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Radiation down-regulates replication origin activity throughout the S phase in mammalian cells

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ABSTRACT

An asynchronous culture of mammalian cells responds acutely to ionizing radiation by inhibiting the overall rate of DNA replication by ~50% for a period of several hours, presumably to allow time to repair DNA damage. At low and moderate doses, this S phase damage-sensing (SDS) pathway appears to function primarily at the level of individual origins of replication, with only a modest inhibition of chain elongation *per se*. We have shown previously that the majority of the inhibition observed in an asynchronous culture can be accounted for by late G_1 cells that were within 2–3 h of entering the S period at the time of irradiation and which then fail to do so. A much smaller effect was observed on the overall rate of replication in cells that had already entered the S phase. This raised the question whether origins of replication that are activated within S phase *per se* are inhibited in response to ionizing radiation. Here we have used a two-dimensional gel replicon mapping strategy to show that cells with an intact SDS pathway completely down-regulate initiation in both early- and late-firing rDNA origins in human cells. We also show that initiation in mid- or late-firing rDNA origins is not inhibited in cells from patients with ataxia telangiectasia, confirming the suggestion that these individuals lack the SDS pathway.

INTRODUCTION

DNA damage-sensing checkpoints function by anticipating whether conditions are appropriate for successful entry into and completion of the DNA replication (S) or chromosome segregation (M) phases of the cell cycle (reviewed in 1). Passage through either one of these cell cycle phases in the presence of strand breaks or other lesions could lead to cell death or genetic instability by chromosome loss and/or rearrangement. A p53-mediated G_1 checkpoint (2,3) and a G_2/M checkpoint (4,5) have been extensively characterized and are controlled by cyclin-dependent protein kinases (reviewed in 6,7). However, neither the G_1 nor the G_2 checkpoint has the potential to protect cells that are already in S phase at the time of DNA damage, which could represent as many as 50% of an asynchronous population.

We and others have focused attention on an S phase damage-sensing (SDS) pathway that responds to a radiation challenge by immediately down-regulating the overall rate of DNA synthesis for 2–3 h, as measured by [^{3}H]thymidine incorporation into DNA (8–13). The SDS response is characterized by a biphasic dose–response curve. The steep initial component is thought to reflect inhibition of initiation at origins of replication, while the less sensitive shallow component has been attributed to an effect on chain elongation (9,10,14). Several studies have lent support to this interpretation, including measurements of changes in the size distribution of nascent DNA after radiation treatment (15) and analysis of the rate of chain growth by DNA fiber autoradiography (14). In addition, we have used a two-dimensional (2-D) gel replicon mapping technique to show directly that moderate dose radiation delivered to an asynchronous culture of CHO cells transiently and completely inhibits all subsequent initiation in the early-firing dihydrofolate reductase (DHFR) origin of replication, with only a modest effect on forks that were in progress at the time of irradiation (8). There is good evidence that the SDS pathway operates *in trans*, since replication of a 5 kb plasmid is completely inhibited by radiation doses that are insufficient to cause breaks in the 5 kb episome itself but which are high enough to damage cellular chromosomes (16,17).

Acute down-regulation of DNA replication occurs in a variety of cells that lack functional p53, including CHO and HeLa cells (18–20); therefore, the SDS pathway (and a possibly distinct G_1/S checkpoint; below) must differ from the p53-mediated mid-G_1 checkpoint (3,21). In *Saccharomyces cerevisiae*, inhibition of replication in response to DNA damage depends on functional MEC1 and RAD53 gene products (22). Interestingly, MEC1 has been shown to be a homolog of the human ataxia telangiectasia (AT) mutant (ATM) gene product (23) and, like MEC1 mutants in yeast, cells from AT patients fail to acutely down-regulate DNA synthesis in response to DNA damage (24).

Certain aspects of the SDS response are rather enigmatic. For example, in an *asynchronous* culture of Chinese hamster ovary (CHO) cells, even moderately high radiation doses (e.g. 8–10 Gy)

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inhibit the overall replication rate by only 50–60% (25). Thus, the SDS pathway does not appear to prevent many forks that were already in progress at the time of radiation from replicating through single-strand lesions in their paths and converting them to potentially lethal double-strand breaks. Furthermore, the 50% inhibition observed in *asynchronous* cultures cannot be recapitulated in *synchronized* S phase CHO cells, since the same radiation doses inhibit overall replication rates by only ~15–25% regardless of when the cells are irradiated after entry into the S period (26).

This apparent dichotomy is partially explained by the observation that cells within ~2 h of entering the S period are prevented from doing so by a radiation challenge; consequently, replication in this fraction of the cells is completely inhibited and, when averaged with the modest effects on S phase cells, could result in 50% inhibition of [^{3}H]thymidine incorporation in an unsynchronized population overall (26). It is not known whether the inhibition of entry into S phase represents a distinct G_1/S cell cycle checkpoint or, rather, a bona fide S phase damage-sensing mechanism that functions at the level of individual origins of replication regardless of when they fire. This raises the possibility that the relatively modest effects of radiation treatment on intra-S phase cells may result solely from a slowing of chain elongation rates, i.e. that origins firing at any time after entry into S phase may not be subject to down-regulation.

In the present study, we have asked whether down-regulation of origins of replication occurs throughout S phase in response to irradiation. We have used a 2-D gel replicon mapping method that can distinguish between effects on initiation and elongation (27) and have characterized the replication pattern of both early- and later-firing origins in two different variants of the naturally amplified human rDNA repeats, which are sequestered in nucleolar organizer regions (Fig. 1). Our data show that moderate dose radiation efficiently down-regulates initiation of DNA synthesis in both early- and later-firing rDNA origins and therefore probably does so at origins firing any time in S phase. However, in AT cells, radiation does not inhibit initiation at either early- or late-firing rDNA origins. Implications of these findings are discussed.

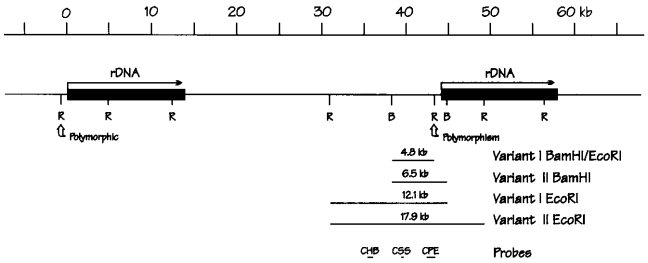

Figure 1. Sequence arrangements of the human rDNA locus. The polycistronic transcription units that encode the 18, 5.8 and 28S rDNA species are indicated by the black rectangles on the linear map. The origin of replication resides in the 31 kb intergenic spacer, with a concentration of start sites centered ~7 kb upstream from the promoter of the rDNA gene (33). The presence or absence of an *Eco*RI site distinguishes two variants of the rDNA repeat in the Wilson cell line (indicated with an arrow below the map), so that a 4.8 kb *Bam*HI–*Eco*RI fragment (Variant I) and a 6.5 kb *Bam*HI fragment (Variant II) are produced in a *Bam*HI/*Eco*RI digest. The hybridization probes used to detect these two variants in 2-D gels are as follows: CHB, a 500 bp *Hin*dIII–*Bam*HI fragment; CSS, a 0.25 kb *Stu*I fragment; CPE, a 0.68 kb *Pst*I–*Eco*RI fragment. Note that only the relevant *Bam*HI sites are shown here.

MATERIALS AND METHODS

Cell culture and cell synchrony

A human AT fibroblastic cell line (TAT2F, AT cell line) was maintained in minimal essential medium (MEM) supplemented with non-essential amino acids and 10% HyClone II serum. The human Wilson cell line was grown in RPMI 1640 medium containing 10% fetal calf serum (FCS). In one experiment, synchronized populations of Wilson cells were obtained by centrifugal elutriation as previously described (28). Alternatively, cultures were pre-treated for 12 h with high thymidine (2.5 mM) to arrest them via feedback on ribonucleotide reductase either in the S phase or at the beginning of S phase; then they were incubated in drug-free medium for 12 h to allow S phase traverse, followed by exposure to 200 µM mimosine for 14 h to collect the population at the G_1/S boundary. Culture media and sera were obtained from Gibco BRL.

Radiation treatment

Cells were irradiated at the times indicated in the text and figure legends. Irradiation was performed with a Varian linear accelerator at a dose rate of 400 cGy/min in a container designed to mimic the conditions of the cell culture incubator (5% CO_2 and 95% air at 37°C).

Determination of replication rates

Cells were grown in 24-well dishes and were labeled with 2.5 µCi [^{3}H]thymidine (80 Ci/mmol, 1 Ci = 37 GBq; Amersham) per ml of culture medium for the intervals indicated in the figure legends. The monolayers were then washed with phosphate-buffered saline and fixed with citric acid; the amount of insoluble radioactivity was determined at the end of the experiment (29).

Preparation of replication intermediates and 2-D gel analysis

Cells growing in 15 cm cell culture dishes were harvested at the indicated times and replication intermediates were purified exactly as described previously (30). Briefly, nuclear matrix halo structures were prepared by using lithium diiodosalicylate to extract histones (31) and matrix-affixed replication intermediates were isolated by digesting away loop (non-replicating) DNA either with an *Eco*RI digest or a *Bam*HI/*Eco*RI double digest (legends to figures). Matrix-bound replication intermediate preparations were digested with proteinase K (Sigma Chemical Corp.), followed by dialysis and ethanol precipitation; the resuspended DNA was fractionated further on benzoylated–naphthoylated DEAE cellulose (Sigma) to select for partially single-stranded DNA. For each sample, intermediates from 10^8 cells were loaded and run on a neutral/neutral 2-D gel exactly as described previously (30). After transfer to HyBond N$^+$ (Amersham), digests were probed with one or more of the following fragments (legends to figures): a 0.25 kb *Stu*I fragment (CSS), a 0.68 kb *Pst*I–*Eco*RI fragment (CPE) or a 470 bp *Hin*dIII–*Bam*HI

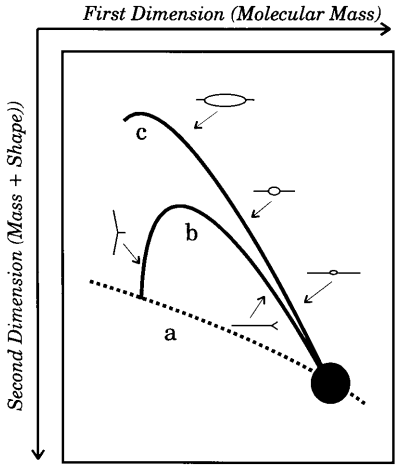

Figure 2. Principle of the 2-D gel replicon mapping method (27). A restriction digest of DNA is electrophoresed in an agarose gel in the first dimension under conditions that separate largely according to molecular mass. The resulting lane is excised, rotated 90° and run in the second dimension under conditions that separate according to both mass and shape. The digest is then immobilized on a membrane and hybridized with appropriate radioactive probes for the fragments of interest. Curve a (diagonal) represents the arc of linear non-replicating fragments from the genome as a whole, which travel approximately the same distance in both directions; curve b corresponds to a fragment with a centered origin or a collection of dispersed origins, some of which are in the center of the fragment; curve c represents a fragment that is replicated passively by forks emanating from an outside origin either upstream or downstream of the fragment. The large black dot corresponds to linear, non-replicating fragments, which constitute the majority of fragments at any one time. In a broad initiation zone, a composite pattern of a bubble arc (curve c) and a stronger single fork arc (curve b) will be observed, since any given fragment will sometimes sustain an internal initiation event but will usually be replicated passively by an outside fork from another site within the zone (30).

fragment (CHB), which recognize both rDNA variants. All probes were labeled with [^{32}P]dCTP by random priming (32).

RESULTS

Principle of the neutral/neutral 2-D gel technique

To analyze radiation effects on origins of replication, we utilized the neutral/neutral 2-D gel method that was developed by Brewer and Fangman to characterize replicons in complex genomes (27). The principle of the method is illustrated in Figure 2. A restriction digest of DNA containing replication intermediates is run in the first dimension gel under conditions that separate largely on the basis of the molecular mass of each fragment, which varies between 1*n* for an unreplicated fragment to ~2*n* for the almost fully replicated fragment. After electrophoresis in the first dimension gel, the lane is excised, rotated 90° and separated in the second dimension gel on the basis of both molecular mass and shape, which vary depending upon whether the fragment contains a single fork (curve b) or a replication bubble surrounding an initiation site (curve c). By transferring the digest to a membrane and hybridizing with appropriate radioactive probes for fragments of interest, it is possible to determine whether a given fragment sustains active initiation events (i.e. displays a bubble arc) or whether it is replicated passively by a single fork entering from either side.

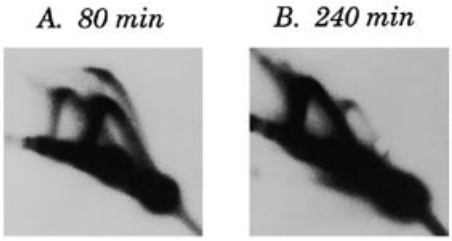

Figure 3. Two different rDNA variants initiate replication at different times in the S period in synchronized Wilson cells. Wilson cells were partially synchronized first with a high thymidine block and then were collected at the G_1/S boundary in mimosine, as described in Materials and Methods. Samples were taken for 2-D gel analysis 80 and 240 min after removal of mimosine. Replication intermediates were purified as described, using a *Bam*HI/*Eco*RI digest to separate matrix-affixed DNA from the loop fraction, as described in Materials and Methods. After transfer to a membrane, the digest was hybridized with a combination of CSS and CPE (Fig. 1) and exposed to X-ray film.

Replication timing of the human rDNA loci

To determine whether γ-radiation down-regulates origin activity throughout the S period, we focused attention on an experimental system in which both early- and late-firing origins can be analyzed and compared on the same 2-D gels. In the naturally amplified rDNA locus in human cells, the basic repeating unit or amplicon is ~44 kb in all 400 copies (28). However, the presence or absence of an *Eco*RI restriction site in the intergenic region distinguishes two different variations of the repeating unit (Fig. 1). The origin in this locus resides in a large intergenic spacer region between transcription units (Fig. 1; 28). Like the early-firing DHFR origin in Chinese hamster cells, the rDNA origins correspond to initiation zones, with nascent strand start sites being chosen from a large number of potential sites distributed throughout the 31 kb intergenic spacer (28). However, the region lying ~5 kb upstream of the transcription start site seems to be preferred (Fig. 1; 33).

When the replication patterns of these two rDNA variants were examined on 2-D gels, we found that they replicate at different times in the S period (Fig. 3). Wilson cells were synchronized at the G_1/S boundary with mimosine as described in Materials and Methods and samples were taken for 2-D gel analysis 80 min (i.e. early S phase) and 240 min (mid to late S phase) after drug removal. Replication intermediates were prepared using a combination of *Bam*HI and *Eco*RI to digest the DNA, the digest was separated on a 2-D gel and was transferred to a membrane. The membrane was hybridized with a combination of probes CSS and CPE, which detect both rDNA variant fragments simultaneously (Fig. 1).

In the early S phase sample (80 min), both a prominent single fork arc and a bubble arc can be detected in the smaller Variant I fragment, while a much less pronounced single fork arc is detected in the larger Variant II fragment. In broad initiation zones such as the DHFR and rDNA origins, any fragment within the zone displays a bubble arc because of internal initiations (as in Fig. 2, curve c), as well as a stronger single fork arc (as in Fig. 2, curve b) resulting from initiations that occurred at other sites in the zone lying outside that fragment (details in 30). In the mid to late S phase sample (240 min), the situation is reversed: the larger

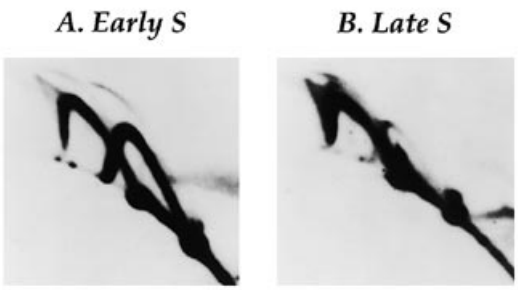

Figure 4. The origins in rDNA Variants I and II are activated in the early and late S periods, respectively, in synchronized cells isolated by elutriation. Unsynchronized Wilson cells in exponential growth phase were elutriated by size as previously described (28) and populations with a DNA content of ~2.0–2.2C (largely in early S phase) and ~3.8–4.0C were isolated. Replication intermediates were isolated as described (30), using a combination of *Bam*HI and *Eco*RI to digest the DNA, and were separated on a neutral/neutral 2-D gel (27). After transfer to a membrane, the digests were hybridized with probe CHB, which detects both variant rDNA fragments.

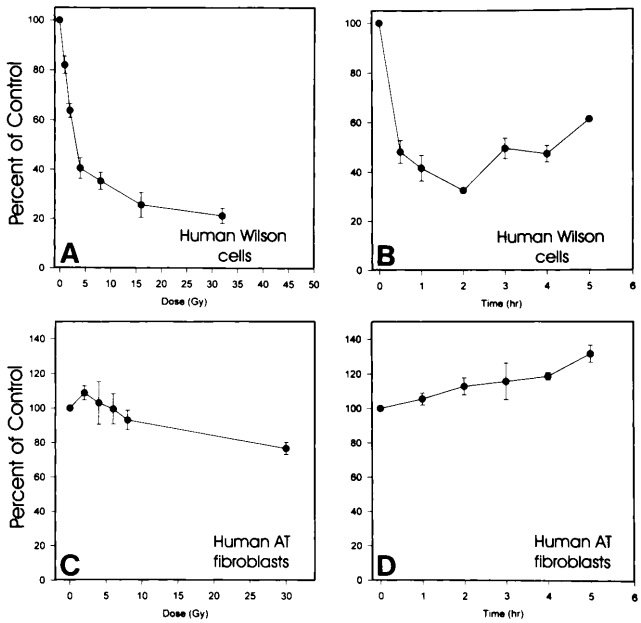

Figure 5. Human Wilson cells, but not human AT cells, display the acute S phase damage response. (**A** and **C**) Dose–response curves. Human Wilson cells (A) or human AT fibroblasts (C) were sham-irradiated or irradiated with various doses of ionizing radiation. The cultures were returned to the incubator for 45 min and then were labeled with 2.5 μCi [^{3}H]thymidine/ml medium for 20 min. Samples were processed to determine the amount of [^{3}H]thymidine incorporated into DNA as previously described (29). Values are expressed as percent of the unirradiated control. (**B** and **D**) The time courses of inhibition of DNA synthesis. Asynchronous Wilson and AT cells were irradiated with 5 Gy and were then labeled with 2.5 μCi [^{3}H]thymidine/ml medium for 20 min at the indicated times thereafter.

Variant II fragment displays a prominent single fork arc and a faint bubble arc (visible on the original film and in the experiment shown in Fig. 4 below), while very few replication intermediates are detected in the smaller Variant I fragment.

The very faint bubble arc in Variant II suggests that by 240 min after removal of mimosine, most initiation had not yet occurred in this variant. This suggestion is confirmed by the experiment presented in Figure 4. Cells with a DNA content of ~2.0–2.2C (largely representing early S phase cells) or a DNA content of ~3.8–4.0C (largely late S phase) were isolated by centrifugal elutriation, replication intermediates were prepared by digestion with *Eco*RI and the digests were then separated on a 2-D gel. After transfer to a membrane, the two rDNA variant fragments were detected with probe CHB (Fig. 1). In the early S phase sample, the smaller Variant I fragment again displays a bubble arc and single fork arc. Note that, unlike the early S phase (80 min) time point in Figure 3, the bubble and fork arcs are only somewhat more pronounced than their counterparts in the Variant II fragment, probably because the sample is contaminated with middle S phase cells. In the late S phase sample, the smaller Variant I has almost finished replicating, while the larger Variant II fragment displays a bubble arc and a pronounced single fork arc. Therefore, we conclude that the origin in rDNA Variant I initiates replication early in the S period, while the origin in Variant II represents one of the few mammalian origins that has been demonstrated to fire in mid to late S phase.

Wilson cells, but not AT cells, display the classic S phase damage response to γ-radiation

The purpose of the present study was to determine whether the origins of replication that are activated in middle or late S phase (i.e. the origin in rDNA Variant II) are subject to down-regulation via the p53-independent SDS pathway. It was therefore important to demonstrate that the SDS pathway is intact in the human Wilson lymphoblastoid cell line.

As shown in Figure 5A, Wilson cells display the typical biphasic dose–response curve that characterizes the SDS pathway, with the overall rates of [^{3}H]thymidine incorporation approaching 70–80% inhibition at very high doses. The response is almost immediate and replication rates are inhibited for 1–2 h before beginning to recover toward pre-irradiation values (Fig. 5B). Note that Wilson cells are somewhat more sensitive to ionizing radiation than are CHO cells, with the inflection point in the biphasic dose–response curve occurring at ~5 and 10 Gy, respectively (8). The immediacy of the response to radiation, as well as the fact that the Wilson cell lines fail to demonstrate a prolonged G_1 arrest, distinguishes this S phase response from the p53-mediated G_1 checkpoint (3,21). As shown in Figure 5C and D, fibroblasts established in culture from an AT individual do not markedly decrease the rate of DNA synthesis in response to moderate dose radiation, in agreement with the suggestion by others that AT patients lack a functional SDS pathway (34–37).

Radiation down-regulates the ribosomal origin throughout S phase in Wilson cells

To determine the generality of the SDS pathway, we examined the response of the rDNA origins in Wilson cells to a radiation challenge. Not only do some of these origins fire in middle and/or late S phase (Figs 3 and 4), but the multiple copies of rDNA amplicons are sequestered into specialized nuclear compartments known as nucleolar organizers. The initiation zone in the rDNA locus corresponds approximately to the intergenic spacer region, which is ~31 kb in length (28). To test whether the origins in both the early- and mid-firing rDNA origins are down-regulated in

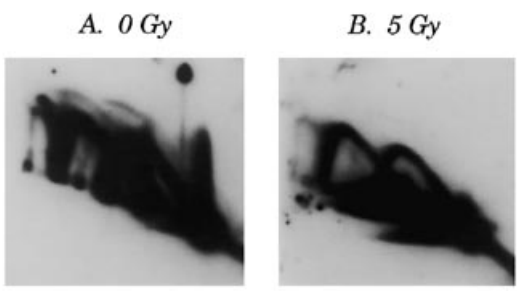

Figure 6. Both early- and mid-firing rDNA origins in Wilson cells are down-regulated by γ-radiation. Asynchronous cultures of Wilson cells were sham-irradiated (**A**) or irradiated with 5 Gy (**B**) and were harvested for 2-D gel analysis 60 min post-irradiation. DNA was digested with a combination of *Bam*HI and *Eco*RI and the replication intermediates were separated on a 2-D gel. The resulting transfer was probed with a combination of probes CSS and CPE.

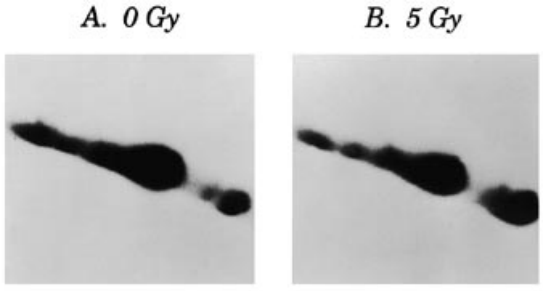

Figure 7. Radiation does not alter the partitioning of replication intermediates between the matrix and loop fractions. Asynchronous cultures of Wilson cells were sham-irradiated (**A**) or irradiated with 5 Gy (**B**) and were harvested for 2-D gel analysis 60 min post-irradiation. Twenty percent of the DNA released from the nuclear matrices by the *Bam*HI/*Eco*RI digestion was processed in parallel with the matrix fraction shown in Figure 6.

response to radiation, an asynchronous population of Wilson cells was irradiated with 5 Gy and cells were sampled 1 h later. Replication intermediates were digested with a combination of *Bam*HI and *Eco*RI, separated on a 2-D gel and transfers of the digests were hybridized with probe CPE, which detects both variant fragments. In the sample from the unirridiated culture (Fig. 6A), pronounced bubble arcs and single fork arcs are detected in both Variants I and II. However, initiation in both the early- and late-firing fragments is almost completely inhibited by a 5 Gy radiation insult (Fig. 6B). As with CHO cells (J.Larner and J.L.Hamlin, unpublished observations), the level of replication forks is also depressed somewhat, presumably because those forks in progress at the time of radiation continue to their destinations, but new initiations are prevented.

Our protocol for 2-D gel analysis involves the purification of replication intermediates by virtue of their association with the nuclear matrix (30). It has also been suggested that DNA damage may be repaired by enzymes that are affixed to the nuclear matrix (38). It was therefore important to show that radiation does not alter the partitioning of replication intermediates with the matrix, rather than having a direct effect on DNA replication *per se*. To determine whether radiation treatment results in a redistribution of replication intermediates from the matrix to the loop DNA, we also analyzed the DNA released from the nuclear matrices by *Bam*HI and *Eco*RI in the experiment pictured in Figure 6 (which constitutes 90–95% of total DNA). In the autoradiograms shown in Figure 7, only the 1*n* spot of unreplicating fragments can be detected with probe CPE. Therefore, the replication bubbles that disappear from the rDNA loci in Figure 6 do not appear in the loop fraction in response to radiation treatment. It could also be argued that radiation alters the affinity of replication intermediates for the BND column used to further purify partially single-stranded replication intermediates from double-stranded non-replicating DNA. However, comparison of the column washes from irradiated and non-irradiated samples also revealed no differences in recovery of replication intermediates from the two samples (data not shown). We conclude that replication bubbles disappear from the irradiated sample in Figure 6 via operation of the SDS pathway and not by radiation-induced detachment from the matrix and/or other artifacts resulting from the method of purification of replication intermediates *per se*. We have additionally shown that radiation does not directly damage replication bubbles, causing them to disappear from their characteristic positions in the 2-D gels (8).

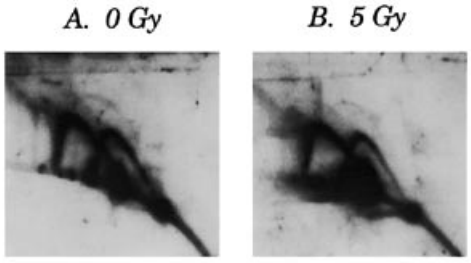

Figure 8. Neither early- nor late-firing rDNA origins are inhibited by radiation in AT cells. Asynchronous cultures of AT cells were sham-irradiated (**A**) or irradiated with 5 Gy (**B**) and were harvested for 2-D gel analysis 60 min post-irradiation. DNA was digested with *Bam*HI/*Eco*RI and the digest was transferred to a membrane and hybridized with a combination of probes CSS and CPE.

γ-Radiation does not inhibit the ribosomal origin in AT cells

Most cells that are propagated as stable cell lines lack functional p53 (39), but most of those that have been tested still retain the SDS pathway (8,10). Exceptions are fibroblasts established in culture from AT patients, which lack not only the p53-mediated G_1 checkpoint (3) and the G_2/M checkpoint (40,41), but also the SDS pathway (Fig. 5C and D; 42). When an asynchronous culture of AT fibroblasts was irradiated with 5 Gy, no significant reduction in the level of either single fork arcs or replication bubbles was detected (Fig. 8). These data directly demonstrate that AT cells are defective in their ability to down-regulate origin function in response to DNA damage at the naturally amplified ribosomal locus and, by inference, probably at all other origins.

DISCUSSION

The SDS pathway, which responds acutely to radiation by down-regulating the overall rate of DNA synthesis, has been studied for almost 25 years (reviewed in 25). The importance of this response to the maintenance of genomic integrity is illustrated by individuals with AT, who suffer a wide range of

disorders, including genetic instability and a greatly increased frequency of cancer (reviewed in 42,43). We are attempting to define the molecular basis of this response with the long-range goal of devising strategies for sensitizing tumors in non-AT individuals to radiation therapy.

Moderate radiation doses result in 30–70% inhibition of overall replication rates, as measured by effects on rates of [^{3}H]thymidine incorporation into DNA (25). Several studies support the view that, at moderate doses, origins of replication are the primary targets of this pathway, while at high doses, effects on the template itself could directly slow chain elongation rates. To study this phenomenon in detail, we have utilized 2-D gel technology, which has the advantage that effects on initiation and elongation can largely be distinguished: if initiation is preferentially inhibited, bubbles in an initiation zone should mature to finished replicons and no new bubbles should reappear for several hours, resulting in the loss of both bubble and single fork arcs. Using this approach, we have shown previously that a moderate radiation dose delivered to unsynchronized CHO cells completely inhibits subsequent initiation at the early-firing DHFR origin of replication, with the number of active replication forks decreasing only after several hours (8). However, when cells are synchronized at the G_1/S boundary with mimosine, radiation delivered either before or immediately after release from the drug has very little effect on either overall replication rates or specifically on the DHFR origin as judged by 2-D gel analysis (8). Presumably, the radiation-sensitive step in the initiation process has already occurred in early-firing origins in the presence of mimosine. Furthermore, when CHO cells are irradiated at various times during the remainder of the S period, overall replication rates are down-regulated by only 15–25% relative to sham-irradiated controls (26). Thus, it was not possible to recapitulate the overall inhibition in an irradiated unsynchronized culture (~50%) with the magnitude of effects when synchronized S phase cells were examined directly.

However, we recently discovered that CHO cells in the last 2–3 h of G_1 are prevented from entering the S period for several hours in response to a radiation challenge (26). The basis of this delay appears to be that the early origins do not fire; thus, replication forks are not commissioned and the overall replication rate is reduced to zero. This subpopulation represents ~15% of the cells that would have been synthesizing DNA within the few hours after radiation treatment in an asynchronous population and, when added to the remainder of cells that were at various other positions in the S period at the time of radiation, would appear to reconstitute the total effect on [^{3}H]thymidine incorporation that is observed in a log culture.

The question then arises why the effect on cells that have already entered S phase is so small. One possibility is that the SDS pathway that operates on the earliest firing origins may not affect mid- and late-firing origins. However, in the present study, we have identified a variant rDNA repeat whose origin fires in the middle and late S periods and we have used the 2-D gel method to show that this origin is also completely down-regulated in response to moderate dose γ-radiation (5 Gy). Furthermore, neither the early- nor later-firing rDNA origins were significantly inhibited in fibroblasts isolated from individuals with AT. This observation lends further support to the suggestion that the ATM gene is a key member of the SDS pathway (34).

Based on these observations, we suggest that the SDS pathway down-regulates all origins regardless of whether they fire in the early S period or at later times in S and regardless of which nuclear compartment they occupy (the exception being mitochondrial DNA origins, which appear not to be affected by γ-radiation; 44). These findings suggest that the consequences to the cell are very different depending upon whether cells are irradiated in the 2 h window prior to entering S phase or in the S period itself. Inhibition of the earliest bank of replicons by γ-radiation in late G_1 has the effect of completely suppressing replication for 1–2 h. Therefore, in cells in late G_1 at the time of radiation, the genes in early-firing replicons are largely protected from replicating through radiation-induced single-strand lesions in the template that could convert them to lethal double-strand breaks. In contrast, cells positioned in the S phase when a radiation challenge is delivered continue to synthesize DNA at most of the forks that were already in operation (amounting to ~80% of pre-irradiation replication rates; 26) and only those replicons in which initiation normally would have occurred in the next hour would be completely inhibited.

What could be the biological significance of a response with these properties? The simplest model is that the ATM protein functions as a sensor of DNA damage and transduces the damage signal via both p53-dependent and p53-independent pathways, leading to a number of coordinately regulated stress responses. The p53-independent SDS pathway, about which very little is known, then ultimately leads to a direct inhibition at origins of replication. Even though the ATM gene product shares homology with lipid PI3 kinases, it (as well as other members of the PI3-like kinase family) has been shown to have protein kinase activity (e.g. ATM activates the nuclear tyrosine kinase, c-abl, by phosphorylating Ser435; 45). A carboxyl terminal fragment of ATM contains the PI3-like kinase domain and restores a normal S phase response to radiation when introduced into AT cells. Thus, it seems likely that the kinase activity of ATM is important in transducing the damage signal to replication origins. On the other hand, the hypersensitivity of AT cells to a radiation challenge cannot be due solely to their inability to down-regulate DNA synthesis, since radiosensitivity and radiation-resistant DNA synthesis can be partially uncoupled experimentally (46). The fact that cells eventually recover normal rates of replication after a radiation challenge argues that the SDS pathway constitutes a bona fide stress response rather than an early read-out of cell death.

It is also conceivable that down-regulation of DNA replication in response to DNA damage may be an indirect consequence of the activation of repair systems: the repair machinery may share some components with replisomes and could redirect these components from replication toward repair activities. The recent identification of nibrin, the protein that is mutated in the Nijmegen breakage syndrome (NBS) gives some support to this possibility (47). NBS not only shares overlapping clinical features with AT, but the two syndromes display similar phenotypes at the cellular level: they each have increased levels of spontaneous and induced chromosomal fragility, are hypersensitive to ionizing radiation, fail to induce p53 and do not down-regulate DNA synthesis in response to ionizing radiation (48,49). Interestingly, nibrin has been shown to be necessary for the relocalization of the human hMre11/hRad50 protein complex to the sites of double-strand breaks (50). Thus, in NBS cells, components common to the repair and replication machinery may not be diverted to damaged sites, with the consequence that replication is not inhibited in response to DNA damage in these cells. In normal cells, competition for proteins common to

replication and repair would have the two-fold effect of preventing further damage in replicons that had not yet fired, as well as immediately initiating repair in damaged ones.

Unlike the p53-mediated checkpoint, the SDS pathway is probably functional in most transformed cells. We have now shown directly that both the early-firing DHFR and rDNA origins, as well as the later-firing rDNA origins, are inhibited in response to DNA damage. By analogy, all chromosomal origins are probably subject to down-regulation by this response. Thus, the SDS pathway may represent an important deterrent to tumor cell killing by chemotherapy and radiation and can potentially be manipulated for therapeutic purposes.

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