

Detection of DNA double-strand breaks through the cell cycle after exposure to X-rays, bleomycin, etoposide and ^{125}I dUrd

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Abstract. Ionizing radiation-induced DNA double-strand breaks (DSBs) are generally more difficult to detect in S-phase cells than in cells from other phases of the cell cycle. To explore the basis for this observation, other double-strand breaking agents were examined: etoposide, bleomycin and ^{125}I dUrd. DSBs induced by these agents in single cells in S, G₁ or G₂/M-phases of cell cycle were measured using the neutral comet assay. Regardless of the nature or location of the DSBs, Chinese hamster V79 cells with S-phase DNA content showed about 2-3 times less damage by all agents than cells with G₁ or G₂/M-phase DNA content. Residual protein content measured after lysis of S-phase cells embedded in agarose did not differ significantly from the protein content of asynchronous cells, and removal of proteins prior to irradiation did not enhance S phase migration. The number of DSBs, the physical nature of the DSB, or the presence of residual proteins, did not appear to influence migration. Therefore, we conclude that differences in DNA structure are responsible for reduced sensitivity for detecting DSBs in S-phase cells.

1. Introduction

DNA from cells in the DNA synthetic (S) phase of the cell cycle generally shows fewer radiation-induced double-strand breaks (DSBs) than DNA from cells in other phases of the cycle. S-phase DNA appears less able to migrate in an electric field (Stamato and Denko 1990, Iliakis *et al.* 1991, Olive *et al.* 1991), less able to elute from filters during neutral filter elution (Radford and Broadhurst 1988, Sweigert *et al.* 1988, Okayasu *et al.* 1988), and more likely to be complexed with other molecules during sucrose gradient centrifugation (Okayasu *et al.* 1988). This effect can present a problem in accurately quantifying DSBs in asynchronous cultures.

S-phase retardation could be the result of the presence of replication-related enzymes tightly associated with DNA which can be resistant to detergents and even proteinase K digestion (Bodnar *et al.* 1983). An alternative explanation, which we favour, is that removal of these proteins during lysis results

in a tangle of partly replicated DNA molecules which resist lysis, hydrodynamic shear, or charge forces involved in filter elution, migration or sedimentation.

Several investigators have shown that replication clusters, identified by anti-BrdUrd staining, appear as granules tightly associated with the nuclear matrix (Nakayasu and Berezney 1989, Nakamura *et al.* 1986, Fox *et al.* 1991). Cells in early S-phase contain 200-500 of these clusters which subsequently disperse as the cells exit S-phase. Such structures could have a much larger effective size and therefore migrate less effectively in the electric field. At a smaller scale, replication forks or bubbles, known to have different mobilities in agarose (Brewer and Fangman 1987), could impede migration of DNA from S-phase cells.

Previous results have shown that differences in DNA migration or elution can be observed even in unirradiated cells (Wlodek and Olive 1992, Olive *et al.* 1991). If S-phase retardation is independent of the nature or location of the DNA DSB, then a similar effect should be observed for other DSB-inducing agents. Three agents were chosen for analysis using the comet assay. Bleomycin, a chemotherapeutic agent, produces DNA DSBs after formation of a complex with DNA, Fe²⁺ and O₂ (Burger *et al.* 1981); there also appears to be sequence specificity associated with damage (Murray and Martin 1985). Previous results by Ostling and Johanson (1987) indicated considerable variability in DNA damage between individual cells exposed to bleomycin which might also be attributed in part to S-phase retardation.

Decay of ^{125}I incorporated in DNA produces DSBs with high efficiency, and the local damage to DNA is more extensive than for X-rays. The use of ^{125}I also provides an opportunity to calibrate the comet assay since incorporated ^{125}I dUrd decay produces DSBs with near unit probability (Charlton 1986), and it is therefore possible to assume that one decay produces one DSB (Radford and Hodgson 1985).

The third agent, etoposide, is a topoisomerase II

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poison that produces DSBs at sites of topoisomerase II binding to DNA (Liu *et al.* 1980, Osheroff 1989). Topoisomerase II is a component of the nuclear matrix of proliferating cells (Earnshaw and Heck 1985, Heck and Earnshaw 1986), and the matrix is also the site of DNA replication (Berezney and Coffey 1976). Topoisomerase II inhibitors like etoposide block the formation of replication forks (Fernandes and Catapano 1991). Evidence suggests that topoisomerases may react preferentially with DNA crossovers (Zechiedrich and Osheroff 1990) perhaps stabilizing the replisomes described above. We therefore examined the hypothesis that etoposide-induced DSBs in matrix-associated DNA could prevent S-phase retardation by selectively damaging the replication clusters which retard migration. If DNA from S-phase cells migrates as efficiently as DNA from G₁ or G₂ cells after treatment with etoposide, this would support the hypothesis that matrix attachment is involved in S-phase retardation.

2. Materials and methods

2.1. Cell culture and drug exposure

Chinese hamster V79-171b lung fibroblasts were maintained as exponentially-growing monolayers by subcultivating twice a week in minimal essential medium (MEM) containing 10% foetal bovine serum (FBS) from Sigma Chemical Co. (St. Louis, MO, USA.) Cells on ice were exposed to 250 Kvp X-rays. Etoposide was purchased from Bristol Canada as a 20-mg/ml solution, and was diluted immediately before use. Bleomycin sulphate was obtained as a clinical formulation from Bristol, and 230 μ M ferrous ammonium sulfate was included in the medium during incubation. Cells attached to Petri plates were incubated for 1 h with etoposide or bleomycin at 37°C, then trypsinized and diluted in ice-cold phosphate-buffered saline (PBS) for the comet assay. ¹²⁵IIdUrd (69.8 TBq/mmol) was incubated with cells for 18 h at a concentration of 18.5 KBq/ml in complete medium containing 2.5 μ M thymidine; cells were chased for 1 h in isotope-free medium and then frozen in liquid nitrogen at a density of 2.5 $\times 10^4$ cells/ml in MEM + 10% FCS (no DMSO) for up to 4 weeks. Cultures without ¹²⁵I, but treated identically, served as controls in the comet assay. This labelling protocol gave a mean of about 350 decays/cell/day as determined by γ counting. Correction was made for the 60-day half-life of the isotope.

For sensitive detection of DNA damage by etopo-

side, we included 0.5 mg/ml proteinase K in the lysis buffer. For damage by bleomycin, X-rays and ¹²⁵IIdUrd, proteinase K in the lysis buffer did not alter sensitivity for detecting DSBs. Bleomycin, however, was much more effective in inducing DNA damage in permeabilized cells, so that we pretreated cells with 0.025% saponin for 2 min at room temperature (Gedik and Collins 1991) before incubation with bleomycin.

2.2. Cell sorting

For some experiments, cells were selected from various phases of the cell cycle by first incubating monolayers with 5 μ M Hoechst 33342 for 30 min at 37°C and then sorting single cells on the basis of Hoechst 33342 fluorescence intensity (Olive *et al.* 1991). Single cells were analysed using a Becton-Dickinson FACS 440 dual laser cell sorter. Windows were selected from various phases of the cell cycle based on Hoechst fluorescence intensity under UV excitation using the 350–360 nm lines from an argon laser operated at 40 mW power. With the G₁ peak in channel 80, G₁ cells were sorted from channels 50 to 85, early S from channels 90 to 104, mid-S from 105 to 120, and G₂/M from 150 to 180. Cells sorted directly into medium were then centrifuged and resuspended in ice-cold PBS for irradiation on ice. This treatment was not found to induce strand breaks in V79 cells, and no difference was observed in the response of cells which were not sorted and those which were passed through the FACS.

2.3. Comet assay

Single cells were centrifuged and resuspended in ice-cold PBS at a concentration of 3 $\times 10^4$ cells/ml. Following irradiation or drug treatment, 0.5 ml cell suspension (1.5 $\times 10^4$ cells) was placed in a 5-ml disposable tube and 1.5 ml 1% low gelling temperature agarose (Sigma type VII boiled in distilled water and held at 40°C), was added to the tube. The contents were quickly pipetted onto a fully frosted microscope slide and allowed to gel for about 30 s on a cold surface. Slides were carefully submerged in lysing solution consisting of 30 mM EDTA, 0.5% SDS, pH 8.0, and the temperature was raised to 50°C for 4 h. Slides were then washed free of detergent in a large volume of TBE buffer (90 mM Tris, 2 mM EDTA, 90 mM boric acid, pH 8.5) for 2–16 h followed by electrophoresis in TBE buffer at 0.55 volts/cm for 25 min. Slides were rinsed and stained

for 1 h in 2.5 $\mu\text{g/ml}$ propidium iodide. Individual cells or 'comets' were viewed using a Zeiss epifluorescence microscope attached to an imaging system (Olive *et al.* 1992). Double-strand damage was quantified as an increase in tail moment, the product of the amount of DNA (fluorescence) in the tail and the distance between the means of the head and tail fluorescence distributions. For each comet, tail moment and total DNA content (total fluorescence per comet in arbitrary units) were recorded.

2.4. Protein measurement

For measurement of residual proteins after cell lysis, 10^7 V79 cells/ml were radiolabelled for 7–8 h with 74 kBq/ml $^3\text{H-L-leucine}$. To obtain enriched populations of S-phase cells, cells were incubated for 15 h with 1.5 mM hydroxyurea, or 3 $\mu\text{g/ml}$ aphidicolin, then released from the block at the G_1/S border by addition of fresh medium. Three and eight hours following release, cells were prepared as plugs in agarose as described below. In all cases, cells were analysed for degree of synchrony by flow cytometry using the Vindelov (1977) staining procedure. The comet assay was also used to determine if S-phase retardation was present after these treatments.

Plugs of cells were prepared by mixing cells 1:3 with 1% low gelling temperature agarose and pipetting the mixture into 100- μl sample holders. After gelling, plugs were removed from sample holders by inverting into buffer. Half of the resulting agarose plugs were individually melted, liquid scintillation fluid added, and radioactivity counted in a LKB 1214 liquid scintillation counter. Following a 4-h lysis at 50°C in 30 mM EDTA, 0.5% SDS, the remaining plugs were washed four times over a 24-h period in 50 ml Tris-EDTA buffer. These plugs were then melted and counted in a liquid scintillation counter. The fraction of protein remaining in the plugs after lysis was determined.

To examine the nature of the proteins remaining in plugs after lysis, V79 cells were labelled for 24 h with 74 kBq/ml $^{35}\text{S-methionine}$ from ICN. Agarose plugs were formed, lysed and rinsed as described above. The plugs were mixed with an equal volume of 2X sample loading buffer, boiled for 5 min, vortexed and loaded into the well of a 7.5% polyacrylamide gel for SDS-PAGE analysis. Following electrophoresis, gels were dried and exposed to photographic film. For comparison with the results obtained using plugs, a sample of $^{35}\text{S-methionine}$ -labelled V79 cells was lysed using a protocol

described by Ben-Ze'ev (1990) to enrich for intermediate filament proteins.

2.5. Elutriation

Cells ($n=10^8$) radiolabelled overnight with 37 kBq/ml $^3\text{H-leucine}$ were suspended in 10 ml cold PBS and loaded into a 5-ml chamber of a Beckman JS-5 elutriator rotor operating at a speed of 1800 rpm. Samples (50 ml) were removed, beginning at a flow rate of 16 ml/min and advancing 1 or 2 ml/min for each successive fraction. Cells were counted and a small part of the sample was prepared for the comet assay while the majority of cells were prepared for assay of residual protein content of cells embedded in agarose plugs as described above. For the comet assay, half of the cells from each fraction received 50 Gy following elutriation and the remainder served as a control. DNA content was measured using the comet assay and by flow cytometry (Vindelov 1977).

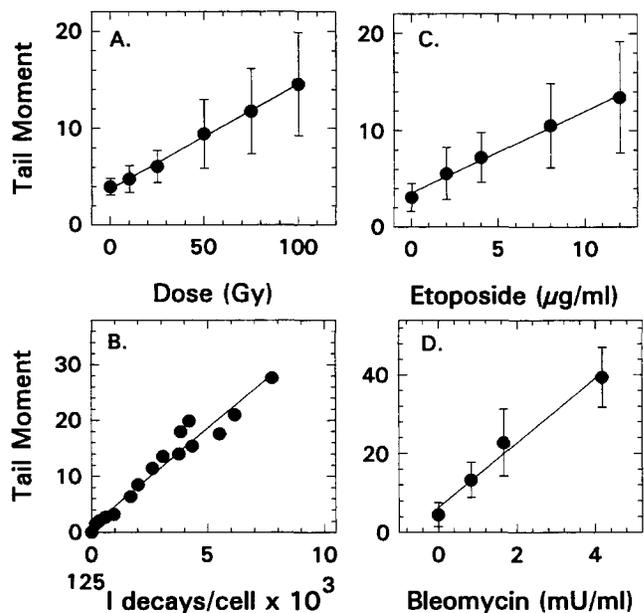


Figure 1. Application of the comet assay to exponentially-growing V79 cells exposed to four DSB agents. (A) X-irradiation on ice in PBS (mean \pm SD for 100 comets); (B) ^{125}I Urd-labelled cells allowed to accumulate decays while frozen in liquid nitrogen. Damage to cells without ^{125}I (mean tail moment for several experiments was 4.1 ± 2.2) has been subtracted, and the mean of 40–100 comets is shown. (C) Cells incubated with etoposide for 1 hr at 37°C (mean \pm SD for 100 comets). (D) Cells permeabilized with saponin were incubated with bleomycin sulphate for 1 hr at 37°C (mean \pm SD for 100 comets).

3. Results

DSBs can be detected in individual cells exposed to X-rays, bleomycin, etoposide, and ^{125}I dUrd when examined using single-cell gel electrophoresis or the 'comet' assay (Figure 1). Dose-response curves are linear for these doses and conditions of electrophoresis. In order to detect significant numbers of DSBs by bleomycin in our V79 cells, and to minimize heterogeneity, it was necessary to permeabilize cells by exposure to saponin; lysophosphatidylcholine (Sidik and Smerdon 1990) also enhanced

damage but in a less reproducible fashion. More damage by etoposide could generally be detected when proteinase K was included in the lysis buffer. Proteinase K and other protein denaturants may reveal more DSBs by digesting topoisomerase II which can mask these breaks (D'Arpa and Liu 1989).

Heterogeneity in DNA damage is evident for all four agents examined, as indicated by bivariate plots (Figure 2) where tail moment is shown versus DNA content. The ^{125}I data were selected from an experiment where the total amount of incorporation

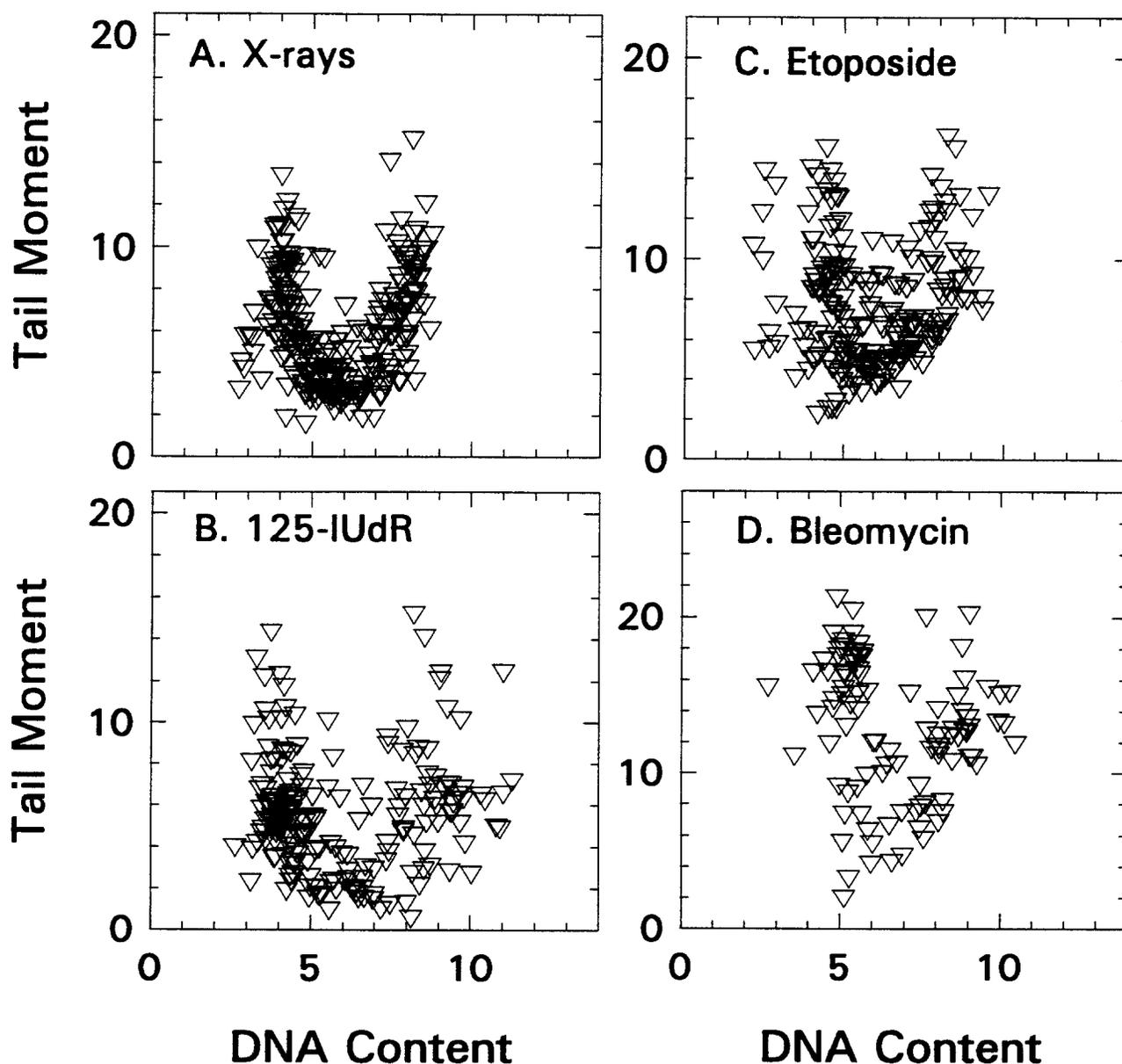


Figure 2. Comparison between DNA content and DNA damage to V79 cells by 4 double-strand breaking agents. Tail moment and DNA content were measured for the same comet. (A) 50 Gy X-rays. (B) 982 decays ^{125}I dUrd/cell. (C) 6 $\mu\text{g}/\text{ml}$ etoposide for 1 hr; (D) saponin permeabilized cells were treated with 1 mU/ml bleomycin for 1 hr.

was low (about 160 decays/cell/day) since higher incorporation levels led to a partial G₂ block during the 24-h exposure. Cells with S-phase DNA content appear as resistant subpopulations with lower tail moments than cells with G₁ or G₂ DNA content. Univariate histograms of observed tail moments (Figure 3) clearly indicate a less sensitive subpopulation for all four agents; migration of DNA from S-phase cells were reduced by about a factor of three. The extent of reduction is similar for all agents, suggesting that it is a property of the S-phase cell, not the nature of the DSB induced in S-phase cells, which is responsible for inhibiting migration of S-phase DNA.

Cell sorting experiments have previously identified early S-phase as the time when DNA migrates least efficiently after X-ray damage (Olive *et al.* 1991). A similar result was obtained using etoposide; cells in early S-phase show the least amount of DNA damage (Figure 4). Like X-rays, there is about a three-fold reduction in tail moment for early S-phase cells compared with G₁ or G₂ cells, and tail moment in unexposed S-phase cells is also significantly reduced.

Residual protein content following lysis of V79 cells embedded in agarose was examined in synchronized cells or in cells separated by centrifugal elutriation. Results (Figure 5, Table 1) indicated that our

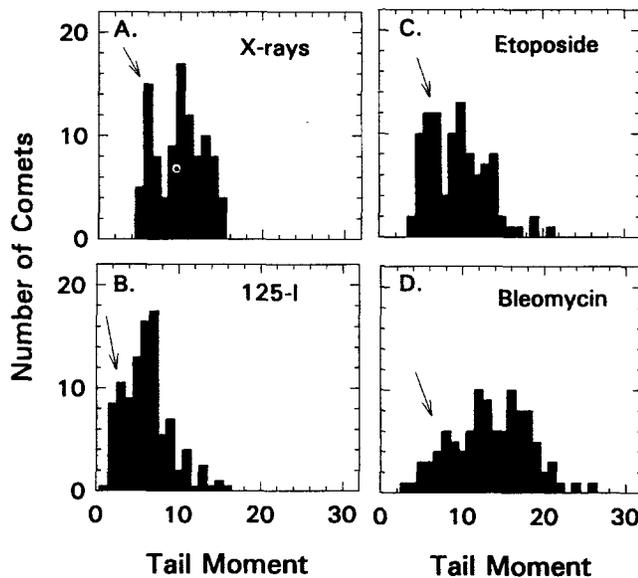


Figure 3. Histograms for 100 comets showing heterogeneity in DNA damage after exposure of V79 cells to 4 double-strand breaking agents. (A) 50 Gy X-rays. (B) 982 decays ¹²⁵I per cell. (C) 6 µg/ml etoposide for 1 hr. (D) 1 mU/ml bleomycin for 1 hr. The arrows point to the population of S-phase cells which show a smaller average tail moment.

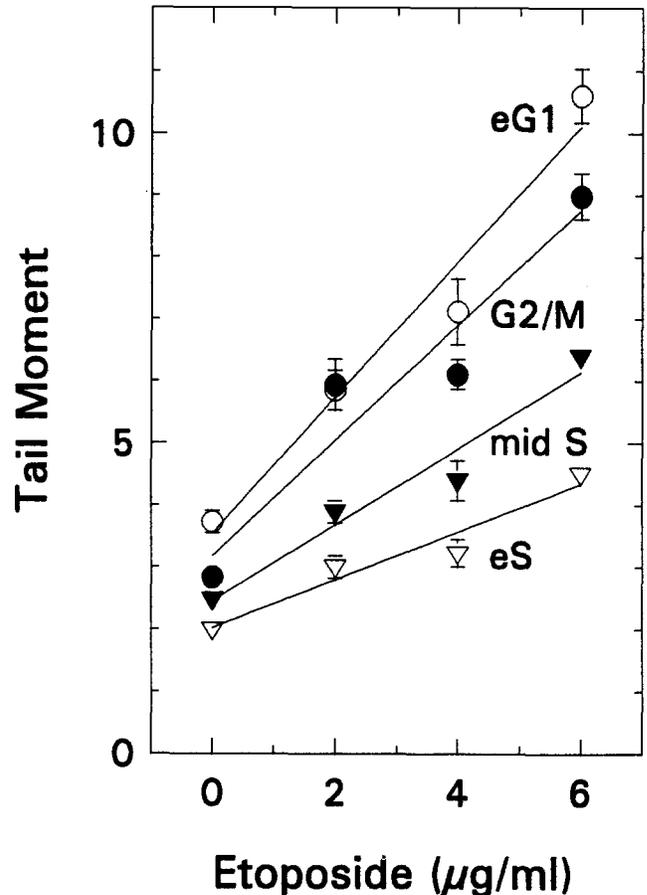


Figure 4. Influence of cell cycle position on DSB induction in V79 cells after treatment with etoposide. Asynchronously growing cells were incubated with 5 µM Hoechst 33342 for 30 min and sorted according to DNA content using a Becton-Dickinson FACS 440 cell sorter. Sorted populations were assayed for DNA damage using the neutral comet assay.

Table 1. Proteins remaining in cells after lysis: influence of position in cell cycle

Position in cycle	Percentage total protein remaining after lysis
Asynchronous	2.05 ± 0.74 (n = 13)
1 h after release (G ₁ /S)	1.80 ± 0.61 (n = 4)
3 h after release (mid S)	1.63 ± 0.61 (n = 5)
8 h after release (G ₂)	1.54 ± 0.47 (n = 4)

V79 cells labelled with ³H-leucine were synchronized using hydroxyurea or aphidicolin prior to release from the block by incubation in drug-free medium. Cells were prepared in agarose plugs for lysis in 0.5% SDS, 30 mM EDTA for 4 h at 50°C followed by a 20-h rinse in Tris-borate buffer. Flow cytometry was used to verify the position of the cells in the cell cycle.

neutral lysis procedure removes about 98% of the protein. When plugs are exposed to alkali or proteinase K, <0.5% of the initial amount of proteins remain in the plug. S-phase cells do not retain more protein than cells from other phases of the cell cycle, and similar amounts of protein were recovered after lysis of agarose plugs of cells prepared from different elutriation fractions (Figure 5 A). Elutriation data also confirm the inability of DNA from S-phase cells,

particularly DNA from cells in early S, to migrate as efficiently as DNA from cells in other phases (Figure 5 B). Mean DNA content of elutriated cells measured using the comet assay compared well with DNA content measured using flow cytometry (Figure 5 C).

Analysis of ^{35}S -methionine-labelled proteins remaining in the plugs following lysis revealed several bands, two of which correspond with the major cytoskeletal proteins, actin (45 kD) and vimentin (57 kD) (Figure 6 A). The method developed by Ben-Ze'ev (1990) to enrich for intermediate filaments produced a remarkably similar banding pattern (Figure 6 B).

S-phase DNA migrates *more* efficiently when cells are lysed in alkali $\geq 0.02\text{ M}$ NaOH prior to alkaline electrophoresis (Figure 7 A). Alkali lysis and electrophoresis result in denaturation of DNA and allow the two strands to migrate independently. The alkali comet assay and other single-strand break detection methods see replication forks as breaks (Rydberg 1980, Graubmann and Dikomey 1983, Olive and Banáth, in press). It could also be argued that alkali releases proteins that retard migration during neutral gel electrophoresis, however, prior exposure of unirradiated cells in agarose to alkali followed by neutralization, neutral lysis and electrophoresis revealed the same degree of S-phase retardation (Figure 7 B). In addition, tail moment in S-phase cells was still lower when cells were lysed in the presence of proteinase K *before* irradiation (Figure 7 C). While DNA is known to be considerably more radiosensitive once proteins are removed (e.g. Stamatou and Denko 1990), our results indicate less sensitivity of lysed compared to intact cells (Figure 1 A) because the Tris-borate buffer present during irradiation acts as an effective radioprotector.

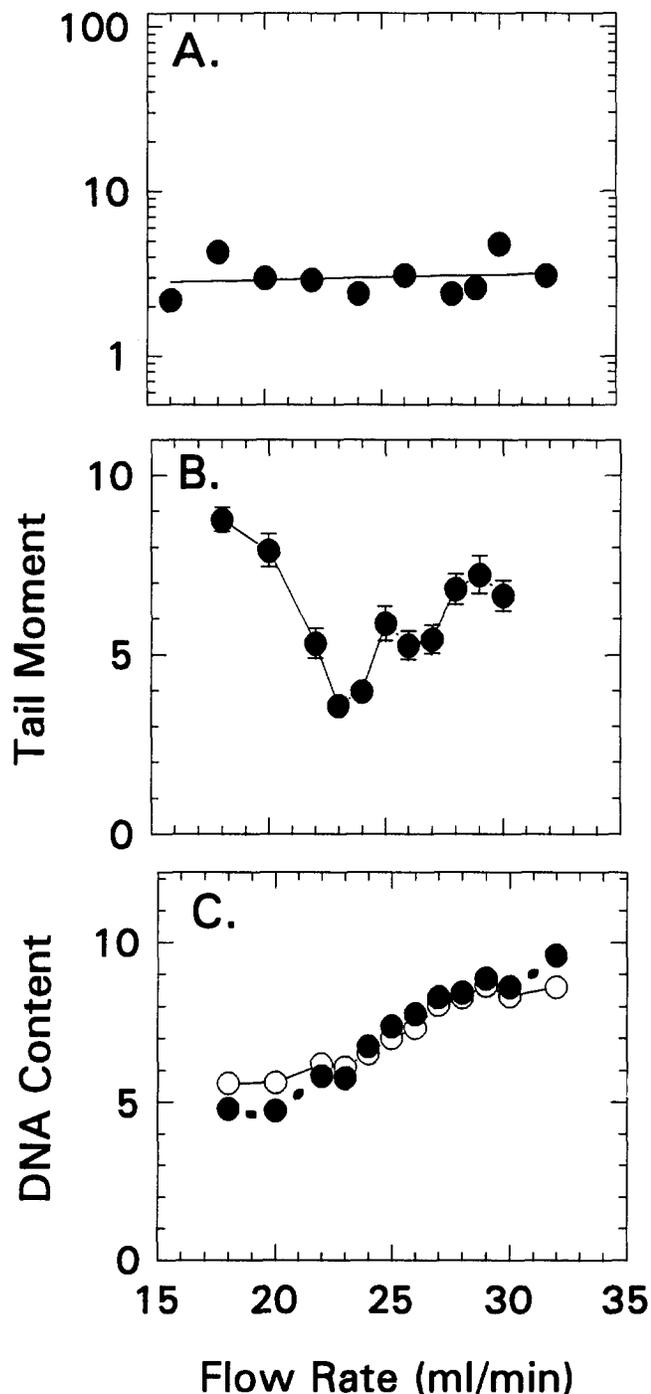


Figure 5. Separation of V79 cells using centrifugal elutriation and analysis of residual protein content and DNA damage by X-rays. (A) Cells recovered by elutriation were embedded in plugs, lysed, and examined for residual proteins (^3H -leucine radioactivity) following lysis. (B) After elutriation, cells were exposed on ice to 50 Gy, then examined for DNA damage using the comet assay. The mean and standard error for 100 comets per fraction are shown after subtraction of background damage measured for each fraction. (C) Cells from each fraction were examined for DNA content (total fluorescence) using the comet assay (closed symbols) or by measuring mean cell fluorescence measured using flow cytometry (open symbols). Flow cytometry measurements were normalized to the same value (7.16) as the comet assay for asynchronous cells. For the comet assay, error bars are smaller than the symbol size.

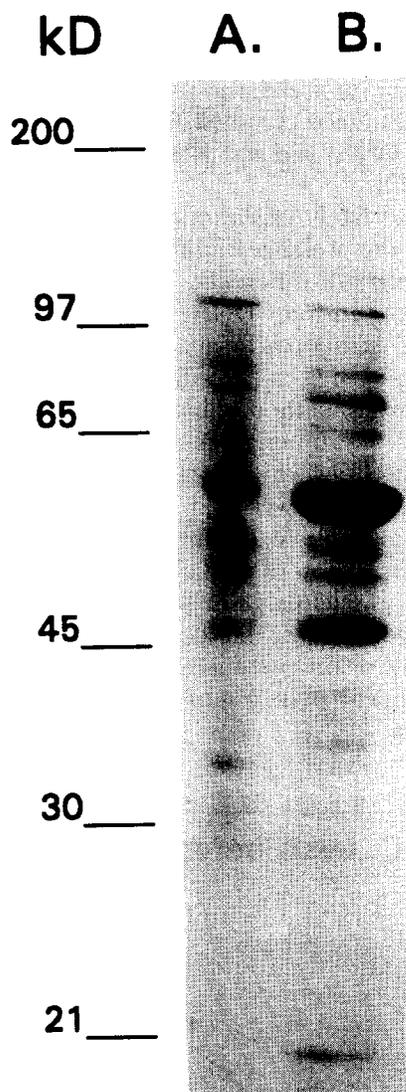


Figure 6. Composition of proteins remaining in agarose plugs following lysis. Lane A: ^{35}S -methionine labelled V79 cells were embedded in agarose plugs, lysed, and examined for residual proteins using SDS-PAGE. Lane B: ^{35}S -methionine labelled V79 cells were enriched for intermediate filament proteins using the method of Ben-Ze'ev (1990) and then analyzed using SDS-PAGE.

4. Discussion

Results using a variety of methods and cell lines have shown that differences in the structural properties of S-phase DNA can influence detection of radiation-induced DSBs. The proximity of DNA to the replication complex appears to influence the rate of its elution through filters during the neutral filter elution assay (Wlodek and Olive 1992). While S-phase cells are generally more resistant to killing by ionizing radiation (Elkind and Whitmore 1967), is there reason to believe that radiation produces

fewer DSBs in S-phase cells? If we assume that one decay of ^{125}I produces one DSB, then the data shown in Figure 1 indicate that the comet assay detects 23 DSBs/cell/Gy in asynchronous cells, in good agreement with results by Iliakis *et al.* (1991) who calculated values of 21–31 DSBs/CHO cell/Gy using pulsed-field gel electrophoresis. If we select data for comets within specific ranges of DNA content to 'calibrate' the comet assay, then estimates of DSBs/cell/Gy are similar for G_1 and S-phase cells.

S-phase retardation is observed for DSBs produced by a variety of other agents including bleomycin, etoposide, and ^{125}I Urd decay. Ostling and Johanson (1987) previously demonstrated a large heterogeneity in DNA damage of single cells exposed to bleomycin, a part of which can be explained by S-phase retardation. However, membrane permeability is a significant problem for this drug (Sidik and Smerdon 1990, Poddevin *et al.* 1991), so that most of the heterogeneity previously observed can probably best be explained by differences in cellular uptake of bleomycin.

Since topoisomerase II, the target for etoposide, is a component of the nuclear matrix and mitotic scaffold (Earnshaw and Heck 1985, Adachi *et al.* 1989), we expected that treatment with etoposide might produce breaks at or near DNA/matrix attachment sites. Since DNA replication is believed to occur in association with nuclear matrix attachment sites (Berezney and Coffey 1976, Dijkwel and Hamlin 1988), breaks at this critical position could allow S-phase DNA to migrate more freely. However, etoposide produced results similar to other agents which could be interpreted to mean that matrix attachment is not directly involved in S-phase retardation. Alternatively, it is possible that topoisomerase II/matrix attachment sites could be more resistant to lysis and proteinase treatment in S-phase cells than in G_1 or G_2 -phase cells. Inhibition of normal topoisomerase II function by etoposide could conceivably increase the complexity of DNA protein interactions, especially in replicating DNA. In addition, evidence now suggests that selectivity of etoposides for matrix attachment sites may be lower than for other types of topoisomerase II inhibitors (Pommier 1993).

Our results for ^{125}I decay using the comet assay are in good agreement with those published by Iliakis *et al.* (1991) using pulsed-field gel electrophoresis while the biological effectiveness of ^{125}I Urd decay does not appear to extend beyond a length of equivalent to a few base pairs in a synthetic oligonucleotide (Martin and Haseltine 1981), the potential range of the M-XY Auger electron produced during decay is larger and might extend to the size of the DNA solenoid (Yasui *et al.* 1988). However, while damage will be

more extensive and more localized for ^{125}I decay compared with damage by X-irradiation, the degree of S-phase retardation was identical.

About 2–3% of cellular proteins remain associated with comets after neutral lysis. These proteins appear to represent largely detergent insoluble proteins, primarily cytoskeletal in composition (Figure 6). The percentage remaining in plugs after lysis was not significantly different for cells in S-phase compared with asynchronous cultures, although variability was high. Alkali and proteinase K treatment reduced the amount of protein in the plugs to <0.5% of the initial amount of protein. While it is possible that a subset of lysis resistant S-phase proteins might inhibit migration, several observations argue against this possibility. More, not less, damage was observed in S-phase cells following exposure to alkali, presumably because of the presence of DNA synthesis initiation sites which behave as single-strand breaks (Figure 7A). Although, alkali might be expected to remove proteins associated with DNA, treatment of cells with alkali, if followed by neutralization and renaturation, did not improve

the ability of S-phase DNA to migrate (Figure 7B). Moreover, removal of proteins by lysis with proteinase K prior to irradiation (which would prevent radiation-induced crosslink formation between proteins and DNA) did not affect S-phase retardation (Figure 7C).

The most likely explanation for S-phase retardation is not the presence of different numbers of DSBs in these cells but differences in the conformation of (largely protein-free) DNA. During S-phase, ‘bubble’ and ‘Y’ structures form as intermediates in DNA replication. Using a two-dimensional agarose gel electrophoresis method, it has been shown that DNA molecules containing forks or bubble shapes show unique mobilities which can be used to map origins of replication (Brewer and Fangman 1987). Even two DSBs per bubble, an unlikely occurrence, would result in formation of fork shapes which would not migrate as efficiently as linear DNA. The nature of the damaged region, the location of damage within the genome, or the presence of replication-associated proteins appear to be minor contributors to S-phase retardation.

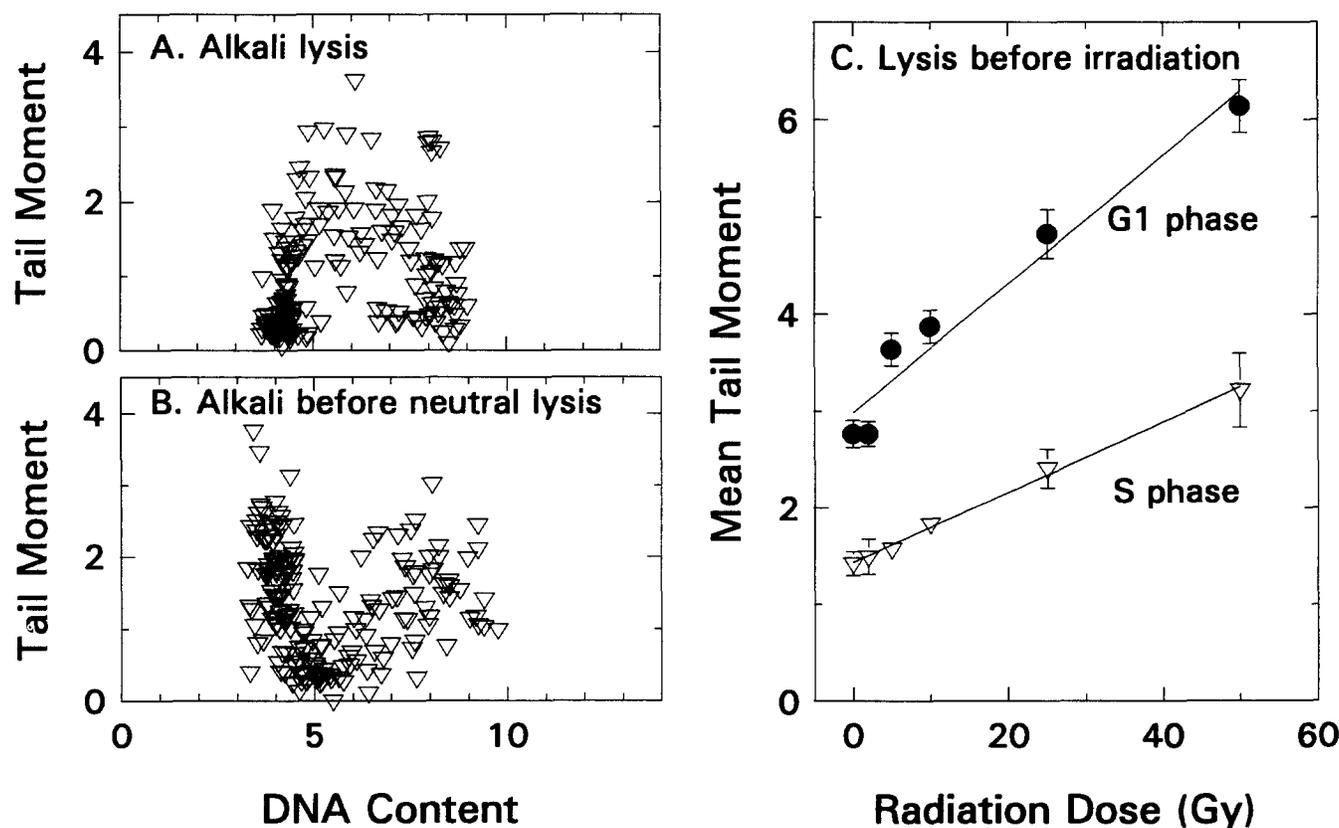


Figure 7. Effect of cell cycle position on tail moment measured following: (A) lysis of unirradiated cells in 0.03 M NaOH, 1 M NaCl prior to alkaline electrophoresis, (B) lysis of unirradiated cells in 0.03 M NaOH and 1 M NaCl prior to neutralization, neutral lysis and electrophoresis, and (C) lysis of cells in neutral lysis solution *prior* to irradiation. Tail moment was calculated for S and G₁ phase cells on the basis of DNA content measured using the comet assay.

Acknowledgements

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