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Radiation Effects on DNA Synthesis in a Defined Chromosomal Replicon

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It has recently been shown that the tumor suppressor p53 mediates a signal transduction pathway that responds to DNA damage by arresting cells in the late G_1 period of the cell cycle. However, the operation of this pathway alone cannot explain the 50% reduction in the rate of DNA synthesis that occurs within 30 min of irradiation of an asynchronous cell population. We are using the amplified dihydrofolate reductase (DHFR) domain in the methotrexate-resistant CHO cell line, CHOC 400, as a model replicon in which to study this acute radiation effect. We first show that the CHOC 400 cell line retains the classical acute-phase response but does not display the late G_1 arrest that characterizes the p53-mediated checkpoint. Using a two-dimensional gel replicon-mapping method, we then show that when asynchronous cultures are irradiated with 900 cGy, initiation in the DHFR locus is completely inhibited within 30 min and does not resume for 3 to 4 h. Since initiation in this locus occurs throughout the first 2 h of the S period, this result implies the existence of a p53-independent S-phase damage-sensing pathway that functions at the level of individual origins. Results obtained with the replication inhibitor mimosine define a position near the G_1/S boundary beyond which cells are unable to prevent initiation at early-firing origins in response to irradiation. This is the first direct demonstration at a defined chromosomal origin that radiation quantitatively down-regulates initiation.

The effects of ionizing radiation on DNA have been studied intensively for decades. An understanding of these effects and the cellular response to them is required to comprehend the genetic basis of radiation-induced mutation and to develop novel approaches to enhance the effectiveness of radiotherapy for the treatment of cancer and other diseases.

Mammalian cells have elaborated complex mechanisms to deal with assaults on the integrity of genomic DNA. It is well known, for example, that cells down-regulate DNA replication ([^{3}H]thymidine incorporation) by 50 to 55% within 30 min of a challenge with high-dose ionizing radiation (25, 31, 38); replication rates return to normal within a few hours (38). Presumably, this transient arrest of DNA synthesis allows the removal of adducts or the repair of strand breaks, or both, before these lesions can be permanently fixed by DNA replication into catastrophic chromosome breaks or mutations. The radiation dose-response curve has a steep initial component at lower doses, which is thought to result from inhibition of new initiations at origins of replication; a much smaller, refractile component is observed at high doses, which is thought to reflect direct effects on chain elongation (25, 31, 40). Because of the consequent high residual rate of [^{3}H]thymidine incorporation by unaffected replication forks, it has been difficult to assess the extent to which initiation is specifically inhibited at any given dose of radiation.

Various models have been proposed to explain how damage to the template itself, or cross-linking of proteins to the template, could act in *cis* to arrest DNA synthesis at the level of initiation (32, 33). However, recent studies suggest that the down-regulation of replication in response to DNA damage is mediated by at least two signal transduction pathways that appear to function in *trans*.

Lamb et al. (16) and Cleaver et al. (6) made the important observations that ionizing radiation inhibits both chromosomal replication and the replication of a resident autonomously replicating plasmid at doses that are not high enough to detectably damage the plasmid template itself. This down-regulation is therefore likely to function in *trans*, and, because DNA synthesis was inhibited within a few hours of the radiation insult (and probably earlier), it is likely to be responsible for the acute-phase response discussed above. Nothing is presently known about any of the intermediates in this proposed regulatory pathway. However, it has been shown that mitochondrial DNA replication is not down-regulated by radiation treatment; this suggests that this pathway responds specifically to nuclear DNA damage (5).

A second damage arrest pathway has been uncovered recently that is mediated by the tumor suppressor, p53, and that arrests cells in late G_1 in response to radiation (12–14). The ultimate target or step of this pathway appears to be a cell cycle checkpoint that prevents traverse of the G_1/S boundary, as opposed to a component that interacts directly with early-firing origins of replication. Since mutations in p53 represent the single most common genetic lesion detected in biopsy specimens of human tumors, the failure of this pathway may be responsible, in at least some cases, for the genetic instability that characterizes tumor cells (20, 22, 34, 37, 42).

There is a third, p53-independent, damage-sensing regulatory mechanism that arrests mammalian cells at the G_2/M interface (12). This pathway has counterparts in both budding and fission yeasts (11, 39) and may be mediated by the Cdc2 protein kinase involved in effecting the G_2/M transition (1).

We are attempting to understand the molecular biology of the first of these pathways, which responds within minutes to a radiation challenge and which ultimately must impinge directly on origins of replication. If the *trans*-acting factor(s) whose interaction with origins is provoked or changed in response to

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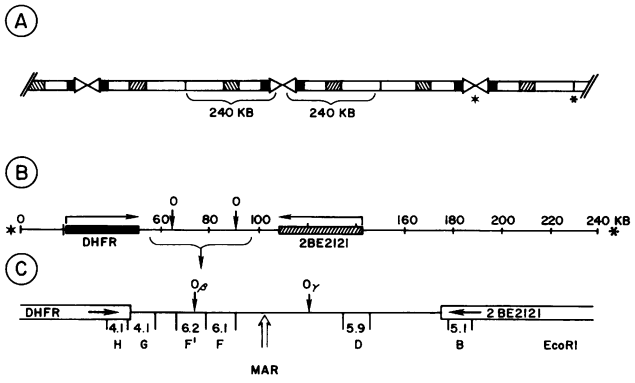

FIG. 1. Map and arrangement of DHFR amplicons in CHOC 400 cells. (A) The major 240-kb amplicons in CHOC 400 cells are arranged as alternating head-to-head (star) and tail-to-tail (asterisk) repeats in the genome. (B) Map of a single amplicon showing the positions of the DHFR and 2BE2121 transcription units. The initiation locus is bracketed below the map. (C) Expanded map of the intergenic region showing relevant restriction fragments and rough locations of ori-β and ori-γ as well as a prominent matrix attachment region (MAR).

DNA damage can be identified, it should eventually be possible to work backward to identify the preceding steps in this signal transduction pathway, including the nature of the initial damage-sensing mechanism itself.

To focus on a defined replicon at the molecular level, we have taken advantage of a methotrexate-resistant Chinese hamster ovary cell line (CHOC 400) that has amplified one allele of the dihydrofolate reductase (DHFR) gene and a local origin ~1,000 times (26). The major repeating units (amplicons) in this cell line are ~240 kb in length and are arranged as tandem head-to-head repeats in stable homogeneously staining chromosome regions (Fig. 1) (23, 24, 26). The *cis*-acting elements (origins) responsible for controlling DNA replication have not yet been identified by genetic means in this (or any other) mammalian locus. However, a variety of studies have shown that initiation of nascent chains is confined to a 50-kb region lying between the DHFR and 2BE2121 genes and probably occurs more frequently near two separate sites within this zone that lie ~22 kb apart (ori-β and ori-γ; Fig. 1B and C) (2, 4, 18). These sites may therefore coincide with or be near to the bona fide *cis*-regulatory origins in this domain.

In the present study, we show that the CHOC 400 cell line rapidly down-regulates DNA synthesis in response to radiation damage with the expected biphasic dose-response kinetics and that replication rates begin to recover within ~3 h. Analysis of replication intermediates in the DHFR domain by a two-dimensional (2-D) gel-mapping technique shows that initiation is virtually completely inhibited within 30 min of irradiation of asynchronous cell populations. However, when cells are blocked with the replication inhibitor mimosine, which has been suggested to inhibit initiation (28), irradiation has little effect on subsequent initiation when the block is removed. These data suggest a model for an S-phase damage-sensing (SDS) signal transduction pathway that ultimately inhibits the first step of a two-step initiation process at origins, the second step of which is inhibited by mimosine.

This study represents the first direct demonstration in a defined chromosomal replicon that radiation quantitatively down-regulates initiation of replication and should open the way to identifying the regulatory *trans*-acting factor(s) that affects origin function in the last step of the SDS pathway.

MATERIALS AND METHODS

Cell lines, cell culture, and synchronizing regimens. The CHOC 400 cell line was developed as previously described (26) and was maintained in minimal essential medium supplemented with nonessential amino acids, 10% fetal calf serum, and 400 μg of methotrexate per ml. The duration of the cell cycle under these conditions is ~22 h. For experiments, cells were grown in methotrexate-free medium on 15-cm plates or in 24-well cluster dishes. Asynchronous cultures were prepared by plating at low density from confluent stocks and refeeding when the cells had reached ~3×10^4 cells per cm^2; irradiation and subsequent manipulations were performed 24 h later. For synchronization, log-phase cultures were arrested in G_0 by incubation in isoleucine-free medium for 45 h (21); they were then released into complete medium containing mimosine and incubated for 14 h, by which time the population had reached the G_1/S boundary (7). Plates were washed once with prewarmed serum-free medium and returned to drug-free complete medium, allowing entry into the S period. All tissue culture media and sera were obtained from GIBCO/BRL.

FACS analysis. Cells were processed for fluorescence-activated cell sorter (FACS) analysis as described previously (28), and analyses were performed in the University of Virginia FACS Facility.

Irradiation. Cells were treated at a dose rate of 70 to 250 cGy per minute. A GE Maximar 250 X-ray machine operating at 200 kVp and 15 mA was used without a filter. All radiation treatments were performed at room temperature in the absence of CO_2.

Radiolabeling protocols. Cells (1×10^5 to 1.8×10^5 per 16-mm well) were incubated with 1.5 μCi of [^{3}H]thymidine per ml (85 Ci/mmol; ICN Biomedicals) and 0.2 μg of unlabeled thymidine per ml for the times indicated in the figure legends, and metabolism was stopped by the addition of 1/10 volume 2.3 M citric acid (29). At the end of the experiment, incorporation into DNA was measured as described in reference 28. Data were plotted by using SigmaPlot Version 4.1; data points represent the means of replicate determinations, and error bars indicate the standard deviation (in some cases, the error bars lie within the area of the symbol).

2-D gel electrophoresis. Replication intermediates were prepared and subjected to 2-D gel analysis as previously described by methods adapted from those originally developed by Mirkovitch et al. (27) and Brewer and Fangman (3), respectively. Each of the samples in the experiments summarized in Fig. 6 to 9 was derived from four 15-cm tissue culture dishes (~10^8 cells). FACS analyses were performed on control cultures to ascertain that the cells were growing asynchronously or were synchronized (as required) at the time of irradiation (data not shown).

RESULTS

Effects of radiation on [^{3}H]thymidine uptake in asynchronous CHOC 400 cultures. To establish that the amplified CHOC 400 cell line displays the acute damage arrest phenotype that typifies other cultured mammalian cell lines (including methotrexate-sensitive CHO cells [31, 38]), overall replication rates after radiation treatment were estimated by measuring the rate of incorporation of [^{3}H]thymidine into DNA.

In the first experiment, asynchronous cultures of CHOC 400 cells were irradiated at doses ranging from 400 to 3,200 cGy. At 90 min later, duplicate cultures were pulsed for 20 min with [^{3}H]thymidine. A typical dose-response curve was obtained

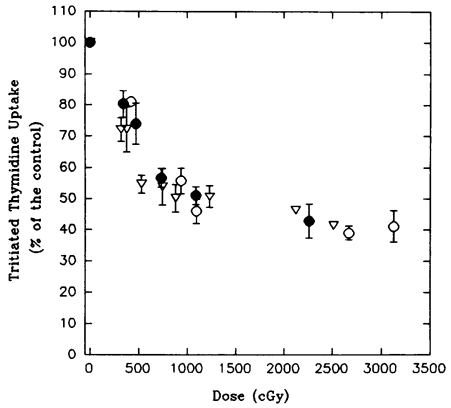

FIG. 2. Effects of different radiation doses on [^{3}H]thymidine incorporation in asynchronous CHOC 400 cells. Asynchronous CHOC 400 cells were irradiated with doses ranging from 400 to 3,250 cGy. At 90 min later, irradiated cultures and sham-irradiated control cells were pulsed for 20 min with a solution containing 1.5 μCi of [^{3}H]thymidine per ml and 0.2 μg of unlabeled thymidine per ml, and incorporation was determined as described elsewhere (28). Results are expressed as the ratio of incorporation by irradiated versus sham-irradiated controls. Error bars represent the standard deviations. Different symbols represent different experiments.

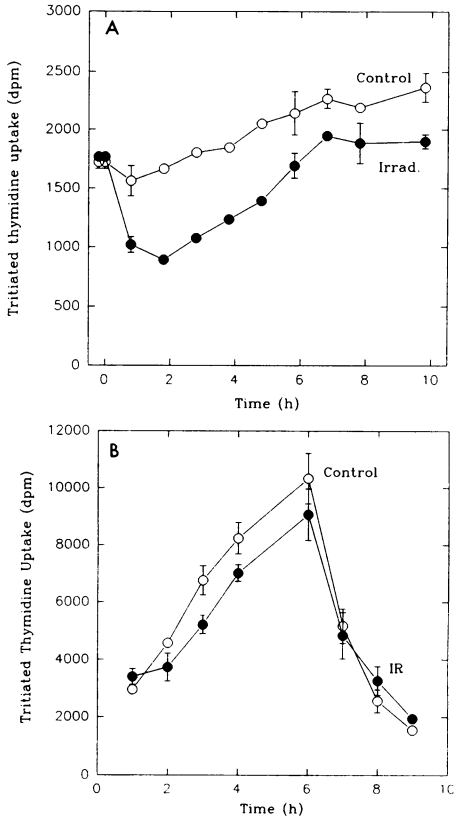

FIG. 3. Time course of radiation effects on asynchronous or early S phase CHOC 400 cells. (A) Quadruplicate samples of asynchronous CHOC 400 cells in multiwell dishes were irradiated at a dose of 1,000 cGy. Immediately following irradiation (time zero) the cells were returned to the incubator, and, starting 40 min later, they were pulsed for 20 min at hourly intervals with a solution containing 1.5 μCi of [^{3}H]thymidine per ml and 0.2 μg of cold thymidine per ml. One-third of the contents of each well was then counted, and results were plotted at the middle of the pulse period. (B) CHOC 400 cells were synchronized near the G_1/S boundary with 200 μM mimosine as described elsewhere (28). Immediately after release from the block, they were irradiated (IR) with 1,000 cGy, quadruplicate wells were pulsed with 1.5 μCi of [^{3}H]thymidine per ml every hour for 20 min beginning 50 min after irradiation, and incorporation was determined (28). Results are plotted at the middle of the pulse period.

that can be interpreted as having a steep (sensitive) component at doses below ~800 cGy (extrapolating to ~1,500 cGy) and a very shallow, insensitive component at higher doses (Fig. 2).

The relative immediacy of the damage arrest response was demonstrated in a time course study. Asynchronous cell populations growing in multiwell dishes were irradiated with 1,000 cGy and were pulse-labeled with [^{3}H]thymidine for 20 min at hourly intervals, beginning 40 min after irradiation. As shown in Fig. 3A, in the 40-min sample the rate of incorporation is inhibited almost maximally. Recovery begins ~3 h later and is nearly restored to preirradiation values by 6 to 7 h. Note that uptake of label is expressed as trichloracetic acid-precipitable disintegrations per minute per well in Fig. 3A, without regard to small differences in cell number that could occur between irradiated and undisturbed log populations in the 10-h period under study owing to the G_2/M arrest point.

Kinetics of cell cycle arrest after irradiation. To determine whether the CHOC 400 cell line exhibits the p53-mediated late G_1 checkpoint or the p53-independent G_2/M checkpoint or both, CHOC 400 cells were irradiated with 1,000 cGy and samples were prepared for FACS analysis at the indicated times thereafter. Results of this analysis are expressed in Fig. 4 as a percentage of the population displaying either the G_0/G_1, S, or G_2/M phase DNA contents.

It is clear that CHOC 400 cells do not arrest in G_1 after irradiation; rather, by 12 h, more than 60% of the population has arrested in G_2. G_1 arrest has never been observed in several other experiments in which samples were taken as late as 36 h postirradiation (data not shown). Thus, by this criterion, it appears that this cell line lacks a late G_1 checkpoint but retains the G_2/M checkpoint.

Analysis of replication intermediates in a defined locus by 2-D gel electrophoresis. To assess the effects of radiation on a defined chromosomal replicon, we analyzed the amplified DHFR domain in CHOC 400 cells by a 2-D gel replicon-mapping method (3). In this technique (Fig. 5), replication intermediates are carefully isolated, digested with an appropriate restriction enzyme, and run on a 2-D agarose gel in such a way as to accentuate the different electrophoretic mobilities of fragments that contain single replication forks (Fig. 5A), replication bubbles (Fig. 5B and C), or doubly forked termination structures (Fig. 5D). The digest is then transferred to a membrane and hybridized with an appropriate probe to detect the fragment of interest.

When the replication pattern of the amplified DHFR domain was analyzed by this technique in previous studies, it was apparent that there is not just one fixed initiation site in this domain (7, 36). Rather, nascent chains initiate at multiple (possibly random) sites scattered throughout the entire spacer region lying between the two genes but probably more often near ori-β and ori-γ (2, 4, 9, 18). The consequence is that a given fragment within this region sometimes sustains an active initiation event and is sometimes replicated passively by a fork that arose from an origin in a neighboring fragment. This results in a composite pattern consisting of a complete bubble arc and a single-fork arc.

The experiment shown in Fig. 6A illustrates this phenomenon, in which replication intermediates in the DHFR initiation

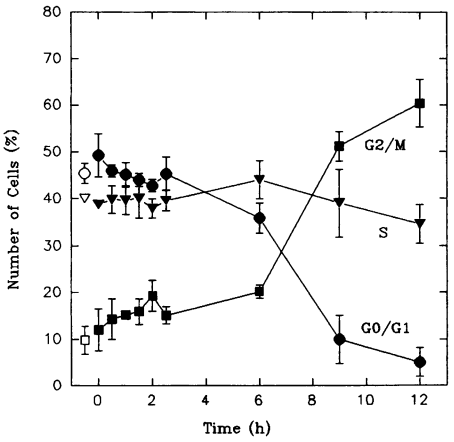

FIG. 4. Kinetics of cell cycle arrest after irradiation. A log-phase population of CHOC 400 cells was irradiated with 1,000 cGy, and samples were taken for FACS analysis at the indicated times thereafter. Results are expressed as the percentage of cells in each cell cycle compartment as a function of time after irradiation. The open signals represent unirradiated control cultures that were sampled at time zero.

locus were examined in samples isolated during the early S period. CHOC 400 cells were arrested in G_0 by isoleucine deprivation and were then incubated for 14 h in complete medium containing 200 μM mimosine. This allows all cells to traverse the G_1 period, but the drug appears to prevent initiation at origins under these circumstances (7, 28).

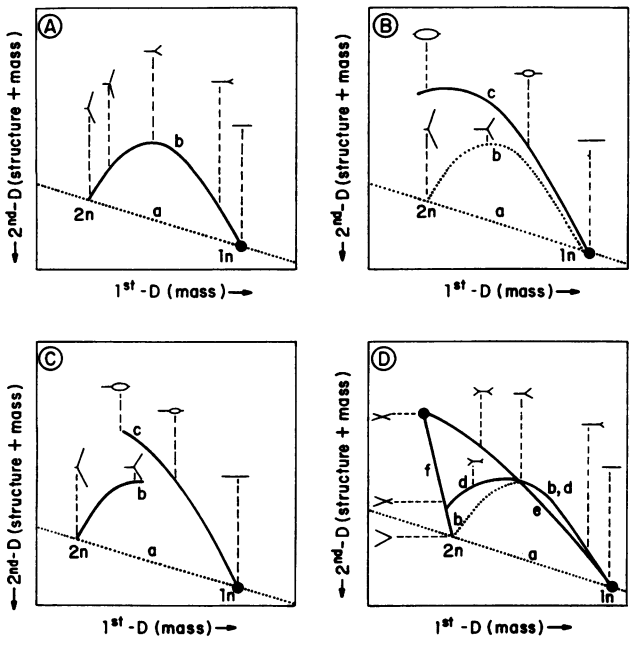

FIG. 5. Principle of the 2-D gel technique. DNA is digested with a restriction enzyme, and the fragments are separated on a neutral agarose gel according to molecular mass. Nonreplicating fragments migrate as a single band, whereas replicating fragments migrate more slowly depending on the extent to which the fragment has been replicated. In the second dimension, the fragments are separated on a 1% agarose gel under conditions in which shape contributes significantly to the migration rate. Each class of replication intermediates traces a characteristic pattern, and fragments that contain initiation bubbles (B and C) can be distinguished from fragments replicated passively by forks entering from flanking regions (A and D).

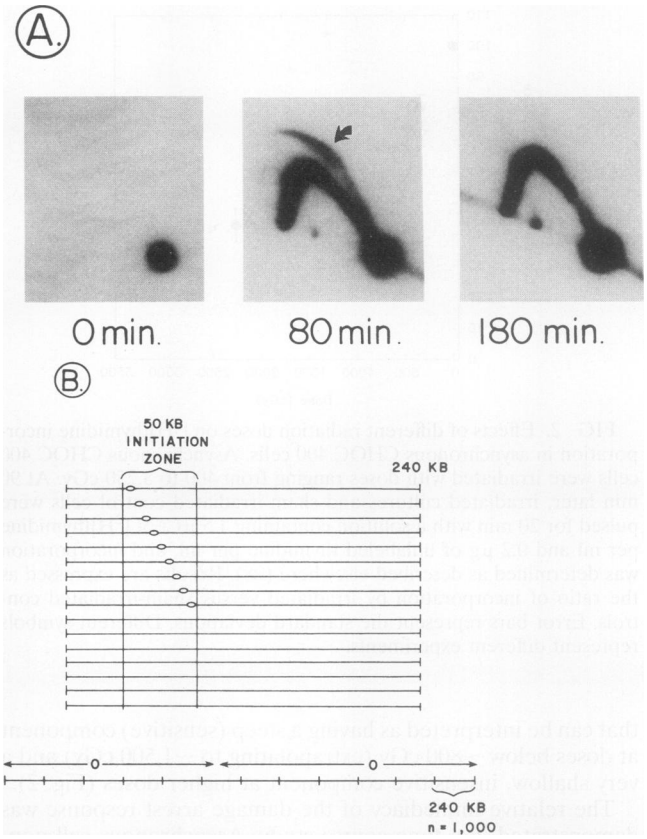

FIG. 6. 2-D gel patterns of replication intermediates in the DHFR initiation locus in the early S period of CHOC 400 cells. (A) CHOC 400 cells were synchronized near the G_1/S boundary with mimosine, and samples were taken prior to drug removal (0 min) or 80 and 180 min after drug removal. Nuclei were isolated, and matrix/DNA halo structures were prepared as previously described (9), using *Eco*RI to remove the DNA loops from the matrix. Replication intermediates associated with the matrix were further purified by fractionation on BND-cellulose (19) and the resulting digests were separated on a 2-D gel. After the digest had been transferred to GeneScreen, the filter was hybridized with a probe specific for the ori-β region (fragment F′ in Fig. 1C). Note the absence of replication intermediates in the time zero sample and the appearance of a composite pattern consisting of a bubble arc and a single-fork arc in the 80-min sample. By 180 min after mimosine removal, the bubble arc (but not the fork arc) has almost disappeared. (Note that the apparent discontinuities in the bubble and single-fork arcs in this experiment resulted from imperfections in the transfer medium.) (B) Diagram illustrating the proposed delocalized initiation that occurs throughout the 50-kb intergenic initiation zone in the 240-kb DHFR amplicon. Note that not all amplicons sustain an active initiation event, so that the initiation zone is sometimes replicated passively by forks emanating from distant amplicons.

When the sample taken prior to drug removal (the zero time point) was hybridized with a probe specific for fragment F′, which is centered over ori-β (Fig. 1), only the 1n spot corresponding to the unreplicated fragment was observed (Fig. 6A, left panel). This confirms our previous observations that mimosine appears to prevent initiation of early-firing origins of replication. However, 80 min after drug removal, a time corresponding to the peak rate of initiation in this locus (7), a composite pattern of a bubble arc and a single-fork arc was

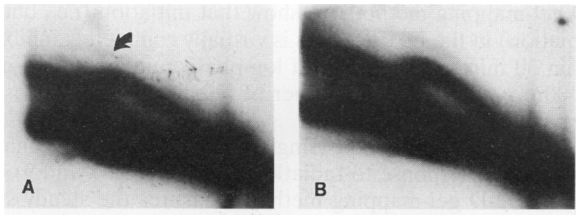

FIG. 7. Effect of irradiation on replication intermediates harvested immediately following treatment. Asynchronous CHOC 400 cells were irradiated with 900 cGy. Immediately thereafter, unirradiated controls (A) and irradiated samples (B) were harvested and the DNA was prepared and analyzed as in the legend to Fig. 5.

detected (middle panel). By 180 min after drug removal, the bubble arc had greatly diminished but a strong single-fork arc was still present (right panel). Thus, initiation in this locus is confined predominantly to the first 2.5 to 3 h of the S period, but the region continues to be replicated passively by forks. In independent experiments, we have shown that the single-fork arc persists for at least the first 6 h of the 10-h S period (7).

The most reasonable interpretation of these data is diagrammed in the model in Fig. 6B. Initiation occurs in the early S period at random sites confined to the 55-kb intergenic initiation zone of the 240-kb amplicon. However, only ~10% of the amplicons actually sustains an initiation event (7), so that the remainder of the amplicons (including their silent initiation zones) must wait to be replicated passively until forks arrive from distant amplicons. One consequence of this phenomenon is that the highest bubble-to-fork arc ratio in the initiation locus is observed in synchronized early S-phase cells, a time when many origins are firing and readthrough of replication forks into adjacent amplicons has not yet occurred (as in Fig. 6A). In asynchronous cultures this ratio is necessarily reduced, since the bubbles from the 10% of amplicons that sustain internal initiations are superimposed on the strong single-fork arc arising from the 90% that are passively replicated.

Radiation effects on the DHFR initiation locus in log cells. To assess the effects of radiation at the level of a single origin, we examined fragment F′ (centered over ori-β; Fig. 1C) by the 2-D gel-mapping technique at various times after irradiating asynchronous cultures of CHOC 400 cells with 900 cGy. This dose of radiation has no discernible direct effect on the integrity of replication bubbles in this locus when the DNA is isolated immediately after radiation treatment (compare patterns in Fig. 7B with those in Fig. 7A; see also Fig. 8). However, as shown in Fig. 8, the bubble arc that can be detected in the time zero sample prior to irradiation (arrow) has completely disappeared within 30 min of radiation treatment. A bubble arc does not reappear for at least 2 h and recovers to ~50% of the pre-irradiation level by 3.5 h. In contrast, the patterns of the single-fork arcs do not appear to change significantly in response to radiation. Replication bubbles were completely lost and then restored with the same time course in a neighboring 6.1-kb *Eco*RI fragment from the initiation locus (Fig. 1C, fragment F; data not shown).

Radiation effects on [^{3}H]thymidine incorporation in early S-phase cells. The data presented above show that in asynchronous cultures, there is a transient and apparently complete down-regulation of initiation in this locus within 30 min of a high radiation dose. Since we failed to detect a G_1 arrest in response to radiation treatment (Fig. 4), it is unlikely that the p53-mediated checkpoint is intact in these cells. Rather, it seems likely that there is an additional regulatory pathway that

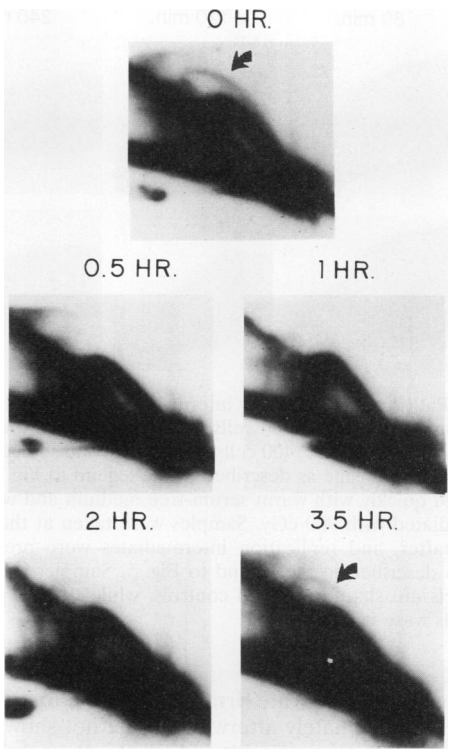

FIG. 8. Pattern of replication intermediates in the DHFR locus after irradiation of asynchronous CHOC 400 cultures. Asynchronous CHOC 400 cells were irradiated at a dose of 900 cGy, the plates were returned to the incubator, and samples were taken for 2-D gel analysis either immediately or at the indicated times thereafter. Note the loss of the bubble arc (arrow) in the 30-min sample and its partial restoration by 3.5 h.

functions within the S period itself at the level of individual origins.

Since this proposed pathway appears to quantitatively inhibit initiation, we reasoned that it should be possible to completely inhibit [^{3}H]thymidine incorporation into DNA by irradiating cells at the time when initiation would normally be occurring; at this time, there should be no refractile component resulting from replication forks already in operation.

To test this hypothesis, CHOC 400 cells were synchronized just beyond the G_1/S boundary with the replication inhibitor mimosine, as described above. At 14 h after releasing cells from G_0 arrest into mimosine, we washed the cultures once to remove the drug and immediately irradiated them with 1,000 cGy. The rate of [^{3}H]thymidine incorporation was then measured during the first 6 h of the 10-h S period by pulsing for 20 min at 1-h intervals (the first pulse began 50 min after irradiation, and results are plotted at the midpoint of the pulse period).

As shown in Fig. 3B, the effect of a 1,000-cGy dose of radiation on [^{3}H]thymidine incorporation is minimal when compared with the effect on log-phase cells treated with the same radiation dose (Fig. 3A). At the first time point (the 50- to 70-min sample), there is no measurable difference between the irradiated sample and the nonirradiated control, compared with a 45% depression in the rate of DNA synthesis in log-phase cells at a comparable time (Fig. 3B). The maximal inhibition achieved is ~12% in this experiment and occurs 4 to 6 h after radiation treatment. (Note that the same results are

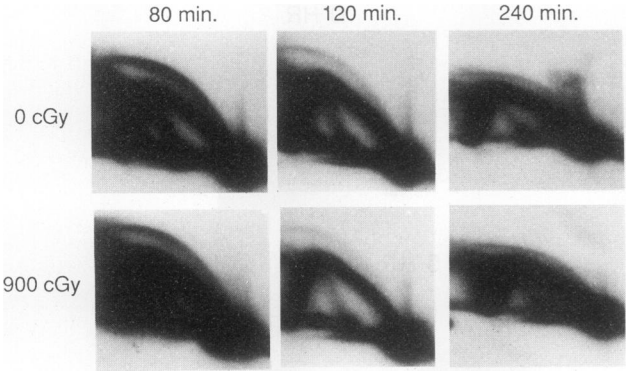

FIG. 9. Pattern of replication intermediates in the DHFR locus after irradiation of CHOC 400 cells synchronized with the initiation inhibitor mimosine. CHOC 400 cells were synchronized near the G_1/S boundary with mimosine as described in the legend to Fig. 3B. Plates were washed quickly with warm serum-free medium and were immediately irradiated with 900 cGy. Samples were taken at the indicated times thereafter, and replication intermediates were prepared and analyzed as described in the legend to Fig. 5. Samples shown in the upper panels are sham-irradiated controls, while the samples in the lower panels were irradiated.

obtained whether cells are irradiated prior to removal of mimosine or immediately afterwards [data not shown].)

Effects of irradiating mimosine-blocked cells on initiation in the DHFR locus. Cells that have traversed the G_1/S boundary but have been prevented from initiating at early-firing origins of replication appear to be relatively refractory to the down-regulation of origin function observed in log cultures. To confirm this suggestion at the molecular level, we used the 2-D gel-mapping technique to analyze the replication intermediates in fragment F′ from the DHFR initiation locus in mimosine-blocked cells.

As shown in Fig. 9, there is only a slight diminution of the bubble arc relative to the single-fork arc at either 80, 120, or 240 min after irradiation with 900 cGy. Thus, cells blocked with mimosine seem to be refractory to the proposed SDS pathway that is manifested when log-phase cultures are irradiated (Fig. 3A and 8).

DISCUSSION

Over the years, there have been several careful studies demonstrating the down-regulation of DNA replication in response to radiation damage (see reference 30 for a critical review). These studies involved incorporation of [^{3}H]thymidine, alkaline sucrose gradient centrifugation, or DNA fiber autoradiography to monitor either the rate of nascent chain elongation or the changing distribution of nascent chain sizes under a variety of circumstances. In aggregate, the results argue that the sensitive component of the dose-response curve corresponds to an inhibition of initiation at origins of replication.

Presumably, the immediate down-regulation of DNA replication is a regulatory phenomenon that allows the repair of breaks or removal of adducts, or both, before they can be fixed into lethal or mutagenic consequences. Our long-term goals are to understand the nature of this regulatory pathway at the molecular level.

CHOC 400 cells display the biphasic dose-response curve that characterizes several other mammalian cell types, including parental drug-sensitive CHO cells (Fig. 2) (38). Using the 2-D gel-mapping method, we show that initiation (i.e., bubble formation) in the DHFR locus is virtually completely inhibited within 30 min of irradiation of log-phase cultures with a dose of 900 cGy; the relative number of bubbles returns almost to normal ~3.5 h later.

The appearance of the single-fork arc does not change detectably in response to radiation damage (Fig. 8 and 9), but since the 2-D gel-mapping method measures the steady-state level of replication intermediates, it cannot differentiate between replication forks that are stationary, slowed, or moving at normal rates. However, after irradiation in log-phase cells, replication bubbles disappear from fragments F′ and F (Fig. 8; unpublished observations), presumably by maturing into larger bubbles; therefore, effects on chain elongation are probably minimal. This is consistent with the inability to inhibit the rate of [^{3}H]thymidine incorporation by more than ~55% even at the highest doses of radiation (Fig. 2).

Since initiation in the DHFR locus appears to be completely inhibited 30 min after irradiation of an asynchronous culture, and since CHOC 400 cells do not exhibit the late G_1 arrest phenotype, down-regulation of replication cannot be explained by the operation of a late G_1 checkpoint. This follows from the observation that initiation in the DHFR locus can be detected as late as 2.5 to 3 h into the S period (i.e., 3 h beyond the end of G_1). Furthermore, the inhibition of incorporation of radiolabel into DNA in asynchronous cultures reflects an effect on all cellular origins, regardless of when they fire in the S period.

One could reasonably ask whether effects observed in the amplified DHFR domain in CHOC 400 cells accurately reflect the situation in the single-copy DHFR locus in parental CHO cells. In this regard, we have only recently been able to increase the sensitivity of the 2-D gel approach enough to examine single-copy loci, and we plan to analyze radiation effects on the DHFR locus in CHO cells in future. However, preliminary experiments suggest that the mode of initiation in the single-copy locus is indistinguishable from that of the amplified DHFR domain in CHOC 400 cells (8). Thus, we assume that the effects observed in the present study will translate to the single-copy locus as well.

In aggregate, our data imply the existence of a damage-sensing pathway that functions within the S period itself to interfere with initiation at origins of replication. It is likely that the plant amino acid mimosine may help to determine at what stage of the initiation reaction down-regulation occurs.

Although this compound was originally reported to be a late G_1 inhibitor (15), several observations indicated that mimosine prevents replication per se, possibly at the initiation step (7, 28): (i) when added to exponential cultures of CHO cells, mimosine inhibits replication with the slow-stop phenotype that characterizes bacterial initiation mutants (41); (ii) when mimosine is added to cells traversing G_1, it inhibits entry into S (e.g., as in Fig. 6); (iii) mimosine inhibits replicating cells at any time prior to the midpoint of the S period but has very little discernible effect on replication beyond that point; and (iv) when added to log-phase cultures, mimosine appears to allow replication bubbles in fragment F′ to mature out of the fragment but does not allow any new initiations to occur. Thus, we assumed that mimosine affects some protein involved directly or indirectly with the initiation reaction. We therefore predicted that cells irradiated in the presence of mimosine and then released from the block would be unable to initiate replication upon entry into the S phase. Instead, the effect on initiation, either globally or at the level of the DHFR origin itself, was minimal (Fig. 3B and 7). We have also irradiated cells 15 min after release from mimosine (prior to the first detectable initiation in the DHFR locus in unirradiated con-

trols) and again observed only a minimal effect on subsequent rates of replication relative to the sham-irradiated control (unpublished observations). Therefore, it is unlikely that mimosine somehow negates the effect of radiation.

Mimosine therefore appears to trap the cells beyond any G_1 checkpoint and probably beyond a target step for the proposed SDS pathway. We suggest the following model for regulation of entry into the S phase, initiation at origins, and the response of these pathways to radiation damage.

In a normal cell cycle, cells must successfully traverse a G_1/S transition point before they can enter the S period. This is followed by a distinct intra-S-phase signal that is required to activate origins of replication; initiation is proposed to occur only during the first half of the S period. It is further proposed that cells must replicate the first bank of replicons before they can initiate at the second bank, etc. (this follows from the observation that the S period cannot be shortened by incubating cells in mimosine for longer and longer times after release from a G_0 block) (7). Origins are proposed to be activated by a T-antigen-like *trans*-acting factor that melts the origin (step 1); this, in turn, activates a prepriming reaction (step 2) that leads to the actual priming and initiation of nascent chains (step 3).

When a log-phase culture of normal cells is irradiated, the three proposed damage arrest pathways function in the following manner. (i) Cells at any position in G_1 will be transiently arrested at a p53-mediated checkpoint in late G_1 until the damage can be repaired (12–14); presumably, this checkpoint either precedes or coincides with the G_1/S transition point itself. (ii) In cells that have already traversed the G_1/S checkpoint and are located anywhere in the first half of S, DNA synthesis will be transiently arrested by the SDS pathway at or before step 1 of the initiation reaction at the next group of origins to fire; since their replication is not completed until after the damage has been repaired, the subsequent bank of replicons is also prevented from initiating. In this model, mimosine would inhibit either step 2 or step 3 of the initiation reaction; thus, mimosine-blocked cells would have already traversed the SDS checkpoint for the first bank of the earliest-firing replicons. (iii) Finally, cells that are in the latter half of S (i.e., after most initiations at cellular origins would have occurred) or in the G_2 period would be transiently arrested at the p53-independent G_2/M checkpoint (12).

This general model requires that the signal that stimulates the G_1/S and G_2/M checkpoints be relatively long-lived; this follows from FACS analysis of irradiated, asynchronous cultures of a variety of cell lines (e.g., RKO colorectal carcinoma, human fibroblast, and myeloid progenitor cells), for which it was shown that by 17 h postirradiation, the majority of the population comes to rest in either late G_1 or G_2 (12). Some cells (e.g., those in early G_1) would therefore have to retain the memory of the signal for the time it takes to arrive at the late-G_1 checkpoint (i.e., 6 to 8 h). Although it was stated that there was little effect on the S period itself in these studies (12), it was evident from the FACS data that some cells moved through the S period extremely slowly in these experiments. This is consistent with the 3- to 4-h SDS arrest that is observed when log-phase cells are irradiated (Fig. 3A) (38).

In contrast, this model requires that the signal that activates the SDS pathway be relatively short lived, since cells irradiated in the presence of mimosine were initiating replication in the DHFR locus even at the earliest time point sampled after irradiation (80-min sample in Fig. 9). Since these cells would have passed both a G_1/S checkpoint and the postulated SDS checkpoint for the earliest-firing origins, they do not appear to "remember" the signal delivered 80 min earlier. However, in log-phase cells, initiation at all DHFR origins is inhibited for at least 2.5 h postirradiation. Presumably, when one bank of origins is prevented from firing by the SDS pathway, the next bank must wait until replication resumes, owing to the usual mechanism that regulates orderly passage through the S period.

The DHFR origins that are down-regulated when log-phase cultures are irradiated could then arise either from cells that were arrested by the G_1/S checkpoint or from cells arrested by the SDS pathway at any time during the first 150 to 180 min of the S period (the time during which DHFR origins normally fire). In this regard, however, we are reasonably sure, for two reasons, that CHOC 400 cells do not contain an active copy of a wild-type p53 gene: (i) in the FACS analyses of irradiated cultures (Fig. 4), CHOC 400 cells arrest at the p53-independent G_2/M checkpoint but fail to arrest at the p53-dependent G_1/S checkpoint; and (ii) CHO cells are capable of amplifying a variety of genes to high copy number in response to selection with appropriate agents (10, 35), and gene amplification has recently been suggested to occur only in cells lacking the functional p53-mediated damage arrest pathway (22, 42). We are presently attempting to clone the Chinese hamster p53 gene and cognate cDNA to formally prove the absence of wild-type p53 activity in CHO cells.

This raises the issue of when in the S period the radiation-sensitive DHFR origins detected in log-phase cells are being down-regulated. In a separate study, we will show that the SDS pathway indeed functions to down-regulate the DHFR origin when radiation is delivered at later times in the S period in synchronized cells (17).

The severity of DNA damage required to fully activate the proposed SDS pathway far exceeds anything that would normally occur in nature. Why, then, has nature elaborated a mechanism that is capable of completely down-regulating DNA replication at all origins of replication in response to high levels of radiation or other serious insults to the integrity of DNA? The kind of damage expected obviously depends on the particular tissue type and its access to deleterious encounters in the immediate environment. However, one can imagine that responses would develop evolutionarily to cope with UV, certain naturally occurring chemicals, heat, hypoxia, and, possibly most importantly, biological damage accruing from errors in the rejoining reactions of ligases and topoisomerases I and II. Perhaps the experimental situation (e.g., irradiation) amplifies a damage arrest response that would not normally achieve such a magnitude, as suggested by Povirk (33). Although much more insight is required to understand the relevance of this pathway to the survival of the species, an understanding of the molecular components of this pathway is nevertheless critical for developing rational approaches to enhance cell killing by radiation. With the amplified DHFR domain as a model, it may be possible to identify the changing distribution or interaction of proteins that occurs at the origin or in replication complexes in response to the operation of the SDS pathway.

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