our early DNA-repair experiments were conducted using 3.5 cm plastic culture dishes and surface-adherent cells [13,14]. This methodology worked well in experiments in which repair was measured over a period of several minutes, but was unsuitable for automation of experiments designed to achieve fast time resolution. The major problem was the erratic behaviour and slowness (5–15 min) of the hyperosmotic permeabilization method which was initially employed. This was used to allow loading of cells with the break-trapping agent ddATP prior to repair experiments, but was plainly not suitable for fast-time-resolved studies. Accordingly, we tested permeabilization by electric shock and, after some successful preliminary experiments, this was adopted (see below). This change required also that suspension cells be used since at that time electroporation could only be used with cells in suspension. HL60 (human leukaemia) cells have subsequently been used in all experiments on the repair of u.v. damage. A further problem encountered in experiments using culture dishes was the need to attain a dose of 1 kJ/m^2 of 351 nm light in order to achieve 50% photolysis of the caged break-trapping agent ([caged- α - ^{32}P]ddATP). The XeF excimer laser then available was capable of giving 100 mJ pulses at 10 Hz in a $1 \text{ cm} \times 2 \text{ cm}$ rectangular beam. After optical processing so that the beam filled the 3.5 cm culture dish, insertion losses reduced the pulse at the target to some 25–50 mJ. Thus 20–40 pulses delivered in 2–4 s were required for photoactivation. This

was regarded as being too restrictive in terms of potential time resolution of the system. The cells were exposed in quartz microcuvettes, since the 351 nm photolytic radiation could be focused on the small face of the cuvette ($0.8 \text{ cm} \times 2 \text{ cm}$) and allow much more rapid photolysis.

The apparatus is required to provide all the operations shown in Scheme 1. It was considered that it would be unlikely that we would wish, or be able, to measure events that take place in less than tens of milliseconds, although the potential time resolution is extremely fast where pulsed lasers are used in single-shot mode.

THE APPARATUS

The general features of the apparatus are shown in Scheme 2. The turntable is constructed from Perspex and has eight stations which accommodate quartz microcuvettes. Since the dose of light required from the 248 nm laser (e.g. 100 J/m^2) is much less than that required from the 351 nm laser, the 248 nm light is applied on the larger face ($1 \text{ cm} \times 2 \text{ cm}$) of the quartz microcuvette. Custom-made cuvettes are used in which the side walls are fabricated from high-quality quartz in order to minimize attenuation of the 248 nm light. The cells ($10^6/\text{ml}$) also cause some light attenuation by scattering and absorption, and this was allowed for by control measurements and calculations which ensured that the nominal dose of 248 nm radiation reached the centre point of the cuvette. Similar considerations apply to the application of the 351 nm light, except that the absorption/scattering is much less. A further advantage of this arrangement is that the 248 nm beam, which is strongly attenuated by the cells, has to traverse only 0.4 cm, the width of the microcuvettes. The 351 nm beam, which is less attenuated, traverses 1 cm, the normal pathlength of the cuvette (see Scheme 2).

The lasers

In early experiments a single laser (Lambda Physik EMG 150) was used. This has two resonant cavities, one designed to act as an oscillator and one as an amplifier. When filled with KrF this arrangement provides up to 1 J of line-narrowed 248 nm radiation, but for our work the natural broader linewidth of the KrF

Scheme 2 Diagram of the automated apparatus

The control interface box drives the turntable motor and supplies signals for the pneumatic control valves which operate the pipettes and electrodes. Details of each part of the apparatus are given in the text.

excimer transition was acceptable. This meant that the two resonant cavities could be used as two separate lasers, the only constraint being that they fire together, since the triggers are common. The laser was thus used with the oscillator filled with KrF and the amplifier filled with XeF to provide both frequencies of light required in the experiments. The KrF laser provided ~ 500 mJ at 248 nm and the XeF laser ~ 100 mJ at 351 nm. The KrF laser was stable for several days, but the XeF laser lost power to $\sim 50\%$ of the initial value in 1–2 h. The subsequent decrease in power was slower, but it was necessary to refill the laser with XeF at least twice a day.

The light beams emerged from the lasers as $1\text{ cm} \times 2\text{ cm}$ horizontal rectangles and so needed optical conditioning to be suitable for irradiation of the microcuvette target. The 248 nm beam was simply expanded with a diverging lens, masked and attenuated to give a beam of 2 cm (height) $\times 1\text{ cm}$ (width) with a pulse energy of ~ 50 – 100 mJ (100 J/m^2), allowing for attenuation by the cuvette wall and the cell suspension. This simple arrangement in which the beam intensity was attenuated by using either quartz neutral-density filters or a nitric oxide cell was possible since there was excess power available. The 351 nm beam was first squared by using a cylindrical lens and then contracted horizontally with a second cylindrical lens to give a beam of $0.4\text{ cm} \times 2\text{ cm}$. This was done so as to achieve maximum efficiency of conversion of the beam geometry, since 1 kJ/m^2 of 351 nm light is required for photolysis of the caged break-trapping reagents. The energy on target was 25–50% of the initial pulse energy and was in the range of 10–25 mJ, which required four to ten laser pulses to deliver 1 kJ/m^2 in 0.4–1 s at 10 Hz. Since both lasers fired coincidentally, it was necessary to provide shutters so that only the beam required at any particular time reached the target. These were computer-controlled, as was the firing of the lasers (see below).

Later, the laser described above was replaced by two Questek excimer lasers, which could be fired independently. The overall experimental set-up was unchanged, except that the shutters were no longer required, the beam quality at both wavelengths

was much improved and the laser filled with XeF gave twice as much light as previously with the output now being stable for several days.

The turntable and pipettes

The general layout of the apparatus is shown in Scheme 2. The turntable, driven by a Geneva mechanism, was constructed from two sheets of Perspex (Lucite) so that the laser beams can access the cuvettes in the space between the Perspex sheets. The mechanism takes approximately 1 s to change adjacent samples, and this time period initially limited the time resolution in experiments using uncaged nucleoside triphosphates (see below).

The pipettes are pneumatically actuated variable-volume-delivery Gilson 'Repetman' models (max. volume 1 ml); these are unfortunately no longer available. The plungers suffer abrasion from dust particles and/or crystals in the medium to be dispensed and have proved prone to leakage; this has necessitated their withdrawal from the market. The first pipette adds 0.9 ml of a suspension of HL60 cells ($10^6/\text{ml}$) to the cuvette. The second pipette adds quencher, making an 0.5 ml addition of 5 M NaOH to the cuvette, after laser irradiation and incubation of the sample. This pipette has proved very troublesome, since the NaOH is both corrosive and abrasive. Regular changes of the plunger seals have proved necessary, and even so, leakage has been difficult to prevent. The pipettes operate in ~ 500 ms, and the quencher is injected into the cuvette with sufficient force to ensure thorough mixing, whereupon the cell suspension undergoes immediate lysis and becomes transparent.

In a number of experiments we have found it convenient to initiate DNA-break trapping by using electroporation, which admits the trapping agents to the cells, rather than by laser-induced decaging of trapping agent. In some experiments it was desirable to be able to add nucleoside triphosphates in order to compensate for any depletion which occurs after electroporation and examine the effects of nucleotide imbalances on the repair kinetics. It was necessary to add 1 mM ATP in some experiments,

but this could not be done before the 248 nm laser pulse, since it would cause extreme attenuation of the light. The addition of nucleotides was achieved with a third automatic pipette which was arranged so that it injects an 0.1 ml aliquot between the electroporation electrodes by means of a thin plastic tube inserted through the plastic insulating block which retains the electrodes. Nucleotides were usually added immediately after the electric discharge, and it was shown that adequate mixing was provided by the removal of the electrodes.

Electroporation

As mentioned above, the most important reason for adopting suspension HL60 cells for these experiments was the desire to be able to use electroporation in order to load the cells with negatively charged DNA-break-trapping reagent. The method adopted follows closely that of Chu et al. [17], which was devised for transfection of mammalian cells. In this method two 540 μ C capacitors are charged to an appropriate voltage and discharged through two (2 cm $\times$ 1 cm) 180 μ m-thick platinum electrodes placed against the sides (4 mm apart) of the 1 ml microcuvettes. Charging (5 s) and discharging (half-time 5 ms) as well as electrode insertion, by means of a pneumatic ram, were controlled by the microcomputer. The cells are suspended in iso-osmotic buffer at 137 mM NaCl, a salt concentration previously shown to be optimal for transfection [17]. This permits the high ($\sim$ 50 A) initial current flow that discharges the capacitors with a half-time of 5 ms. An initial discharge voltage of 900 V/cm has usually been used, since this was determined to be optimal for maximum permeabilization as measured by the ingress of the dye Trypan Blue.

The computer-control system and programming

The apparatus is controlled by a PC (Amstrad 1512) with an Amplicon PC 36 interface which provides 48 digital input/output lines to a dedicated control box. This gives control of all aspects of the experiment. The software was written and compiled in Boreland Turbo Pascal running under DOS 3. The system allows timings to be specified with an accuracy of 1 ms. An important feature is the use of a single-wheel position for all operations on one sample; this permits a generalized programming system where events can be ordered in any sequence. A graphics display shows the progress of the experiment and a printout provides a permanent record of it.

Subsequent analysis of samples

While the DNA-single-strand-break-trapping process could be automated as described above, unfortunately the analysis of quenched samples has remained intensively manual (see [14]). In most experiments [α - 32 P]ddATP, [3 H]TTP or their caged variants [13] have been used, and the analysis involves trichloroacetic acid precipitation, filtration on to glass filters and washing, followed by liquid-scintillation counting of radioactivity. The incorporation of radiolabel into the DNA of an irradiated sample relative to that of a control is a measure of the repair-induced DNA polymerization. In some samples the DNA content of the precipitate has been isolated and it has been shown that the vast majority of the label resides in this component of the precipitate (W. Meaking and R. A. Meldrum, unpublished work).

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