Oncogene (2001) 20, 3486–3496
© 2001 Nature Publishing Group All rights reserved 0950–9232/01 $15.00
www.nature.com/onc

Detection of repair activity during the DNA damage-induced G2 delay in human cancer cells

Gary D Kao[1], W Gillies McKenna[1] and Tim J Yen*[,2]

[1]Hospital of the University of Pennsylvania, Department of Radiation Oncology, 2 Donner, 3400 Spruce Street, Philadelphia, Pennsylvania, PA 19104, USA; [2]Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania, PA 19111, USA

All eukaryotic cells manifest cell cycle delay after exposure to DNA damaging agents. It has been proposed that such cell cycle checkpoints may allow DNA repair but direct evidence of such activity during the radiation-induced G2 delay has been lacking. We report here that cells arrested in G2 by radiation (2–3 Gy) and etoposide incorporate bromodeoxyuridine (BrdU) at discrete foci in the nucleus. We detected G2 cells with CENP-F, a nuclear protein maximally expressed in G2. Caffeine and okadaic acid, both established radiosensitizers, inhibit the incorporation of BrdU in G2 cells. Radioresistant HT29 and OVCAR cells demonstrate BrdU foci formation more frequently during the G2 delay when compared to the more radiosensitive A2780 cell line. The repair foci formed during G2 may be followed through mitosis and observed in daughter cells in G1. Taken together, these observations are consistent with the detection of DNA repair activity during the radiation-induced G2 delay after relatively low doses of radiation. *Oncogene* (2001) **20,** 3486–3496.

Keywords: DNA damage; G2; DNA repair; BrdU

Introduction

Arrest at specific points in the cell cycle is an almost universal response to DNA damage in eukaryotic cells. The most commonly studied checkpoints have been at G1 and G2. Many human cancer cell lines that lack p53 do not show the DNA-damage induced G1 delay but still block prominently in G2. A number of factors have been implicated as components of the G2 block induced by DNA damage that include transiently decreased $p34^{cdc2}$/cyclin B1 kinase activity (Lock, 1992; Nishii *et al*., 1996; O'Connor *et al*., 1993; Tsao *et al*., 1992), inhibitory phosphorylation of the $p34^{cdc2}$/cyclin B complex, increased cyclin B1 mRNA instability, decreased cyclin B1 protein levels, exclusion of cyclin B1 and cyclin B1-associated kinase activity from the nucleus (Li *et al*., 1997; Jin *et al*., 1998; Maity *et al*., 1995; Kao *et al*., 1999). The G2 DNA damage checkpoint is only partially abrogated by ectopic expression of cyclin B1 (Kao, 1997), constituitively active form of p34cdc2 (Jin *et al*., 1998), or cyclin B1 tagged with a nuclear localization signal (Li *et al*., 1998).

It has been postulated that the G2 cell cycle checkpoint after DNA damage in eukaryotic cells helps ensure the integrity of the genome (Hartwell *et al*., 1994; Featherstone and Jackson, 1999). This could be achieved by allowing time to repair DNA damage-induced lesions, or by preventing cell division and subsequent distribution of damaged DNA or improperly segregated chromosomes to daughter cells. Direct evidence supporting or contradicting either of these hypotheses is scarce, but circumstantial evidence suggests a relationship between the G2 delay and the ability to survive DNA damage. For example, cells treated with agents that shorten the G2 delay, such as caffeine, okadaic acid, or pentoxyphylline, increase radiosensitivity (Jung and Streffer, 1992; Lau and Pardee, 1982). Yeast rad9 mutants do not manifest a G2 delay after DNA damage, and are radiosensitive. To our knowledge, direct evidence of DNA repair during the G2 delay has yet to be demonstrated.

The use of BrdU uptake to mark proliferating cells is well established. Other investigators have pulse-labeled cells exposed to DNA damaging agents with BrdU to obtain evidence of DNA repair (Kalle *et al*., 1993; Selden *et al*., 1993; Balajee *et al*., 1998). However, since both normal S phase cells as well as those engaged in the unscheduled DNA synthesis (UDS) after DNA damage will take up BrdU, the data is difficult to interpret because of the difficulty in ascertaining the cell cycle status of the DNA damaged cells.

In this report, we show unambiguously that cells arrested in G2 after low doses of ionizing radiation and brief etoposide treatment incorporate BrdU in discrete foci. Identification of cells in G2 is made possible by the use of a G2-specific marker protein, CENP-F. BrdU foci were sensitive to radiosensitizers, and could be tracked through recovery from the DNA damaged-induced G2 delay.

*Correspondence: TJ Yen
Received 17 November 2000; revised 2 March 2001; accepted 7 March 2001

Results

CENP-F as a marker for cells in G2 and mitosis

CENP-F is a kinetochore protein that is first detected in the nuclear matrix of cells in the G2 phase of the cell cycle but is then recruited to kinetochores in prophase, where it remains throughout mitosis, and is degraded after cells exit mitosis (Liao *et al*., 1995). As mitotic cells are easily distinguished from interphase cells, we wished to confirm the reliability of using CENP-F nuclear staining as a marker of G2 cells. HeLa cells that were uniformly blocked at the G1/S boundary by thymidine/aphidicolin double block were harvested at 9–10 h after release, when the majority (>90%) had reached G2, with a few cells having entered mitosis. Cyclin B1 expression is also known to be maximal in G2/M (Pines and Hunter, 1994; Juan *et al*., 1997). G2 cells stained brightly for cyclin B1 in the cytoplasm, while the CENP-F were entirely nuclear (Figure 1a). In early prophase cells (in which the chromatin is beginning to condense), cyclin B1 translocated into the nucleus and is colocalized with CENP-F, while mitotic cells also stain brightly for cyclin B1 but CENP-F is concentrated at kinetochores (as discrete pairs of foci). Finally, cells that have just divided and are in G1 no longer expressed CENP-F and cyclin B1.

Three additional experiments were performed to validate CENP-F as a specific marker of G2 cells. To exclude the possibility that these patterns of expression could be an artifact of synchronization, staining was performed on exponentially growing asynchronous cells. As shown above, there was excellent correlation between cells expressing cytoplasmic cyclin B1 and nuclear CENP-F staining (Figure 1b).

To further examine relative expression patterns of proteins known to be cell-cycle regulated, we compared cyclin E and CENP-F. Cyclin E is a nuclear protein expressed in late G1 and S phase cells, and is destroyed prior to onset of G2 phase (Ohtsubo *et al*., 1995). As expected, cyclin E and CENP-F produced mutually exclusive staining patterns (Figure 1c). Cells exhibiting CENP-F staining were negative for cyclin E, while cyclin E positive cells were CENP-F negative.

Lastly, cell-cycle specific expression of CENP-F was examined via dual-parameter flow cytometry, simultaneously probing for CENP-F and DNA content (via Propidium Iodide). This technique allows identification of the protein expression status of individual cells while also identifying where they lie in the cell cycle. In asynchronous cells (Figure 2a), G2/M cells were 2N DNA content show high levels of CENP-F expression, while G1/S phase cells show low levels, consistent with the specific patterns noted via immunofluorescent microscopy. Cells arrested in G2 after 5 Gy ionizing radiation show high levels of CENP-F (Figure 2b).

The combined results demonstrate that *in situ* expression patterns of these proteins are useful markers of the cell-cycle status of individual cells in both synchronized and asynchronous cultures. G2 cells strongly express nuclear CENP-F (as well as cytoplasmic cyclin B1), but cyclin E is absent. In contrast, late G1 and S phase cells show nuclear cylcin E, but not CENP-F, while early G1 cells express none of these proteins. Furthermore, we found CENP-F can be reliably used to identify G2 cells by flow cytometry.

CENP-F staining is not detected in S phase cells

By definition S phase cells are actively replicating DNA and so readily incorporate BrdU, which in turn is readily detectable by immunofluorescence. We next studied the relationship between BrdU uptake, which labels S phase cells, and CENP-F staining patterns in synchronized HeLa cells. Cells were harvested 1 h after release from thymidine/aphidicolin block and then pulsed with BrdU for 1 h and harvested. At this time, the majority of the cells had not yet incorporated sufficient BrdU for detection. These cells also did not exhibit detectable CENP-F (Figure 3a).

When synchronized cells were labeled 3 h after release, they showed robust BrdU incorporation, but still expressed no detectable CENP-F (Figure 3b). Flow cytometry showed that the majority (>90%) of cells at this point had entered S phase. To examine cells that had reached late S and early G2 phase, synchronized cells were prepared as in Figure 3a,b, but BrdU was added 7 h after release, and harvested after 1 h of labeling. By this time, flow cytometry showed most (>90%) of cells had duplicated their DNA, suggesting that S was completed or near so in most cells at this time-point. Far fewer cells showed BrdU uptake, and those that did showed few and limited areas of BrdU uptake (Figure 3c), in contrast to the robust BrdU uptake in early-mid S phase cells in Figure 3b. Most notably, at this cell-cycle stage, cells that showed BrdU uptake still expressed undetectable CENP-F (cell at the right aspect of Figure 3c), while cells strongly expressing CENP-F no longer incorporated BrdU, suggesting that these respectively represent cells in late S and G2.

Foci of BrdU incorporation in G2 cells after exposure to irradiation and etoposide

BrdU is incorporated during replication, but we reasoned that repair of DNA damage would likely also require nucleotide incorporation and therefore may take up BrdU. It would likely be difficult to use BrdU incorporation as a marker for DNA damage during S phase because of the high background BrdU uptake in actively replicating cells. However, as noted above BrdU uptake normally ceases by G2 and therefore repair of DNA damage during G2 could result in detectable BrdU incorporation. In a control experiment, HeLa cells synchronized in G2 were mock irradiated and then pulsed with BrdU for 1 h. As expected, these cells were BrdU negative but CENP-F positive (Figure 4). Parallel samples were irradiated with 2 Gy, and pulsed with BrdU. We selected this dose of irradiation as it has been established to result in the survival of at least half of the irradiated HeLa

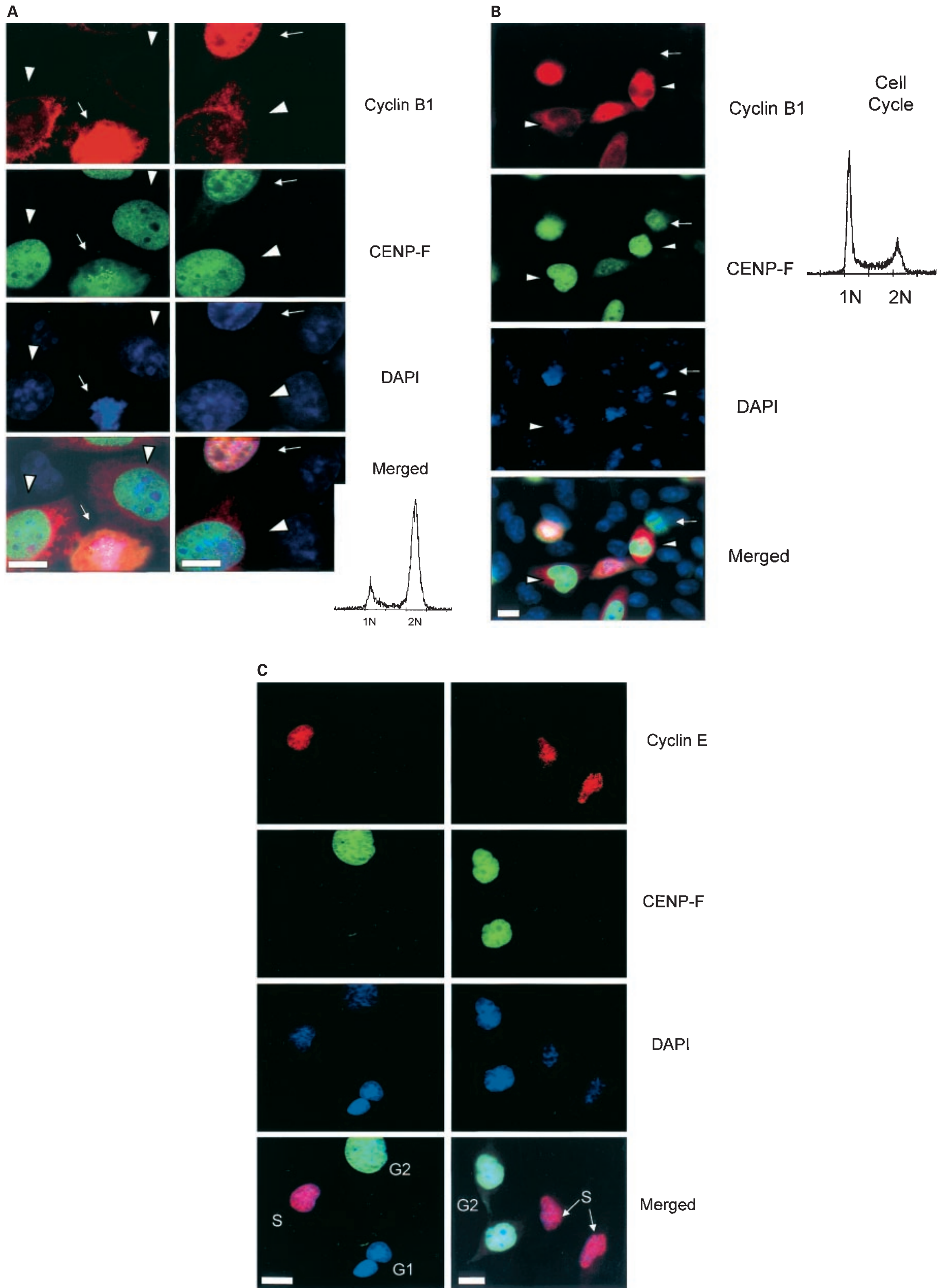

Figure 1 CENP-F is highly expressed by G2 cells. (**a**) Diffuse nuclear staining of CENP-F marks G2 cells. Synchronized HeLa cells harvested in G2/M were stained for CENP-F, cyclin B1, and DAPI. Arrowheads denote G2 cells, which show cytoplasmic cyclin B1 and nuclear CENP-F. Arrows indicate mitotic cells, which show colocalization of cyclin B1 and CENP-F. The arrow in the right panel indicates a late G2/early prophase cell while the left panel arrow indicates a cell in metaphase. A parallel tissue sample of cells synchronized and handled in an identical manner was harvested simultaneously for flow cytometry, and the cell cycle profile shown in the corner histogram. (**b**) CENP-F marks asynchronous G2 cells. Unsynchronized HeLa cells growing in

cells (Nagasawa *et al*., 1994) and so should allow DNA repair activity to occur in a substantial proportion of cells. These irradiated G2 cells again showed strong CENP-F expression, but in contrast to mock-irradiated control cells, discrete foci of BrdU incorporation were readily detectable after irradiation (Figure 4, center panels), many of which were in pairs.

In common with ionizing radiation, treatment of cells lacking wild-type p53 function with etoposide results in a G2 delay (Barratt *et al*., 1998), and is thought to likewise result in double-strand DNA breaks (Kauffmann and Kies, 1998). We therefore assessed the effect of etoposide treatment on BrdU uptake in synchronized G2 cells. Cells treated with etoposide readily showed BrdU uptake during G2 (Figure 4, right-hand panels), in accord with results after irradiation.

We completed two other measures of verifying the specificity of our observations. First, we verified that our observations were not an artifact related to the synchronization procedure, and second, we confirmed that the observations were independent of the type of anti-BrdU antibody used. Asynchronously growing HeLa cells were either mock irradiated, or irradiated with 3 Gy. Cells were returned to the incubator for 2 h before BrdU was added. After 1 h of exposure to BrdU, all cells were harvested. G1 cells showed neither BrdU nor CENP-F staining. In both irradiated and unirradiated cells, strong and diffuse BrdU staining and undetectable CENP-F expression easily identified S phase cells (Figure 5). At the modest dose of radiation used, BrdU uptake is not noticeably changed in S phase cells. Strong CENP-F expression and the

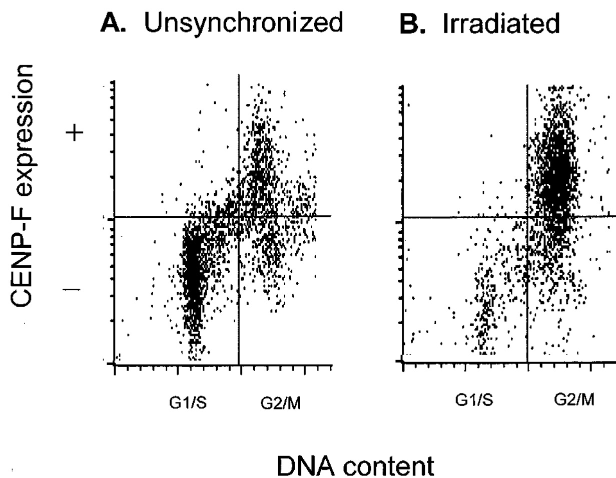

Figure 2 CENP-F expression is maximal in G2/M cells as measured by dual-parameter flow cytometry. (**a**) Asynchronous HeLa cells were simultaneously analysed for CENP-F and DNA content. Permeabilized and paraformaldehyde-fixed cells were probed with Anti-CENP-F antibody, followed by anti-rabbit, FITC-conjugated secondary antibody, while DNA was stained with Propidium Iodide. G1 and early S phase cells (left half of scattergram) show only background CENP-F expression. CENP-F expression is increased in G2/M cells (right half of scattergram). (**b**) Cells were prepared as in (**a**) with the exception that the cells were irradiated with 5 Gy and allowed to accumulate in G2 for 12 h before harvesting. The majority of cells are blocked in G2 (accumulated in the right half of the scattergram), and show high levels of CENP-F expression. The small proportion of residual G1 and S phase cells continue to show low levels of CENP-F expression

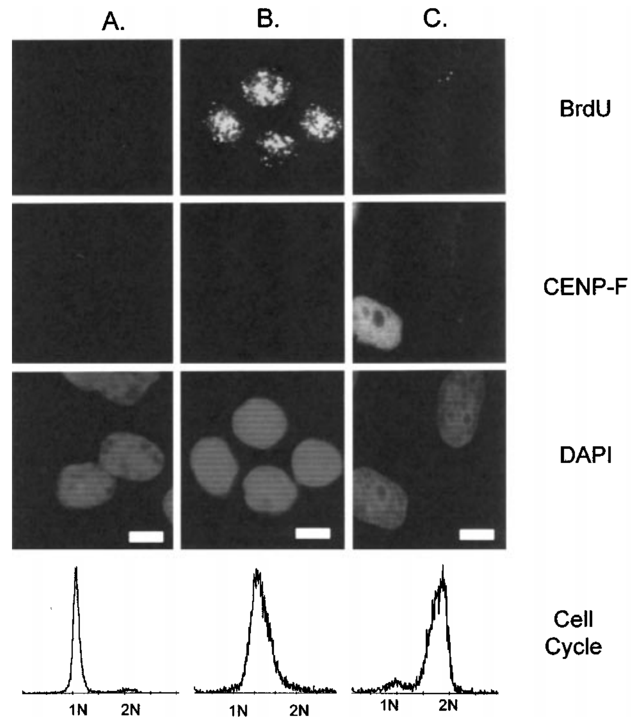

Figure 3 BrdU uptake and lack of CENP-F expression in G1 and S phase cells. (**a**) Synchronized HeLa cells were released, immediately pulsed with BrdU for 1 h, harvested, and stained for BrdU, CENP-F and DAPI. No BrdU and CENP-F is detectable. A parallel tissue sample of cells synchronized and handled in an identical manner was harvested simultaneously for flow cytometry, and the cell cycle profile shown in the histogram. (**b**) HeLa cells were prepared harvested and stained as in (**a**), except that 4 h after release the cells were pulsed with for BrdU for 1 h. A parallel tissue sample of cells synchronized and handled in an identical manner was harvested simultaneously for flow cytometry, and the cell cycle profile shown in the histogram. (**c**) HeLa cells were prepared harvested and stained as in (**a**), except that 7 h after release the cells were pulsed with BrdU for 1 h. A parallel tissue sample of cells synchronized and handled in an identical manner was harvested simultaneously for flow cytometry, and the cell cycle profile shown in the histogram. Bar, 10 μm

exponential phase were harvested and stained for cyclin B1, CENP-F, and DAPI as in (**a**) Arrowheads indicate G2 cells expressing both cyclin B1 and CENP-F, while the arrow points to a mitotic cell in anaphase, in which cyclin B1 has disappeared while CENP-F is fading. A parallel sample of cells handled in an identical manner was harvested simultaneously for flow cytometry, and the cell cycle profile as shown in the histogram. (**c**) Cyclin E and CENP-F expression are mutually exclusive. Unsynchronized HeLa cells growing in exponential phase were harvested and stained for cyclin E, CENP-F, and DAPI. Differential staining patterns of G2, S, and G1 phase cells are as indicated in the merged images. Bar, 10 μm

absence of uniformly diffuse BrdU staining (as seen in S phase cells) marked G2 cells in both irradiated and unirradiated samples. Irradiation resulted in a greater proportion of G2 cells and fewer G1 cells compared to mock-irradiated cells, reflecting the induction of the G2 checkpoint. More importantly, many irradiated G2 cells showed the punctate foci of BrdU uptake similar to those seen in irradiated synchronized cells, confirming our earlier observation was not due to artifact of cell synchronization restricted to irradiated synchronized cells.

Secondly, in order to exclude the possibility that our observations were dependent on the specific type of anti-BrdU antibody used, we compared two different commercial preparations. HeLa cells were irradiated and pulsed with BrdU as in previous experiments. Cells were harvested and probed with either anti-BrdU from Pharmingen (clone 3D4) or Becton-Dickinson Immunocytometry (antibody number 34758083). Results were similar with either antibody (data not shown), supporting the specificity of our observations.

Time course of formation of BrdU repair foci during the radiation-induced G2 delay

In order to determine the time course of formation of radiation-induced BrdU foci, we harvested samples at serial time points after irradiation of synchronized cells. G2 cells were irradiated with 3 Gy and BrdU was immediately added to the media, and samples were harvested (1, 2, 4, 7, and 10 h after irradiation). As expected, irradiation resulted in activation of the G2 checkpoint and cells were inhibited from progression into mitosis (Figure 6). At 15 h after release from the G1/S block, 85% of irradiated cells were still in G2, compared to only 16% for unirradiated cells. There appeared however to be a lag in the formation of BrdU repair foci. One hour after irradiation, even though the majority of cells had reached G2 phase, few showed BrdU repair foci. BrdU repair foci were evident in a considerable proportion of cells at 2 h after irradiation, and the proportion of G2 cells showing BrdU repair foci steadily increased through the G2 delay.

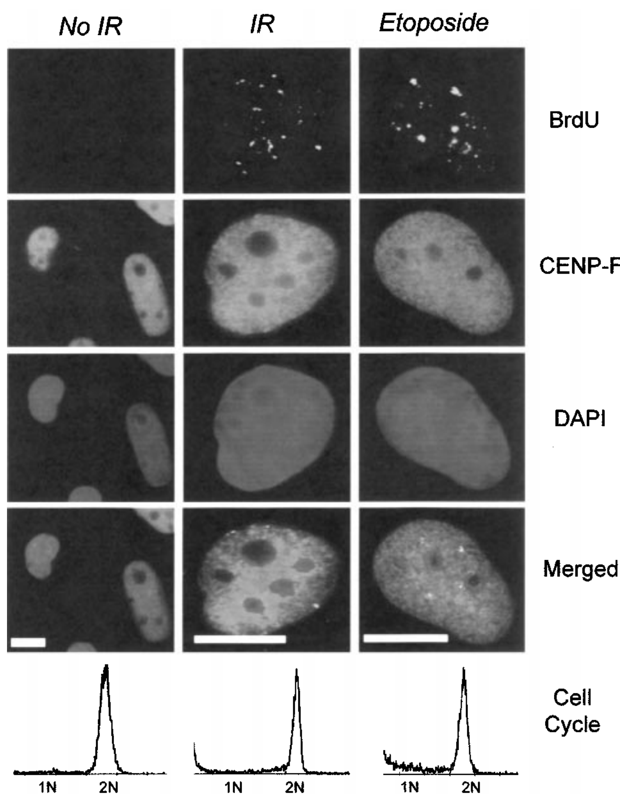

Figure 4 BrdU incorporation is detectable in irradiated and etoposide-treated G2 cells. Synchronized HeLa cells were irradiated with 2 Gy or treated with etoposide (20 ug/ml) when the cells had reached G2 (8 h after release) pulsed with BrdU for 1 h, and stained for BrdU, CENP-F and DAPI, and with parallel samples harvested for cell-cycle analysis via flow cytometry. The etoposide was removed after 15 min incubation and the cells washed twice with PBS prior to replacement with media. Strong CENP-F staining and flow cytometry confirmed that the cells were in G2. In the absence of irradiation, no BrdU is detectable, while irradiated and etoposide-treated cells readily show foci of BrdU uptake. Bar, 10 μm

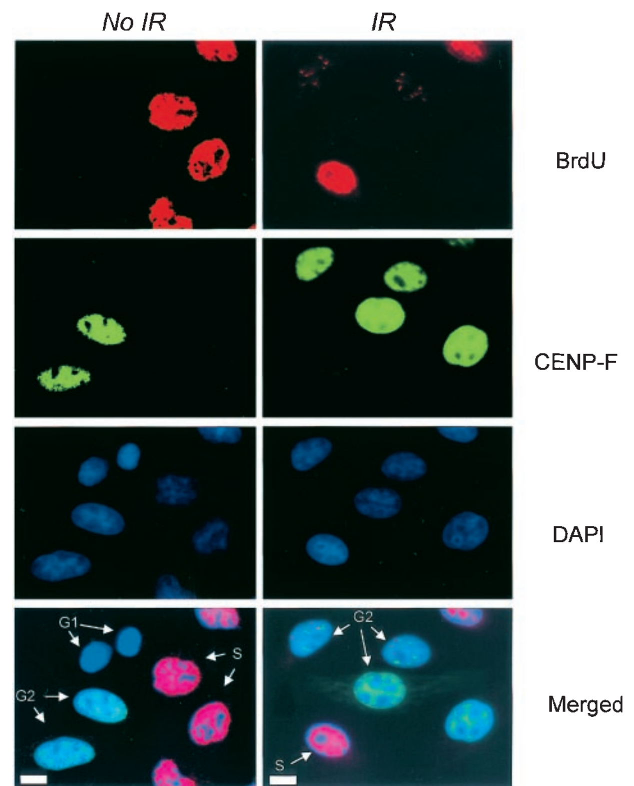

Figure 5 Specificity of BrdU repair foci after irradiation: detection in unsynchronized cells (right hand panels). Unsynchronized HeLa cells were either mock irradiated (left-hand panels), or irradiated with 3 Gy ionizing irradiation. Cells were returned to the incubator for 2 h before BrdU was added. After 1 h of exposure to BrdU, all cells were harvested. Cells in respective phases of the cell cycle are indicated by arrows in merged images (bottom-most panels). G1 cells showed neither BrdU nor CENP-F staining, while strong and diffuse BrdU staining and low levels of CENP-F expression easily distinguished S phase cells. After irradiation, many (but not all) G2 cells show discrete BrdU repair foci (right bottom-most panel). Bar, 10 μm

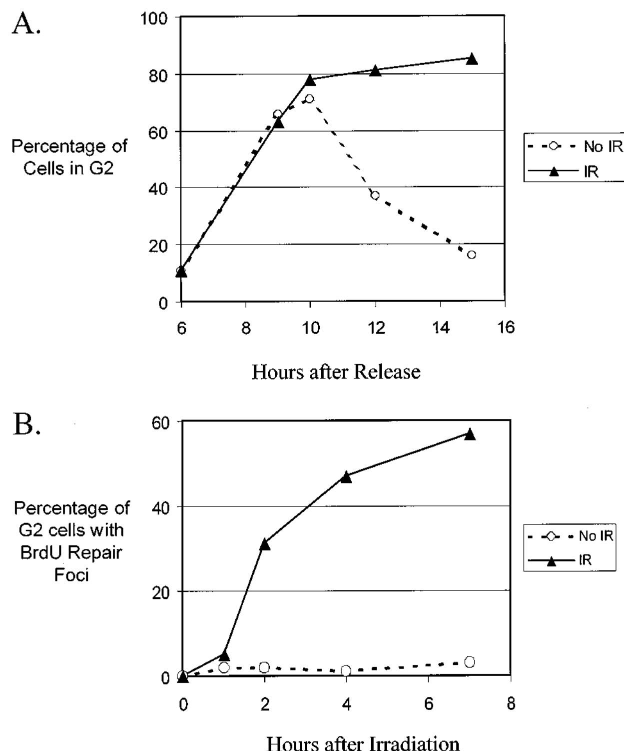

Figure 6 Time course of formation BrdU repair foci after irradiation. (**a**) Synchronized HeLa cells were harvested at serial time points after irradiation. A sample was harvested at 6 h to establish a baseline, and cells were irradiated at 8 h after release with 3 Gy. In all samples G2 cells were determined by CENP-F expression and lack of chromatin condensation (see Figures 1, 2 and 4). Irradiated cells were delayed from exit out of G2 and entry into mitosis compared to unirradiated controls. (**b**) Synchronized cells were irradiated with 3 Gy 8 h after release. BrdU was added immediately after irradiation. Samples were harvested at time points after irradiation as indicated. At each time point the percentage of G2 cells which show foci or BrdU incorporation was determined. A sample was harvested at the time of irradiation (time = 0) to establish a baseline. These experiments were performed three times with similar results

Foci of BrdU incorporation in irradiated G2 cells correlates with radioresistance

A number of agents such as caffeine and okadaic acid (OA) are known to increase radiosensitivity (Murnane, 1995; Rieber and Rieber, 1997; Jacquet *et al*., 1995). While these agents are likely to have myriad intracellular effects, including alterations of kinase activities, we hypothesized that the net result may be inhibition of DNA repair. To test this hypothesis, G2 cells were irradiated (3 Gy) and 200 uM okadaic acid was added at the same time as BrdU and cells were harvested after 1 h. We found that OA profoundly inhibited the formation of foci of BrdU incorporation (Figure 7a, right hand panels) that is usually apparent in control irradiated cells (Figure 7a, center panels). Cells treated with OA but not irradiated also showed no BrdU (Figure 7a, left hand panels) incorporation. Treatment of irradiated cells with 2 mM of caffeine also inhibited irradiation-induced BrdU foci formation (data not shown). In contrast to its inhibitory effects on BrdU incorporation by cells irradiated in G2, caffeine and okadaic acid did not interfere with BrdU incorporation in S phase cells (Beetham and Tolmach, 1982; Jacquet *et al*., 1995; and data not shown).

We wished to determine whether other human cell lines incorporate BrdU in response to irradiation during G2. We reasoned that if BrdU incorporation during irradiation in G2 phase was consistent with DNA repair activity, it might be possible to detect differences between cell lines that reflect their respective degree of radiosensitivity. A recent report measured the radiosensitivities of a panel of cell lines after synchronization (Biade *et al*., 1997). Each cell line was irradiated with 3 Gy 7 h after release from G1/S phase block, pulsed 2 h later with BrdU, harvested, and stained for BrdU and CENP-F. The proportion of G2 cells of each cell line showing foci of BrdU incorporation was recorded. The average (and standard deviation) of three experiments is listed and plotted in Figure 7b and representative images of the majority of cells of each cell line shown in Figure 7c. HT29, HeLa, OVCAR, A2780 show respectively decreasing proportion of cells showing BrdU foci; this order also corresponds respectively to their decreasing established radioresistances. As noted above, the radiosensitizers caffeine and okadiac acid markedly reduced the foci formation in HT29 cells.

Dual parameter flow cytometry detection of BrdU uptake by G2 cells after irradiation

The pattern of BrdU foci in irradiated G2 cells differ from the pattern seen in S phase cells in several ways. As shown in Figures 3 and 5, S phase cells show diffuse staining of BrdU with a 1 h exposure to pulse BrdU, and do not show the discrete paired foci of BrdU that are often characteristic of irradiated G2 cells. Additionally, S phase cells do not exhibit CENP-F staining. Nonetheless, it remains a formal albeit unlikely possibility that BrdU foci formation in irradiated G2 cells may reflect cells regressing back into S phase where they re-replicate some of their DNA. In order to exclude this possibility, we performed flow cytometry to assay simultaneously for BrdU uptake and DNA content to determine where each cell lies in the cell cycle and whether it has taken up BrdU (Hoy *et al*., 1993; Wang and Ellem, 1994). As expected, S phase cells exhibited strong BrdU signal, while unirradiated G2 cells show minimal BrdU uptake (Figure 8b,c). in contrast, irradiated G2 cells with a 2N DNA content showed robust uptake of BrdU (Figure 8d). These data support the findings on immunofluorescence of BrdU foci formation in cells blocked in G2 after irradiation.

DNA damage repair foci may be detected in cells that have recovered from the DNA-damaged induced G2 delay

Many cells that are irradiated with the modest doses of radiation used in this report are eventually able to overcome the G2 checkpoint and enter mitosis. Having

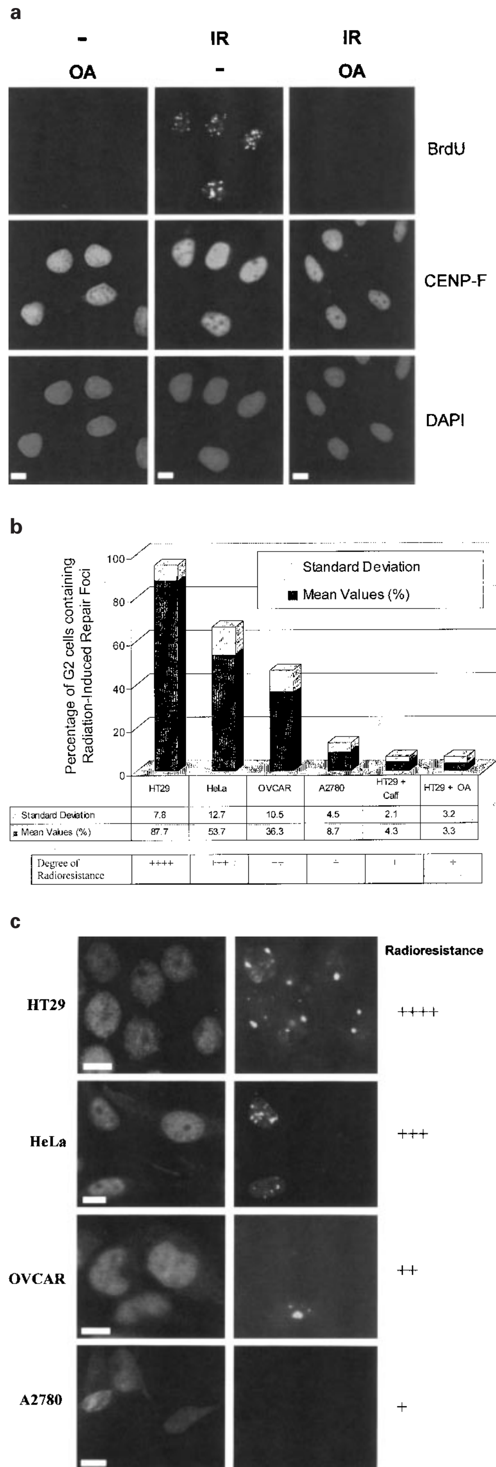

Figure 7 BrdU foci after irradiation correlates with radioresistance. (**a**) Synchronized HeLa cells were released, irradiated, and pulsed with BrdU as in Figure 4. The radiosensitizer okadaic acid (200 nm) was added at the same time as BrdU. Coverslips were stained for BrdU, CENP-F and DAPI as indicated. Okadaic acid treatment of unirradiated cells has no effect on BrdU uptake (left-hand panels), while irradiated cells readily show foci of BrdU (center panels). In contrast, okadaic acid treatment markedly inhibit BrdU uptake after irradiation. (**b**) The indicated cell lines were synchronized, irradiated with 3 Gy during G2, pulsed with BrdU, and stained for CENP-F and BrdU. At least 300 cells were counted and the percentage of G2 cells of each cell line showing BrdU foci of the total cells was recorded. Plotted values are the average count and error bars show standard deviation. Relative radioresistance of each cell line is derived from Chapman *et al*. (**c**) Representative images of irradiated G2 cells of the cell lines analysed in Figure 6b are shown. Cells were stained for CENP-F and BrdU. The proportion of G2 cells (marked by strong CENP-F) showing BrdU uptake correlated with the established radioresistance. Images shown were representative of the greater than 80% of cells in each cell line. Bar, 10 μm

established that DNA repair foci can be visualized during the G2 delay, we wished to determine whether these foci of BrdU could be detected as cells enter and complete mitosis. Cells were synchronized, irradiated in G2 with 2 Gy (to allow survival of most cells), and pulsed with BrdU for 1 h followed by removal of the BrdU and chase with 0.2 mM thymidine. Instead of harvesting the cells during the G2 block as in previous experiments, cells were allowed to complete mitosis and progress into G1 (20 h after release). BrdU-labeled foci can still be readily detected. Interestingly, occasional foci are localized outside the nucleus (Figure 9a) and between daughter cells (indicated by arrowheads in Figure 9b,c) and which visibly colocalize with DAPI-stained chromatin. It appears the DNA repair activity that resulted in BrdU incorporation at these sites of DNA damage may not have been sufficient to result in nuclear retention of these fragments of chromatin. These observations are consistent with previous reports of the expulsion of portions of the chromatin from the nucleus following exposure to DNA damage (Haaf *et al*., 1999). It remains to be determined whether these may in fact represent incomplete or unsuccessful repair of DNA damage. Nonetheless, the techniques used here allow one to track the fates of individual sites of DNA damaged with radiation through the cell cycle.

Discussion

We have employed a number of techniques that allows detection of the cell-cycle status of individual cells. Synchronization enables a population of cells more uniform in cell cycle status. CENP-F expression allowed us to distinguish G2 from S phase cells. BrdU uptake, normally absent in G2 cells that have completed replication, was induced by DNA damage in the form of clearly defined foci. The BrdU foci induced by irradiation during the G2 checkpoint is distinguishable from the diffuse staining noted in S phase cells, partially co-localize with BRCA1, and may be tracked through the completion of mitosis. The observations presented here provide evidence that DNA damage repair occurs during the DNA damage-induced G2 delay.

Patterns of BrdU incorporation have been extensively characterized in proliferating cells (Kill *et al*., 1991; O'Keefe *et al*., 1992; Nakayasu and Berezny, 1989; Van Dierendonck *et al*., 1989; Fox *et al*., 1991). Using synchronized cells blocked in S phase with hydroxy-urea, O'Keefe *et al*. (1992) identified five

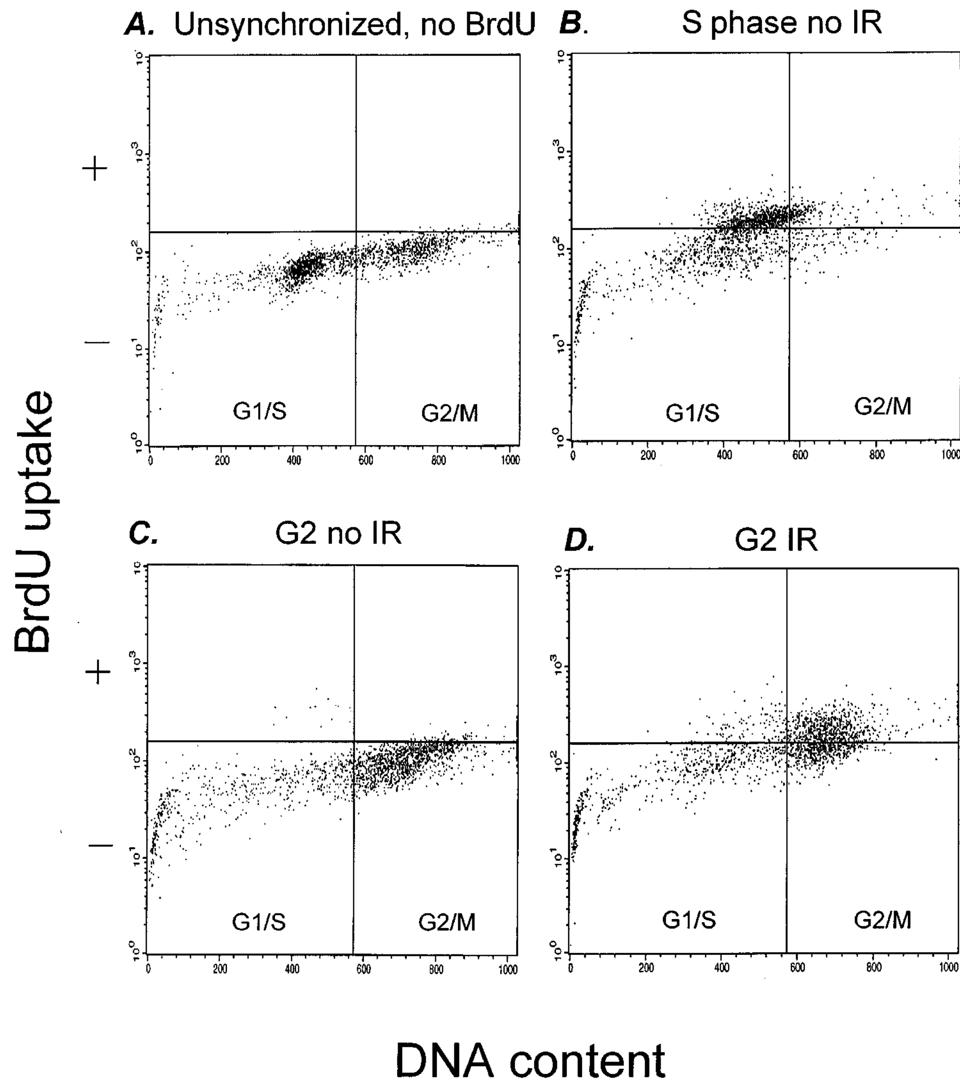

Figure 8 Two parameter flow cytometry: G2-specific uptake of BrdU after irradiation. (**a**) shows unsynchronized control cells not pulsed with BrdU. (**b**) shows synchronized control cells pulsed with BrdU for 1 h in S phase and harvested. (**c**) shows synchronized HeLa cells harvested in G2 at the same time as (**d**), in which synchronized HeLa cells were irradiated in G2, pulsed with BrdU, and harvested. All samples were processed for BrdU uptake and DNA content as described in Materials and methods

distinct patterns of DNA replication during S phase. Immediately after release, cells in early-mid S phase show diffuse staining throughout the nucleus, while late S cells show discrete but large areas of replicating DNA. We have also seen these different patterns in proliferating HeLa cells (data not shown), but these patterns appear distinct from the foci of BrdU uptake noted in irradiated HeLa cells blocked in G2. In the report by O'Keefe *et al.*, (1992) immunoelectron microscopy was additionally used to localize areas of BrdU uptake to submicroscopic structures in hydroxyurea-synchronized cells, confirming that early replication is localized to euchromatin, followed by localization to the peripheral heterochromatin, including electron-dense areas. It remains to be seen whether techniques such as immunoelectron microscopy may localize DNA repair foci to specific submicroscopic nuclear structures.

The detection of DNA damage by measuring BrdU uptake has been well-established (Beisker and Hittelman, 1988; Cohn and Lieberman, 1984; Vijg *et al.*, 1984; Stefanini *et al.*, 1982; Zelle *et al.*, 1980; Ahmed and Setlow, 1977; Richold and Arlett, 1972). These efforts, however, are limited by the inability to reliably distinguish S phase cells from those engaged in unscheduled DNA synthesis (UDS) as a result of damage. More recently, BrdU incorporation after DNA and DNA content have been measured simultaneously with dual-parameter flow cytometry (Wang and Ellem, 1994; Hoy *et al.*, 1993). This enabled the tracking of individual and actively replicating cells, but is still limited by the inability to reliably distinguish G2 from mitotic cells, as well as detecting patterns of localization of the incorporated BrdU in the nucleus. The use of a marker specific for G2 cells and visible via immunofluorescence such as CENP-F should therefore

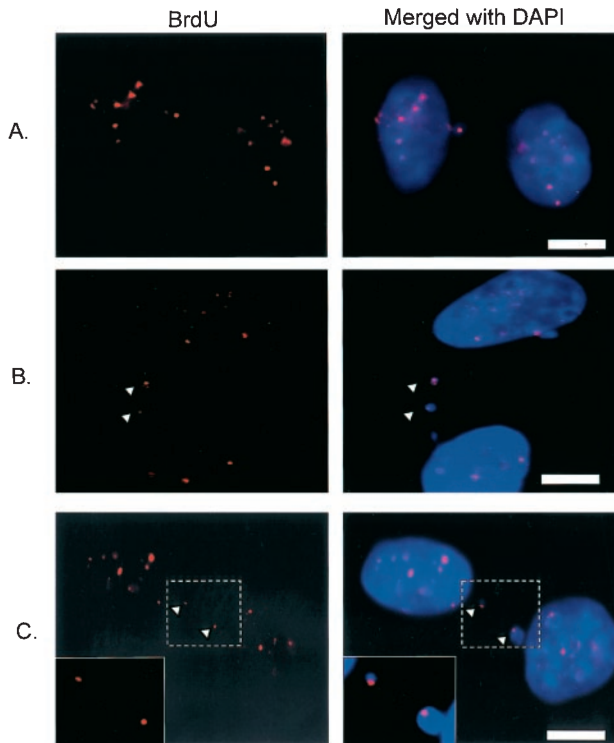

Figure 9 BrdU repair foci can be detected in irradiated cells that have recovered from the G2 delay. Synchronized HeLa cells were irradiated with 2 Gy and pulsed with BrdU in G2. Cells were allowed to complete mitosis and divide into daughter cells (re-entering G1 at 20 h after release) before harvest and staining for BrdU (left-hand panels) and DAPI (BrdU and DAPI merged in right-hand panels). BrdU repair foci are readily apparent in each case. Occasional repair foci appear to be excluded from the nucleus following mitosis (**a**), and stranded between daughter cells (arrowheads (**b**,**c**)). The region represented by the dashed box is magnified in **c**. Bar, 10 μm

be useful in studying cell-cycle specific events by helping define the cell cycle state of individual cells.

As noted above, BrdU uptake has traditionally been used to distinguish S phase cells from G1 and G2 cells. However, incorporation of BrdU by G2 cells in the process of repairing DNA damage implies that BrdU uptake alone is insufficient to distinguish S phase cells from G2 cells that are undergoing DNA repair. The simultaneous detection of CENP-F as a G2-specific marker therefore allows this distinction and establishes with greater certainty the existence of repair activity in G2 cells. It should be noted that our findings of DNA repair activity during the G2 checkpoint do not exclude the possibility that DNA damage repair also occurs in other parts of the cell cycle. Indeed, a number of observations suggest that repair activities may occur in S phase as well. BRCA1 foci have been reported to form in S phase cells after exposure to DNA damage (Scully *et al*., 1997; Paull *et al*., 2000; Zhong *et al*., 1999; Wang *et al*., 2000)). It has been hypothesized that DNA repair by non-homologous end-joining predominates in G1-early S phase cells, while recombinational repair is preferentially used in late S-G2 cells (Takata *et al*., 1998). Techniques such as those presented in our studies may be useful in distinguishing such cell-cycle specific activities.

In summary, the observations reported here are consistent with the notion that cell cycle checkpoints may allow the cell additional time to repair DNA damage. We have further found a correlation between the established radiosensitivity of a number of cell lines with the proportion of cells that demonstrate DNA repair foci during the G2 checkpoint. These findings teleologically may help explain why tumor cells employ a multiplicity of seemingly redundant mechanisms to effect the G2 DNA damage checkpoint. These results further suggest that measures targeting the G2 checkpoint may increase the therapeutic efficacy of DNA-damaging treatments targeting tumor cells, many of which already lack the G1 checkpoint due to inactivation of wild-type p53 function.

Materials and methods

Cell lines, culture conditions, synchronization, irradiation, and BrdU pulse-labeling

HT29, HeLa, OVCAR and A2780 cells were obtained from American Type Culture Collection (ATCC). All synchronization was performed via the double thymidine/aphidicolin sequential block as previously described (Kao *et al*., 1999), which results in synchronization of >90% of cells. Cells blocked at the G1/S transition were released and fixed at times indicated. Cells were irradiated while attached to culture dishes with Cesium-137 gamma rays from a Shepherd 81-14R Panoramic Irradiator (JL Shepherd and Associates) at a dose rate of 1.35 Gy/min. Etoposide was ordered from Sigma-Aldrich (St. Louis, MO, USA). After irradiation, etoposide treatment, or mock-irradiation, cells were immediately returned to the incubator and allowed to equilibrate for at least 1 h before subsequent processing. BrdU pulse-labeling was performed with a cell proliferation kit from BD Immunocytometry.

Antibodies

Rabbit polyclonal antibodies specific for CENP/F were prepared and characterized as previously described (Liao *et al*., 1995), and used at 1 : 1000. Anti-BrdU antibodies were from BD Immunocytometry (San José, CA, USA, Cat. No. 34758083) and Pharmingen (San Diego, CA, USA, clone 3D4, Cat. No. 33281A), and used at concentrations of 1 : 10 and 1 : 1000 respectively. Anti-cyclin B1 ((GNS-1) Pharmingen) and anti-cyclin E ((HE111) Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibodies were used at 1 : 1000.

Immunofluorescence

Immunofluorescence was performed via a modification of the methods used by Sherwood *et al*. (1994). Briefly, cells were grown on glass coverslips, synchronized using the same procedure described above, and treated as indicated in the figure legends. At the indicated times they were washed in PBS, and fixed by immersion in ice-cold absolute methanol/acetone (1/1 v/v) for 5 min. Coverslips were incubated at room temperature for 1 – 2 h with the respective antibodies in KB buffer. The coverslips were washed in PBS containing

0.2% Triton after which fluorescein-labeled secondary antibodies (Molecular Probes, Oregon) were applied for 1 h. Coverslips prepared in the same way, in which the primary antibody was omitted, showed no immunofluorescence. The secondary antibody was removed and the coverslips again washed in PBS prior to counterstaining nuclei and chromosomes were stained with 4′,6-Diamidino-2-Phenylindole (DAPI) at 2.5 μg/ml. Photographs were taken using a Nikon Microphot SA equipped with epifluorescence optics.

Flow cytometry

All flow cytometry were performed on a Becton Dickinson (Sunnyvale, CA, USA) flow cytometer following as described in Dolbeare *et al.* (1983). Briefly, during flow cytometry, cells were excited at 488 nm. Red fluorescence from propidium iodide was collected through a 600 nm-long wavelength pass filter and recorded as a measure of total DNA content. Dual parameter flow cytometry was performed according to the method of Juan *et al.* (1997), and green fluorescence from fluorescein was also collected through a 514 nm bandpass filter. Ten thousand cells were analysed for each sample.

Acknowledgments
We would like to thank members of the Yen lab for invaluable discussions and comments on the manuscript, and to D Chapman and C Stobbe for assistance with the irradiation. Ms Roseanne Diehl superbly assisted the preparation of this manuscript. TJ Yen was supported by grants from the NIH, PO1 core grant CA06927, and an Appropriation from the Commonwealth of Pennsylvania.

References

Ahmed FE and Setlow RB. (1977). *Cancer Res.*, **37,** 3414 – 3419.
Balajee AS, May A and Bohr VA. (1998). *Mutation Res.*, **404,** 3 – 11.
Barratt RA, Kao G, McKenna WG, Juang J and Muschel RJ. (1998). *Cancer Res.*, **58,** 2639 – 2645.
Beetham KL and Tolmach LJ. (1982). *J. Cell. Physiol.*, **113,** 385 – 397.
Beisker W, Hittelman WN. (1988). *Exp. Cell. Res.*, **174,** 156 – 167.
Biade S, Stobbe CC and Chapman JD. (1997). *Radiat. Res.*, **147,** 416 – 421.
Cohn SM and Lieberman MW. (1984). *J. Biol. Chem.*, **259,** 12463 – 12469.
Dolbeare F, Gratzner H, Pallavicini MG and Gray JW. (1983). *PNAS,* **80,** 5573 – 5577.
Featherstone C and Jackson SP. (1999). *Curr. Biol.*, **9,** R759 – R761.
Fox MH, Arndt-Jovin DJ, Jovin TM, Baumann PH, Robert-Nicould M. (1991). *J. Cell. Sci.*, **99,** 247 – 253.
Gowen LC, Avrutskaya AV, Latour AM, Koller BH, Leadon SA. (1998). *Science,* **281,** 1009 – 1012.
Haaf T, Raderschall E, Reddy G, Ward DC, Radding CM and Golub EI. (1999). *J. Cell. Biol.*, **144,** 11 – 20.
Hartwell LH and Kastan MB. (1994). *Science,* **266,** 1821 – 1828.
Hoy CA, Carswell C and Schimke RT. (1993). *Mutat. Res.*, **290,** 217 – 230.
Jacquet P, de Saint-Georges L, Barrio S, Baugnet-Mahieu L. (1995). *Int. J. Radiat. Biol.*, **67,** 347.
Jin P, Hardy S and Morgan D. (1998). *J. Cell. Biol.*, **141,** 875 – 885.
Juan G, Li X and Darzynkiewicz Z. (1997). *Cancer Res.*, **57,** 803 – 807.
Jung T and Streffer C. (1992). *Int. J. Radiat. Biol.*, **62,** 161 – 168.
Kalle WHJ, Haekamp-van Dokkum AM, Lohman PHM, Nataranjan AT, van Zeeland AA and Mullenders LHF. (1993). *Anal. Biochem.*, **208,** 228 – 236.
Kao G, McKenna WG, Maity A, Blank K, Muschel RJ. (1997). *Cancer Res.*, **57,** 753 – 758.
Kao GD, McKenna WG and Muschel RJ. (1999). *J. Biol. Chem.*, **274,** 34779 – 34784.
Kaufmann WK, Kies PE. (1998). *Mutat. Res.*, **400,** 153 – 167.
Kill IR, Bridgger JM, Campbell KHS, Maldonado-Codina G and Hutchinson CJ. (1991). *J. Cell Sci.*, **100,** 869 – 876.
Lau CC and Pardee AB. (1982). *PNAS USA,* **79,** 2942 – 2946.
Li J, Meyer A and Donoghue D. (1997). *PNAS USA,* **94,** 502 – 507.
Liao H, Winkfein RJ, Mack G, Rattner JB and Yen TJ. (1995). *J. Cell Biol.*, **130,** 507 – 518.
Lock RB. (1992). *Cancer Res.*, **52,** 1817 – 1822.
Maity A, McKenna WG and Muschel RJ. (1995). *EMBO J.*, **14,** 603 – 609.
Murnane JP. (1995). *Cancer and Metastasis Reviews,* **14,** 17 – 29.
Nagasawa H, Keng P, Harley R, Dahlberg W and Little JB. (1994). *Int. J. Radiat. Biol.*, **66,** 373 – 379.
Nakayasu H and Berezney R. (1989). *J. Cell. Biol.*, **108,** 1 – 11.
Nishii K, Kabarowski JH, Gibbons DL, Griffiths SD, Titley I, Wiedemann LM and Greaves MF. (1996). *Oncogene,* **13,** 2225 – 2234.
O'Connor PM, Ferris DK, Pagano M, Draetta G, Pines J, Hunter T, Longo DL and Kohn KW. (1993). *J. Biol. Chem.*, **268,** 8298 – 8308.
Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM and Pagano M. (1995). *Mol. Cell. Biol.*, **15,** 2612 – 2624.
O'Keefe RT, Henderson SC and Spector DL. (1992). *J. Cell. Biol.*, **116,** 1095 – 1110.
Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M and Bonner WM. (2000). *Curr. Biol.*, **10,** 886 – 895.
Pines J and Hunter T. (1994). *EMBO J.*, **13,** 3772 – 3781.
Richold M and Arlett CF. (1972). *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.*, **21,** 127 – 136.
Rieber MS and Rieber M. (1997). *DNA Cell Biol.*, **16,** 121 – 125.
Scully R, Chen J, Ochs RL, Keegan K, Hoekstra M and Feunteun J. (1997). *Cell.*, **90,** 425 – 435.
Selden JR, Dolbeare F, Clair JH, Nichols WW, Miller JE, Kleemeyer KM, Hyland RJ and DeLuca JG. (1993). *Cytometry,* **14,** 154 – 167.
Sherwood S, Rush D, Kung A and Schimke R. (1994). *Exp. Cell Res.*, **211,** 275 – 281.
Stefanini M, Reuser A and Bootsma D. (1982). *Somatic Cell Genet.*, **8,** 635 – 642.

Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, Yamaguchi-Iwai Y, Shinohara A and Takeda S. (1998). *EMBO J.,* **17,** 5497–5508.

Tsao Y-P, D'Arpa P and Liu L. (1992). *Cancer Res.,* **52,** 1823–1829.

Van Dierendonck JH, Keyzer R, van de Velde CJH and Cornelissse CJ. (1989). *Cytometry,* **10,** 143–150.

Vijg J, Mullaart E, van der Schans GP, Lohman PH and Knook DL. (1984). *Mutat. Res.,* **132,** 129–138.

Wang XQ and Ellem KA. (1994). *Exp. Cell. Res.,* **212,** 176–189.

Wang Y, Cortez D, Yazdi P, Neff N, Elledge SJ and Qin J. (2000). *Genes Dev.,* **14,** 927–939.

Zelle B, Berends F and Lohman PH. (1980). *Mutat. Res.,* **73,** 157–169.

Zhong Q, Chen CENP-F, Li S, Chen Y, Wang CC, Xiao J, Chen PL, Sharp ZD and Lee WH. (1999). *Science,* **285,** 747–750.