Visualisation of altered replication dynamics after DNA damage in human cells

Catherine J. Merrick¹, Dean Jackson² and John F. X. Diffley¹∗

¹ Cancer Research UK
   London Research Institute Clare Hall Laboratories
   Blanche Lane
   South Mimms
   Potters Bar
   Herts, EN6 3LD

² Department of Biomolecular Sciences
   UMIST
   PO Box 88
   Manchester
   M60 1QD

* Corresponding author: Tel: +44 (0)20-7269-3869
   Fax: +44 (0)20-7269-3801
   Email: John.Diffley@cancer.org.uk
Eukaryotic cells respond to DNA damage within S phase by activating an intra-S checkpoint: a response which includes reducing the rate of DNA synthesis. In yeast cells this can occur via checkpoint-dependent inhibition of origin firing and stabilisation of ongoing forks, together with a checkpoint-independent slowing of fork movement. In higher eukaryotes, however, the mechanism by which DNA synthesis is reduced is less clear. We have developed strategies based on DNA fibre labelling that allow the quantitative assessment of rates of replication fork movement, origin firing and fork stalling throughout the genome by examining large numbers of individually labelled replication forks. We show that exposing S phase cells to ionising radiation induces a transient block to origin firing but does not affect fork rate or fork stalling. Alkylation damage by MMS causes a slowing of fork movement and a high rate of fork stalling, in addition to inducing a block to new origin firing. Nucleotide depletion by HU also reduces replication fork rate and increases stalling, moreover, in contrast to a recent report, we show that HU induces a strong block to new origin firing. The DNA fibre labelling strategy provides a powerful new approach to analyse the dynamics of DNA replication in a perturbed S phase.
Introduction

Many types of DNA damage can cause mutations in the genome of a cell, not only by direct mutagenesis but also by generating lesions which are processed into mutations when DNA is replicated during S phase. Mechanisms which guard against this include multiple DNA repair systems and also cell cycle checkpoints which coordinate cell cycle progression with the DNA damage response (1). One such checkpoint acts within S phase to reduce the rate of DNA synthesis, presumably minimising the risk of damage being fixed into potentially dangerous mutations before it can be repaired.

The reduction in rates of DNA synthesis in the intra-S checkpoint may be due to any of a combination of parameters: the overall number of active origins, the temporal programme of origin firing, the rates of movement of all active forks and the occurrence of ‘fork stalling’ events. Any or all of these parameters may be affected by DNA damage, either as a direct physical result of DNA lesions or via the action of checkpoint proteins. This issue has been addressed in some detail in the budding yeast *Saccharomyces cerevisiae*, in which replication from specific origins has been examined after treatment with methyl methane sulphonate (MMS) and hydroxyurea (HU) using a combination of Southern blot, 2D gel and density transfer analyses of replication intermediates. These techniques can separate effects on origin firing from effects on fork rate, at least on a population level, and they have shown that origin firing is blocked in response to MMS or HU (2,3) and that rates of fork movement are also reduced after MMS damage (2).

The block to origin firing in yeast depends on the checkpoint kinases Mec1 and Rad53 whereas the reduction in fork rate appears to be independent of these kinases. Mec1 and Rad53 (homologues of human ATM/ATR and Chk2, respectively) are also central to several other aspects of the S phase checkpoint: the induction of a transcriptional programme of
damage response genes (4,5); the prevention of irreversible fork stalling after MMS damage (2,6,7) and the increase of dNTP levels in the cell after damage (8). It is not clear, however, whether these additional checkpoint responses actually affect the rate of DNA synthesis.

S phase responses to DNA damage have also been examined extensively in human cells. However, by contrast to the techniques described above, the standard assay for an S phase checkpoint response in mammalian cells, the radioresistant DNA synthesis (RDS) assay, simply measures rates of overall DNA synthesis by pulse-labelling a population of cells with tritiated thymidine after DNA damage. Because it only measures bulk synthesis, this assay cannot distinguish effects on origin firing from those on either fork movement or fork stalling. Moreover, it is affected not only by intra-S-phase changes to DNA synthesis but also by inhibition of the G1-to-S transition. In addition, in order to correlate the incorporation of tritiated thymidine with DNA synthesis it is necessary to assume that the specific activity of the endogenous dNTP pools remains constant. These pools may, however, be affected by changes in the rates of de novo nucleotide synthesis and/or nucleotide salvage after damage. Therefore, in the absence of a good range of efficient, sequence-defined early and late origins in mammalian genomes (which might facilitate the use of the same techniques employed to study S.cerevisiae), various alternative assays have been used to further investigate specific aspects of the mammalian S phase checkpoint.

Size separation of ³H-labelled DNA on an alkaline sucrose gradient after treating cells with ionising radiation (IR) led to the inference that origin firing is blocked since the proportion of small DNA fragments – assumed to represent recently fired origins – is reduced after IR damage (9,10). Longer fragments of labelled DNA – assumed to represent ongoing forks - were also found to be reduced but only after much higher doses of IR. A similar block to origin-firing was observed after MMS and UV damage, with fork movement again being affected to a lesser extent and only after longer time periods (11,12). The response to both IR
and UV was found to be deficient in ATM cells. It is important to note, however, that alternative interpretations of much of this data could be made since it is not possible to tell how the large and small DNA fragments actually originated and, like the RDS method, this assay could be skewed by changes to dNTP levels as well as cell cycle effects outside S phase. Indeed, a subsequent investigation of $^3$H labelling of DNA in asynchronous versus synchronised cell populations showed that at least 50% of the reduction in $^3$H labelling which follows exposure to IR in an asynchronous population was due to the complete prevention of S phase entry via a G1/S checkpoint, as opposed to any intra-S-phase change in replication dynamics (13). Nevertheless, the existence of a block to origin firing which is genuinely intra-S phase and ATM-dependent has been corroborated by a second method: 2D gel analysis of replication in rDNA (one of the few areas in the mammalian genome showing sequence-defined ‘early’ and ‘late’ replication). This showed - at least qualitatively - that unfired origins could be blocked following IR damage within S phase while fork movement appeared to be minimally affected, at least after moderate IR doses (14).

Replication dynamics, and their dependence on checkpoint proteins, were not tested by the 2D gel method after other forms of DNA damage such as alkylation by MMS. However, an alternative approach has been used to examine origin firing after aphidicolin or HU treatment - drugs which stall replication. This technique, involving the fluorescent labelling of characteristic patterns of ‘early S’ and ‘late S’ foci in CHO cells, revealed an ATR/Chk1-dependent block to the appearance of late replication patterns when the cells are treated with aphidicolin (15,16). This was interpreted as a checkpoint-dependent block to origin firing, however the method does not yield quantitative data on the numbers or proportions of affected origins in the labelled foci, nor can it address other parameters such as fork rate or fork collapse.
Finally, all of the techniques described above – including those used to examine replication in yeast – rely on examining replication intermediates in populations of cells. Thus, all such approaches may miss important information which can be obtained by examining individual replication forks.

In order to integrate all these different pieces of information using a single experimental system, a DNA fibre-labelling strategy has been developed in which all the various parameters determining DNA synthesis during S phase can be assessed individually, on the level of single replication forks as opposed to whole cell populations. This method measures DNA synthesis across the entire genome, independently of sequence or structure; it is quantitative and the results can be subjected to statistical analysis. The technique has been used in a systematic investigation of both the immediate and longer-term changes to replication dynamics which occur after a variety of DNA-damaging and replication-stalling stimuli.
Experimental Procedures

Cell Culture and Synchronisation

Hela cells were grown as monolayers in DMEM+10% FCS. Synchronisation was carried out by adding 0.17[M] nocodazole (from stock solution 3.4mM in DMSO). After 4-5hrs, rounded mitotic cells were shaken off into pre-warmed PHEM buffer (60mM PIPES, 25mM HEPES, 10mM EDTA, 2mM MgCl₂, pH 6.9), collected with minimal centrifugation (~130g 5mins) and replated in fresh medium. DNA damaging treatments were applied 15-16hrs after replating, when the majority of cells were in early S phase.

The experiment in Fig.4e was carried out in unsynchronised IMR90 cells, also grown in DMEM+10% FCS.

Flow Cytometry

Cell samples were prepared by trypsinising, washing in cold PBS and fixing for at least 2hrs in 70% ethanol at 4°C. Cells were then washed in complete PBS and incubated for 30mins in 0.5ml complete PBS containing 40µg/ml propidium iodide and 0.5mg/ml RNase A. Flow cytometry was carried out using a Becton Dickenson FACScan.

DNA Damaging Treatments

MMS (100% solution, Sigma) was added directly to the culture medium at final concentrations of 0.005%-0.03% (0.59-3.54mM). After 20min treatments the MMS was removed, washing cells twice with MMS-free medium before incubating in further fresh medium.
IR exposures were carried out at between 1 and 10 Grays (~25-250s: control cells were removed from the incubator for the same time period).

HU (Sigma) was dissolved in water and added to the culture medium at 20\(\mu\)M-2mM final concentrations.

**Replication Labelling and DNA Fibre Spreads**

Cells were single-labelled with 50\(\mu\)M IdU for 10-60mins, or, for double-labelling, 10\(\mu\)M or 20\(\mu\)M IdU for 10mins, then 100\(\mu\)M CldU for 20mins. In the experiments in Fig.5, cells were pulsed with 20\(\mu\)M IdU for 10mins directly before DNA damage, then incubated with 50\(\mu\)M thymidine for 15mins to wash out the IdU, then kept in fresh medium before double-labelling 1.5-4.5hrs later.

DNA spreads were made as in (18), with certain modifications. Briefly, cells were trypsinised and resuspended in ice-cold PBS at 2.5\(\times\)10\(^5\) cells/ml. Labelled cells were diluted 1:8 in unlabelled cells and 2.5\(\mu\)l of cells were mixed with 7.5\(\mu\)l of spreading buffer (0.5% SDS in 200mM Tris-HCl pH 7.4, 50mM EDTA) on a glass slide. After ~8mins the slides were tilted at ~15\(^\circ\), the resulting DNA spreads were air-dried, fixed in 3:1 methanol/acetic acid and refrigerated overnight.

**Immunolabelling**

Slides were treated with 2.5M HCl for 1hr, washed several times in PBS and blocked in 1% BSA, 0.1% Tween.

Slides were then incubated at RT with the following antibodies, rinsing 3x in PBS and then washing 3x20mins in blocking buffer between each incubation. 1) Overnight in 1:2000 rat anti-CldU (detects CldU) (OBT0030F ImmunologicalsDirect) 2) 2hrs in 1:1000 Alexafluor 633-conjugated anti-rat (A-21094 Molecular Probes) 3)
2hrs in 1:500 mouse anti-BrdU (detects IdU) (MD5100 Caltag) 4) 2hrs in 1:1000 Cy3-conjugated anti-mouse (C-2181 Sigma). Slides were then counterstained for 20mins with 1:20 000 YOYO-1 in PBS (Molecular Probes) before rinsing 3 times in PBS and mounting in PBS/glycerol.

Microscopy was carried out using a Zeiss LSM Meta 510 confocal microscope.
Results

*S phase progression is slowed by IR, MMS and HU*

Many techniques used to synchronise cells in S phase, such as aphidicolin, mimosine or double thymidine blocks, interfere with replication forks and are likely to activate DNA damage responses. Therefore, in this study, HeLa cells were synchronised by nocodazole arrest, mitotic shakeoff and release for 16 hours – at which point most cells are in early S phase. Initially, we used such synchronised cells to examine the effects of various treatments on overall S phase progression. Firstly, cells were treated with 20 minute pulses of 0.001%-0.03% MMS, the MMS was removed and S phase progression was followed by flow cytometry over the next 12hrs. Fig.1a shows that S phase was slowed in a dose-dependent manner, ranging from a mild effect after 0.005% MMS to near-complete arrest over 12 hours after the 0.03% treatment. Secondly, S phase progression was followed after exposure to 1 Gray (Gy) or 5 Gy of IR (exposures which should cause approximately 36 and 180 double strand breaks per cell respectively (17)). 1 Gy did not cause a detectable slowing of S phase but 5 Gy resulted in a moderate slowing of S phase progression (Fig.1b). Thirdly, 5-100 mM HU was added to the cells in early S phase. Again, a dose-dependent slowing of S phase was observed (Fig1c); 5µM HU having little effect, 20µM causing a significant slowing of S phase and 100µM leading to arrest with a near-2C DNA content.

*Fork movement is reduced by MMS and HU but not by IR damage*

The DNA fibre labelling (DIRVISH) technique (18) has been adapted in this study such that two distinguishable modified nucleotides, IdU and CldU (19), could be used to label replication within a single S phase. In this technique (itself adapted from the classical DNA fibre autoradiography technique (20) in which newly replicated DNA is labelled with tritiated thymidine) cells are pulse-labelled with halogenated nucleotides, then collected and lysed on a
glass slide. By tipping the slide, DNA from the cells is spread out in the form of single fibres. This DNA is subsequently fixed, denatured and immuno-labelled to detect the halogenated nucleotides. In these experiments, all DNA was then counterstained in a third colour with YOYO-1 DNA dye, allowing the exclusion of any broken or tangled fibres. Consecutive pulse-labelling of S phase cells with IdU and then CldU yields double-fluorescently-labelled tracks on the DNA which can be interpreted unambiguously as either ongoing forks, newly-fired origins, terminations or fork stalling events (Fig.2). The length of any track after a given labelling period is proportional to its fork rate, while counting the relative numbers of different track forms can determine changes in the rates of origin firing or fork stalling after DNA damage.

DNA fibre assays were carried out after each of the three treatments examined in Fig.1 in order to establish which parameter(s) of DNA synthesis contributed to the overall slowing of S phase seen by flow cytometry. In order to quantify any change in fork rates after DNA damage, cells were exposed to 20min pulses of MMS (0.005-0.03%) and then, after removal of the MMS, immediately labelled with IdU for 10-60mins before preparing DNA fibre spreads. The mean length of at least 50 IdU-labelled tracks was calculated for each time period. Fig.3a shows that fork rates were reduced for at least 60 minutes after more severe MMS treatments. The severity of slowing was correlated with the MMS dose but slowing was only observed above ~0.01% MMS.
Fig. 3b shows that reduction of cellular dNTP pools by treatment with hydroxyurea also slows replication forks in a dose-dependent manner. When cells were treated with sufficiently high levels of HU (above \( \sim 100 \mu M \)), forks were essentially stalled and very little progression occurred over several hours (data not shown).

By contrast to MMS and HU, IR did not cause detectable fork-slowing, even at doses which do reduce overall S phase progression. Fig. 3c shows no significant change in the mean lengths of tracks labelled after IR exposures of up to 5 Gy.

**Origin firing is rapidly inhibited after IR, MMS or HU**

DNA fibre labelling can be used to distinguish newly-fired origins from ongoing forks using the experimental protocol outlined in Fig. 4a. Active replication forks prior to damage were labelled with IdU (red), cells were then treated with damage and the IdU was replaced by CldU (green). During the subsequent 20 minutes, any newly-fired origins will generate tracks labelled along their entire length with CldU and these can be counted against the number of double-labelled (ongoing) forks which were tagged with IdU prior to damage.

Fig. 4b shows that origin firing was inhibited in response to MMS and that the severity of inhibition was dose dependent over the range tested (20 min pulses of MMS at 0.005%-0.02%). Exposure to IR also inhibited origin firing but unlike the response to MMS, this may show a threshold between 1 and 2.5 Grays (Fig. 4c). No further decrease in origin firing was then seen after IR exposures up to 10 Gy (data not shown). This damage-insensitive subset of initiation events - seen after the maximum doses of both IR and MMS damage - may represent the proportion of the total origins which are already committed to fire within this 20 minute labelling period at the time of damage.
The response of cells to HU was also tested in this origin-blocking assay, since nucleotide depletion has been shown to inhibit origin firing via the S phase checkpoint in *S.cerevisiae* (2,3,21). In higher eukaryotes the S phase checkpoint response to HU has not been tested but aphidicolin, which stalls replication by inhibiting DNA polymerases, does inhibit the appearance of late S-phase foci in CHO cells (16).

Replication forks were pre-labelled for 10mins with IdU as before, then the IdU was replaced with CldU together with 250µM HU. The accumulation of new (CldU-labelled) origins was then counted against the IdU-tagged ongoing tracks over the subsequent 2-6hrs. Fig.4d shows that origin firing is greatly reduced, such that it takes 6 hours to accumulate the same number of origin firing events which occur in control cells in less than 1 hour. It is unlikely that many new origins did fire but were simply not labelled due to nucleotide depletion, since most existing forks were able to progress, incorporating CldU, for a further 1-2µm over the 6hrs of HU arrest. In order to confirm this, the experiment was repeated using only 50µM HU – a concentration which allows existing forks to elongate more extensively, growing by 3-4µm over 3hrs. As before, new origin firing was severely inhibited (Fig.4d). Because of a recent report indicating an increase in origin firing after treatment of a modified hamster fibroblast cell line with HU (22), this experiment was repeated using primary human fibroblasts instead of HeLa cells and a similar inhibition of new origin firing was observed (Fig.4e).

**Origin firing recovers at different rates after IR, MMS and HU**

A modified version of the origin-firing assay described above was used to assess recovery in the rate of firing over longer periods after DNA damage (Fig.5a). As in Fig.4, active replication forks were tagged with a pulse of IdU prior to DNA damage (Fig.5a, tracks labelled ‘a’), then the IdU was washed out before MMS or IR were applied. This generates
exclusively-IdU-labelled (red) tracks representing the number of active replication forks before DNA damage. At timepoints from 1.5 to 4.5hrs later, cells were then double-labelled with consecutive pulses of IdU (red) and CldU (green). This protocol distinguishes any new origins actually firing at each timepoint (exclusively green or green at both ends: labelled ‘c’ in Fig.5a) from ongoing replication forks (red-then-green: ‘b’ in Fig.5a). These new origins were counted against the exclusively-red tracks which form an internal control since they had been tagged identically in all the cells before any DNA damage.

Fig.5b shows that a 20min pulse of 0.01% MMS (grey bars) elicited a sustained block to origin firing when compared to the levels occurring in undamaged cells (white bars): origin firing recovered to only a very limited extent during at least 4.5hrs after the MMS treatment. In comparison, 5Gy IR (Fig.5c) caused a much more transient block to origin firing with significant recovery after only 1.5hrs. By 3hrs post-IR exposure, origin firing had returned to normal levels.

The efficiency of origin firing recovery was also assessed after release from an HU arrest. As before, replication forks were pre-labelled with IdU, then completely arrested by adding a high level of HU for 1-4hrs. Upon release from HU, the IdU was replaced with CldU and new origins fired within 1hr were counted against the pre-labelled tracks. By comparison to either IR or MMS damage, origin firing recovered relatively well after a brief (1hr) HU arrest, but recovery became progressively less efficient after longer periods (2-4hrs) (Fig 5e). This is unlikely to be an artefact due to under-detection of CldU-labelled tracks after HU release, since the nucleotide balance within the cells recovered sufficiently fast to allow the origins which did fire to elongate by $\sim 3\mu m$ within 30mins and $6\mu m$ within 60mins (data not shown).
Replication forks stall at an elevated rate after MMS and HU but not after IR

The slowing of replication forks after MMS damage which was documented in Fig.3 could result from at least two distinct modes of altered fork progression. DNA damage may provoke a pan-nuclear change to a slower mode of replication, for example by modification of all replication forks or a change to a different polymerase. Alternatively, there could simply be a series of transient stalling events at each fork in isolation as it encounters successive DNA lesions.

If such fork stalling does occur within the timeframe of a double-labelling experiment (see Fig 4a), it should be detectable in the form of IdU-labelled tracks which fail to incorporate the subsequent 20-minute pulse of CldU because they are currently stalled. These events will therefore appear as an elevated number of red-only tracks (Fig.2).

When the percentage of these red-only tracks was counted, a significant level of fork stalling was indeed found after higher MMS treatments (Fig.6a), supporting the hypothesis that fork slowing occurs via stochastic stalling events. By contrast, IR did not cause significant fork stalling, consistent with the lack of overall fork slowing after IR damage (Fig.6b).

In the case of HU treatment, all forks are essentially stalled by sufficiently high levels of HU. In lower levels of HU, however, replication does proceed at reduced speed (Fig 3b) and in this situation there is elevated fork stalling, detectable in as little as 5µM HU and increasing in a dose-dependent fashion to very high levels when S phase cells are subjected to 20 or 50µM HU (Fig 6c).

Discussion

This work comprises the first systematic investigation of all the various parameters which determine the rate of DNA synthesis in mammalian cells during S phase and the ways in which these parameters are affected by DNA damage. The fibre labelling technique developed
here is an improvement on other methods that have been used to investigate S phase checkpoint responses because it unambiguously separates changes in the rate of origin firing from changes in the rates of fork movement and fork stalling. Using this technique, each of these parameters can be examined quantitatively and under comparable conditions - using the same experimental method throughout. (Labelling cells with short pulses of modified nucleotides does not in itself perturb S phase (23) or activate the S phase checkpoint in yeast (24), so the technique should measure only changes in DNA synthesis which are induced by IR, MMS or HU.) Fibre labelling also offers the advantage of revealing replication dynamics on the level of individual forks rather than as an average of an entire cell population. It does not allow any analysis of replicon clustering in relation to higher-order chromatin or nuclear structure, but it does allow subtle yet potentially important effects on a minority of individual forks to be detected and quantified.

**Effects of IR, MMS and HU on replication dynamics: mammalian cells compared to S.cerevisiae.**

This study shows that different forms of DNA damage affect replication in different ways. Moderate levels of ionising radiation, alkylation by MMS or nucleotide depletion by HU can all slow down the overall progression of S phase. In the case of IR, this slowing appears to be entirely due to a rapid but fairly transient block to origin firing. Alkylation by MMS elicits a similar block to origin firing but this persists for much longer after the removal of the drug than does the block to origin firing after IR. MMS also causes additional changes to replication: a general slowing of fork movement and the stalling of many forks for significant periods, phenomena that are not observed after levels of IR which block origin firing to a similar extent. Finally, nucleotide depletion by HU reduces fork movement (as might be expected since HU blocks RNR and therefore depletes the cell of dNTPs) and this is
accompanied by elevated levels of fork stalling. HU treatment also blocks origin firing but unlike the block elicited by MMS, this is relatively rapidly reversible: there is good recovery of initiation events within 1hr after a brief HU arrest, although the efficiency of recovery declines after longer arrests.

Most of the experiments described were carried out in HeLa cells which lack functional p53. However, the intra-S phase checkpoint is believed to be p53-independent and since all experiments were carried out in synchronised S phase cells, any p53-dependent G1/S checkpoint defects should not be relevant. HeLa cells have been previously shown to downregulate their DNA synthesis in response to both IR and MMS damage (as measured by reduced \(^3\)H thymidine incorporation), supporting the existence of a functional S phase checkpoint in these cells (11,25).

The S phase responses to DNA damage observed here in HeLa cells are thus essentially similar to the responses observed in checkpoint-competent \(S.\text{cerevisiae}\). Exposure of synchronised yeast cells to IR during S phase results in an extension of S phase, probably due to reduced origin firing (26). The response to MMS or HU involves reduced fork movement and fork stalling as well as blocked origin firing (2,3,7): all the same phenomena as are observed here in mammalian cells.

In \(S.\text{cerevisiae}\), the relative checkpoint dependence of each of the phenomena described above has been established: blocked origin firing depends on the Mec1 and Rad53 checkpoint kinases (3,21) and the same proteins are responsible for increasing dNTP levels and for maintaining stalled forks in a stable state (2,6,7). By contrast, the slowing of fork movement is independent of Mec1/Rad53 and has been proposed to be a direct physical result of replisomes encountering alkylated bases or their repair intermediates on DNA (2). It will be of interest to determine if the fork slowing in yeast is a consequence of high rates of fork stalling as appears to be the case in human cells.
Since HeLa cells should be proficient in the intra-S-phase checkpoint, they would therefore be expected to show a full range of checkpoint-dependent as well as checkpoint-independent DNA damage responses and this work would suggest this is indeed true. A degree of ambiguity remains, however, as to which replication phenomena are actually dependent on which, if any, of the mammalian checkpoint proteins. Regarding the two mammalian Mec1 homologues, ATM and ATR, radioresistant DNA synthesis (RDS) is known to occur in ATM deficient cells (9) and there are more recent suggestions that the different sub-pathways acting downstream of ATM play distinct roles in origin firing and fork elongation (27). By contrast, the direct study of the replication role of ATR has until recently been hindered by the fact that ATR is an essential protein. However, cells deficient in the ATR-pathway proteins Hus1 (28) and Chk1 (15) have been studied and ATR has thus been implicated, albeit indirectly, in the inhibition of origin firing after UV and aphidicolin treatment. Future work will use the fibre labelling technique described here, together with recent advances in recombinational knockout and/or siRNA technology, to make a direct comparison of each replication phenomenon separately in checkpoint-competent versus specifically checkpoint-compromised mammalian cells.

**Further insights into replication dynamics obtained from DNA fibre labelling**

The fibre labelling technique used in this study offers a quantitative assessment of replication dynamics with detailed time resolution: it therefore lends itself to the analysis of both dose-dependent and time-dependent effects. This has revealed several aspects of the S phase response to DNA damage which were not apparent from RDS experiments, nor from the population-level studies previously carried out in *S.cerevisiae*.

The slowing of replication forks after MMS treatment, for example, has a non-linear dose-dependence. It is possible that this is due to a thresholded checkpoint response which acts
in trans to slow down all ongoing forks once a critical level of DNA damage is detected, however, a threshold at comparable levels of MMS was not detected in the origin-blocking response (suggesting that if a checkpoint is responsible for both origin-blocking and fork-slowing, the two must at least be differently thresholded). Since in S.cerevisiae the slowing of forks is entirely independent of Mec1/Rad53 (2), it is likely that fork slowing is similarly checkpoint-independent in mammalian cells, being instead a direct result of replication forks encountering DNA lesions. After lower levels of MMS damage, these lesions may be cleared by methods such as base excision repair sufficiently fast that they are not detected by the subsequent fibre-labelling assay, although Fig.4 would suggest that they do generate a sufficient checkpoint signal to inhibit origin firing. At higher levels of MMS, however, repair may become saturated and alkylated lesions and/or repair intermediates may therefore accumulate on the DNA. This would be consistent with the slight recovery in fork rates seen at later times after intermediate MMS treatments, since the accumulated fork-blocking lesions would presumably be progressively removed over time.

Ionising radiation differs from MMS in that no fork-slowing was detected after IR doses of up to 5Gy. If approximately 35 double-strand breaks are induced per Gy (17) then these would be far too infrequent to be detected as fork-blocking lesions, and broken DNA strands are in any case excluded when the data is collected. However, IR is also thought to cause many single-strand breaks and other more minor DNA lesions. In striking contrast to the persistent lesions caused by MMS, it would appear that any single-strand lesions induced by IR are either also too sparse to be detected, or that they do not impede fork movement for any significant length of time, or that they are repaired extremely rapidly. It will be interesting to investigate whether this is still the case – and whether fork slowing after MMS damage is also altered - in cells lacking specific checkpoint pathways: for example, do checkpoint proteins
have secondary roles in promoting specific pathways of damage repair, as well as simply slowing down the cell cycle?

Regarding origin firing, the results presented here differ markedly from recent findings regarding the response to HU in Chinese hamster lung fibroblast cells (22). In these cells, origin firing was not simply inhibited by HU: the firing of a particular ‘dominant’ origin became less efficient but this was accompanied by the activation of normally dormant origins and an increase in the overall density of origin firing. By contrast, in both of the cell types tested here, HeLa cells and primary fibroblasts, origin firing throughout the genome was severely inhibited, even by 20-fold lower HU than the amount used by Anglana et al. (50[M compared to 1mM). This difference may be explained by the fact that the Chinese hamster cells had been selected for resistance to coformycin, an inhibitor of adenylate deaminase2, and this may have selected for cells with a mutated checkpoint response to nucleotide depletion and/or adaptations to reduced nucleotide concentration allowing them to tolerate HU. Consistent with this, fork-slowing exhibited by the Chinese hamster cells in response to HU was also different: in HeLa cells or IMR90 cells, 1mM HU is more than enough to completely arrest cells in early S phase with little or no fork progression. The Chinese hamster cells, however, were reported to show significant S phase progression and great heterogeneity in replication track lengths. This again implies that these cells may have adapted to tolerate disturbed nucleotide metabolism: for example they may possess a mutated form of ribonucleotide reductase which has some resistance to HU. It is also possible that the origins examined by Anglana et al are somehow regulated differently from the majority of genomic replication origins.

It is notable that while initiation events are blocked after both IR and MMS damage, the response to IR appears to be thresholded between 1 and 2.5 Gy whereas the response to MMS increases linearly. This may be due to the fact that IR damage is transduced via ATM and
MMS damage via ATR (29). It has recently been proposed that ATM is activated via a very rapid and sensitive signalling cascade involving intermolecular autophosphorylation and dissociation of ATM dimers, perhaps after the protein senses a global change in chromatin structure induced by double strand breaks (DSBs) (30). The mechanism of ATR activation is presently unknown but if the lesions sensed by ATR do not induce any global changes to chromatin, and/or if they require processing by a replication fork or damage repair pathway before detection, then a comparable all-or-nothing checkpoint response involving ATR might not be expected. In budding yeast and Xenopus, checkpoint activation by MMS requires active replication forks (31-33). Experiments in yeast indicate that the activation of Rad53 in response to HU and MMS requires some threshold number of forks (34). However, above this threshold there is capacity for differential activation of Rad53 depending on the number of stalled replication forks (32).

The allocation of different types of DNA damage to ATM and ATR may also explain the different rates of recovery from IR, MMS or HU. Origin firing appears to recover relatively well after a brief HU arrest, but less efficiently after longer periods of arrest. It also recovers much faster after IR than after MMS. There are at least two possible explanations for these observations. Firstly, recovery from a short HU arrest may be rapid because the transient depletion of nucleotides causes little actual DNA damage and the stimulus for checkpoint signalling would therefore be removed as soon as dNTPs were restored and stalled forks were able to restart. After progressively more time in HU, it may take longer to restore dNTP levels to normal; and/or stalled forks may begin to lose their integrity or be processed via recombinational repair. It has been proposed that recombination is directly responsible for the slowing of fork progression which is elicited by UV or cisplatin, treatments which also cause potentially fork-stalling lesions on DNA (35). If forks stalled by HU are channelled into the same pathway then they may not restart so efficiently after HU release, and may themselves be
sensed as DNA damage, resulting in a more persistent checkpoint signal in the form of stalled forks and/or damage repair structures.

At the opposite end of the spectrum, MMS may cause the greatest number of persistent lesions, generating an ongoing checkpoint signal for longer than do the lesions caused by IR. However, it is not clear that IR-induced lesions are any less persistent than MMS lesions: in fact, recent studies of the persistence of H2AX foci suggest that at least a subset DSBs can remain unrepaired for many hours after irradiation (36), and exposure of HeLa cells to 5Gy in early S phase results in a G2 arrest in most cells up to 40 hours later (data not shown). A second – and not mutually exclusive – hypothesis is therefore that the checkpoint signal during S phase is ‘turned off’ at different rates after IR and MMS damage, perhaps because ATM ceases to respond to persistent DSBs faster than ATR becomes insensitive to any persistent MMS-induced lesions.

Relevance of the S phase checkpoint to cancer therapy

The study of the S phase checkpoint is particularly important in view of its potential role in determining the efficacy and specificity of cancer therapy. If the same checkpoint deficiencies which sensitise tumour cells to chemo- or radio-therapy also allow them to continue replicating regardless of damage and simultaneously reduce their efficiency of DNA repair, then any DNA-damaging therapy could carry a double risk of allowing further dangerous mutations to accumulate in any tumour cells which survive the treatment.

Therefore it is important to consider the actual S phase responses of particular tumours to particular forms of damage. For example, IR-induced DSBs may not themselves prevent the DNA on either side of a break from being correctly replicated and if any other IR-induced lesions – such as SSBs - are rapidly repaired and/or non-fork-blocking, IR may actually be
relatively benign during S phase and lethal only if the cell reaches G2/M with unrepaired chromosome breakage.

By contrast, alkylation by MMS does appear to cause multiple fork-blocking lesions on DNA, giving alkylating drugs a much greater impact on cells within S phase. If the fork stalling caused by such drugs is irreversible in checkpoint-deficient tumour cells (as in checkpoint-mutant yeast strains) (2,6,7), then all of the stretches of DNA between collapsed forks would remain unreplicated at the end of S phase. Catastrophic chromosome breakage and rearrangement would result in any cells which survived mitosis from such a state.

An understanding of all the replication phenomena which are induced by DNA damage, and of the proteins which control these phenomena, may thus lead to the development of better chemotherapeutic drugs – perhaps causing different spectrums of DNA lesions or activating specific aspects of the checkpoint response. In this regard, future work will include the study of replication responses to clinically relevant chemotherapeutic drugs including alkylating agents and topoisomerase inhibitors such as etoposide, whose mode of action on a molecular level remains debatable (37). In the longer term, the information gained from these studies may also contribute to the development of targeted cancer therapies designed on the basis of known checkpoint deficiencies in individual tumours.
Figure Legends

**Figure 1: S phase progression is slowed by IR, MMS and HU**

a) Cells were synchronised by mitotic shakeoff and treated in early S phase with 0.005%-0.03% MMS for 20mins. Cell cycle progression was followed over the next 12hrs by flow cytometry.

b) Cells as in A, exposed to 1-5Gy IR in early S phase.

c) Cells as in A, with 5-100μM HU added to the medium in early S phase.

**Figure 2: Replication track forms visualised by fibre labelling**

a) Schematic of double-labelled replication tracks.

b) Example of labelled replication tracks.

c) Schematic showing alterations to replication tracks as a result of DNA damage.
**Figure 3: Reduced fork progression after DNA damage**

a) Cells were treated with 0.005%-0.03% MMS for 20mins, then MMS was removed and cells were labelled with 50μM IdU for 10-60mins before preparing DNA fibres. The mean length of at least 50 replication tracks is plotted for each timepoint.

b) Synchronised cells in early S phase were labelled with 10μM IdU for 10mins, then 50-100μM HU was added and the IdU was replaced with 100 μM CldU. The mean length of track extension in CldU over the subsequent 6hrs is plotted for each HU concentration.

c) Synchronised cells in early S phase were labelled with 10μM IdU for 10mins, exposed to 1-5Gy IR, then labelled with 100μM CldU for 20mins. The mean total lengths of at least 50 unidirectional (red-then-green) tracks are plotted with the white portions of each bar representing the CldU-labelled length (replicated after IR exposure).

**Figure 4: Inhibition of origin firing after DNA damage**

a) Outline of protocol for measuring origin firing within 20mins of DNA damage.

b) The protocol in A was used to quantify origin firing after 20min pulses of MMS (0.005%-0.02%). The graph shows pooled data from two independent experiments; at least 100 replication tracks were counted in each experiment for each MMS level.

c) As in B, using 1-5Gy IR instead of MMS.

d) As in B, pre-labelling with 20μM not 10μM IdU and adding 50 or 250μM HU together with the CldU directly after this pre-label. Newly fired (CldU-labelled) origins were then allowed to accumulate for 30-50mins (control: white bars), 1-3hrs (50μM HU: grey bars or 2-6hrs (250μM HU: black bars) and were quantified, as before, as a percentage of ongoing tracks.

e) As in D, using unsynchronised IMR90 cells instead of HeLa cells.
**Figure 5: Recovery of origin firing after DNA damage**

(a) Outline of protocol to measure origin firing at timepoints up to 4.5hrs after DNA damage. New origins are counted as a percentage of tracks pre-labelled with IdU before damage.

(b) The protocol in A was used to quantify origin firing 1.5-4.5hrs after a 20min pulse of 0.01% MMS (grey bars), and also in undamaged cells (white bars). The graph shows pooled data from two independent experiments; at least 100 replication tracks were counted in each experiment for each MMS level.

(c) As in B, using 5Gy IR instead of MMS.

(d) Outline of protocol to assess recovery of origin firing after 1-4hrs of HU arrest.

(e) The protocol in D was used to quantify origin firing within 30 or 60mins of release from 2mM HU.

**Figure 6: Fork stalling after DNA damage**

(a) From the labelling protocol outlined in Fig.4A, tracks labelled with IdU-only were counted as a percentage of the total number of tracks after MMS damage. (A proportion of these will represent terminations (see Fig1) but any significant increase over the control percentage is taken as evidence of fork stalling.)

(b) As in A, using 1-5Gy IR instead of MMS.

(c) As in A, labelling cells after 3hrs of replication in low levels of HU.
References


28


Merrick et al. Fig. 1

(a) Control vs. MMS (20 min pulse at 16 hrs)

(b) Control vs. IR at 16 hrs

(c) Control vs. HU at 15 hrs
Merrick et al Fig.2

a

1: Ongoing forks

2: Newly fired origins

3: Terminations

b

Fork slowing:

Undamaged

After damage

Blocked origin firing:

Undamaged

After damage

Fork stalling:
a
Synchronised cells in early S

10min pulse IdU

+/- IR, MMS or HU

20min pulse CldU

Count new origins (CldU tracks)

Merrick et al. Fig. 4

b
d

new origins as percentage of ongoing tracks

% MMS

0 0.005 0.01 0.02

0 2 4 6 8 10

0 1 2 3 4 5 6

new origins as percentage of ongoing tracks

Time (hrs)

0 1 2 3 4 5 6

0 5 10 15 20 25 30

Grays IR

0 1 2.5 5

Time (hrs)
Synchronised cells in early S

10min pulse IdU (a)

+/- IR or MMS, wait 1.5-4.5hrs
10min IdU, 25min CldU

Count CldU (c) against IdU (a)

---

New origins as percentage of tracks labelled before MMS

Time after MMS (hrs)

---

New origins as percentage of tracks labelled before IR

Time after IR (hrs)

---

Synchronised cells in early S

10min pulse IdU

+ 2mM HU, wait 1-4hrs
Wash out HU & IdU, add CldU

Count CldU tracks after 30-60mins
Merrick et al. Fig. 6

(a) 

Percentage Red-only tracks

% MMS

0 0.005 0.01 0.02

(b) 

Percentage Red-only tracks

Grays IR

0 1 2.5 5

(c) 

Percentage Red-only tracks

μM HU

0 5 20 50
Visualisation of altered replication dynamics after DNA damage in human cells
John F.X. Diffley, Dean Jackson and Catherine J. Merrick

J. Biol. Chem. published online February 23, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400022200

Alerts:
- When this article is cited
  - When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts