EVIDENCE THAT X-IRRADIATION INHIBITS DNA REPLICON INITIATION IN CHINESE HAMSTER CELLS

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SUMMARY: Analysis of DNA replicated 20-30 min after x-irradiation showed that the relative size distribution of single-strand DNA was deficient in DNA $< 4 \times 10^7$ daltons and enriched in DNA $> 4 \times 10^7$ daltons. During this time, depressed thymidine incorporation into total DNA could be accounted for by failure to replicate DNA $< 4 \times 10^7$ daltons, with relatively little effect on larger DNAs. At 2 hr post-irradiation, synthesis of the entire size spectrum of DNA was depressed. By 4 hr post-irradiation, recovery of synthesis of all sizes of DNA was complete. The data indicate that x-irradiation preferentially inhibits replicon initiation, with relatively little effect on elongation.

INTRODUCTION

Radiation-induced reduction of precursor incorporation into DNA of mammalian cells has been known for many years, but very little progress has been made concerning the mechanisms involved. Radiation-induced expansion of the acid-soluble deoxyribonucleoside triphosphate pools has been suggested as a possible cause of reduced precursor incorporation into DNA (1). However, we have recently shown in Chinese hamster cells that neither the size nor the specific activity of the dATP, dGTP, and dTTP pools was affected by radiation and that the anomalous stimulation of deoxycytidine incorporation after x-irradiation was the result of dCTP pool changes unrelated to DNA replication (2). Moreover, we have shown that the bulk of DNA replication in irradiated S-phase cells terminated at a time and rate very similar to control cells (within 0.7 hr), although the S phase, assayed autoradiographically, was lengthened by ~ 3 hr (2). We suggested from these findings that the lengthened S phase might result if the synthesis of only a limited number of replicons was temporarily inhibited by radiation (3). Results presented in this report indicate that radiation preferentially inhibits the initiation of replicon synthesis.

METHODS

Chinese hamster cells (line CHO) were grown exponentially in suspension culture and x-irradiated as previously described (2). Following irradiation,

the cells were pulse-labeled 30 min with (methyl-^{3}H)thymidine (55 Ci/mmole, New England Nuclear) at 2 μCi/ml (3 μM final thymidine concentration) for DNA specific activity determinations. Cells to be analyzed by alkaline sucrose gradient centrifugation were labeled for 5 or 10 min with 100 μCi/ml (methyl-^{3}H)thymidine (4.8 μM final thymidine concentration).

The specific activity of labeled DNA was determined by a modified Schmidt-Thannhauser procedure, as previously described (2). Alkaline sucrose density gradient centrifugation was performed by the method of Cleaver (4) with the following modification. Cells (2 x 10^5 in 0.1 ml) were added to a layer containing 1 mg/ml heparin and 1% sodium lauroyl sarcosine. After 15 min, alkaline lysing solution was added and the gradient allowed to stand for an additional 1.5 hr. This procedure, which will be described elsewhere (manuscript submitted), permits a large reduction in the time required for cell lysis, DNA deproteinization, and DNA denaturation prior to centrifugation. Fractions (1.2 ml) were collected from the top on Millipore glass fiber filters (Catalog No. AP2504200), washed with suction five times with cold 5% trichloroacetic acid and 6 times with cold absolute ethanol, and counted as previously described (2).

RESULTS

The data in Fig. 1 show the effect of x-irradiation on (^{3}H)thymidine incorporation into total unfractionated DNA. With increasing dose, incorporation was reduced in a biphasic manner, the fast component predominating at doses $\leq$ 800 rads and the slow component predominating at doses > 800 rads (Fig. 1A). Incorporation into DNA in cells irradiated with 800 rads was depressed within 0.5 hr and recovered to control levels by 4 hr post-irradiation (Fig. 1B).

To examine more closely the effect of x-irradiation on DNA replication, cells were exposed to 800 and 1600 rads, pulse-labeled with (^{3}H)thymidine for 5 and 10 min at varying times after x-ray, and the DNA analyzed on alkaline sucrose gradients. We routinely obtained the peak structure shown in Fig. 2.* This figure shows an example of the profiles obtained from 10-min pulses administered at 20 min (Fig. 2A), 2 hr (Fig. 2B), and 4 hr (Fig. 2C) post-800 rads. Table 1 summarizes the data obtained from the distribution of radioactivity within the gradients. These data provide some interesting comparisons. DNA replicated at 20-30 min post-irradiation (Fig. 2A) was quite deficient in molecules < 4 x 10^7 daltons (e.g., fractions 1-10) but had more DNA, on a relative basis, > 4 x 10^7 daltons (e.g., fractions 11-30) than the unirradiated control. Similar profiles were obtained routinely in experiments in which the pulse time was reduced to 5 min. It can also be seen that 20 min after irradiation incorporation into DNA < 4 x 10^7 daltons was reduced

*The short lysis time attainable with heparin and Sarkosyl provides much improved resolution of bulk, nonreplicating DNA (manuscript submitted) and may account for the increased resolution of newly replicated DNA seen here.

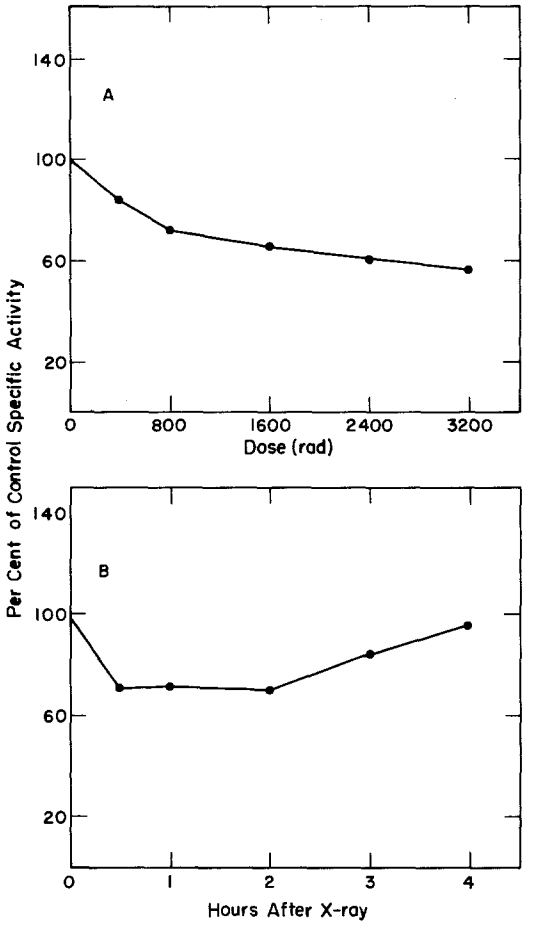

Fig. 1. Effect of x-irradiation on (^{3}H)thymidine incorporation into DNA. (A) Dose-response curve of cells irradiated with increasing doses, then pulse-labeled 30 min beginning 6 min post-irradiation. (B) Time-course of recovery of (^{3}H)thymidine incorporation after exposure to 800 rads. Cells were pulse-labeled for 30 min at the appropriate time post-irradiation. DNA was then isolated and specific activity determined by a modified Schmidt-Thannhauser procedure.

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by 52-58% for both 800- and 1600-rad exposures (Table 1, column 4), while incorporation into DNA > 4 x 10^7 daltons was reduced by only 2-7% after 800 rads (Table 1, column 5). Incorporation into DNA > 4 x 10^7 daltons after 1600 rads was reduced by 9-17% (Table 1, column 5). It should be noted that the overall radiation-induced reduction of incorporation across the entire gradient (Table 1, column 6) agreed very well with that obtained from unfractionated DNA (Figs. 1A and 1B). Hence, nearly all depressed radioactive

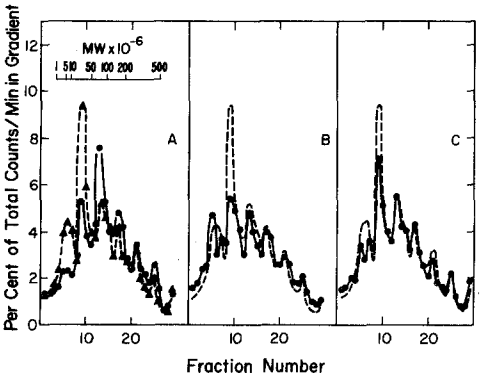

Fig. 2. Alkaline sucrose density gradients of DNA from irradiated cells. Cells were pulse-labeled 10 min with (^{3}H)thymidine at varying times post-800 rads exposure. (A) Labeled at 20 min post-irradiation: (-●-) irradiated and (-▲-) control. (B) Labeled at 2 hr post-irradiation: (-●-) irradiated and (---) control. (C) Labeled at 4 hr post-irradiation: (-●-) irradiated and (---) control. Gradients were centrifuged in a SW-27 rotor in a Beckman L3-50 ultracentrifuge for 6.25 hr at 15°C and 22,000 rpm. Gradients were calibrated with λ and T_2 phage DNA. DNA labeled with (^{14}C)thymidine (0.05 μCi/ml) for 36 hr and chased 12 hr sedimented at fraction 25 (~159S, mol. wt. 4.8×10^8) with a peak width at half-maximum of ~3 fractions.

incorporation 20 min after irradiation with 800 rads and most depression after 1600 rads involve molecules < 4×10^7 daltons.

It is clear from the data in Fig. 1B that, after 800 rads, (^{3}H)thymidine incorporation into unfractionated DNA was still maximally depressed 2 hr post-irradiation. Although the reduction of incorporation into total DNA 2 hr post-irradiation was the same as that at 20 min (see Fig. 1B and Table 1, column 6), there were distinct differences between the replication patterns of the 20-min and 2-hr samples: (1) at 2 hr the relative distribution of DNA across the gradient was similar to the control (Fig. 2B), unlike that seen at 20 min (Fig. 2A); (2) incorporation into DNA < 4×10^7 daltons was less depressed in the 2-hr sample (35%, Table 1, column 4) than the 20-min sample; and (3) at 2 hr incorporation into DNA > 4×10^7 daltons was much more depressed (27%, Table 1, column 4) than at 20 min (2-7%, Table 1, column 4).

The incorporation data from unfractionated DNA (Fig. 1B) and total DNA in the gradient (Table 1, column 6) indicate that, by 4 hr post-800 rads, DNA synthesis had recovered to near control levels. The relative distribution of radioactivity across the gradient was almost identical to the control (Fig. 2C), and incorporation into DNA both less than and greater than 4×10^7 daltons was at or near control levels (Table 1, columns 4 and 5).

TABLE 1. Distribution of Radioactivity from (^{3}H)Thymidine in Alkaline Sucrose Gradients[a]

Exposure (rads)	Pulse Length (min)	Time of Pulse after X-Ray	% Reduction into DNA $< 4 \times 10^7$	% Reduction into DNA $> 4 \times 10^7$	% Reduction into Total DNA
800	5	20 min	58	2	30
800[b]	10	20 min	52	7	23
800[b]	10	2 hr	35	27	30
800[b]	10	4 hr	11	0	4
1600	5	20 min	57	9	32
1600	10	20 min	55	17	35

[a]The % reduction was calculated from: $\left[1 - \frac{\Sigma \text{ cpm/fraction (x-ray)}}{\Sigma \text{ cpm/fraction (control)}}\right] \times 100$. DNA $< 4 \times 10^7$ daltons is in fractions 1-10; DNA $> 4 \times 10^7$ daltons is in fractions 11-30; and total DNA is the sum of all fractions.

[b]Data taken from gradients in Fig. 2.

DISCUSSION

We assume that (1) the percentage distribution of radioactive DNA across the alkaline sucrose gradients (Fig. 2) provides us with a relative measure of the number of DNA molecules of a particular size class containing newly replicated DNA and (2) the amount of radioactivity associated with particular regions of the gradients provides a measure of the incorporation rate into particular size classes of DNA (Table 1). The rate of replication has been measured in a number of mammalian cells (5-8) and is approaching, on the average, 1 μ/min per growing point (see ref. 9). This corresponds to the synthesis of $\sim 1 \times 10^6$ daltons of single-strand DNA per growing point per minute. Thus, a 10-min radioactive pulse is insufficient time to fully label a replicon of $\sim$ 30 μ average size (6,10). It is clear then that the bulk of the label across the gradients (Fig. 2) must represent incorporation into DNA strands whose replication had already begun at the time of pulse-labeling (see ref. 11). From these considerations, we can make some interesting predictions concerning the radiation effect on DNA replication. If replicons either about to initiate or in the process of initiation were prevented from doing so by x-irradiation, then in samples pulse-labeled for 10 min beginning 20 min post-irradiation we should see a preferential reduction of precursor incorporation into DNA molecules in the size range from 0 to approximately

3×10^7 daltons [e.g. (10^6 daltons per growing point per min) x (30 min)]. In fact, the data show that there were fewer molecules $< 4 \times 10^7$ daltons (Fig. 2A), and the 30% depression of incorporation into total DNA could be almost entirely accounted for by the large depression of incorporation into DNA $< 4 \times 10^7$ daltons (Table 1).

Using the same reasoning as above, we can suggest that irradiation does not affect the chain elongation process or ligation into higher molecular weight DNA species to any significant extent at the exposures used here. Again, any replicon that had just initiated synthesis at the time of irradiation would have had the opportunity to grow to $\sim 3 \times 10^7$ daltons by 30 min post-800 rads. Thus, those molecules in varying stages of completion at the time of irradiation would be $> 3 \times 10^7$ daltons. With a small effect of 800 rads on elongation and ligation, we would predict a higher relative proportion of DNA molecules $> 3 \times 10^7$ daltons (Fig. 2A) and very little effect on radioactive incorporation into DNA $> 3 \times 10^7$ daltons. Again, this is what we observed (Fig. 2A and Table 1).

Although there does not appear to be any significant difference between 800 and 1600 rads on incorporation into DNA $< 4 \times 10^7$ daltons (Table 1), there is a slightly greater depression of incorporation into DNA $> 4 \times 10^7$ daltons after 1600 rads. This suggests that the radiosensitive portion of the biphasic dose-response curve (Fig. 1A) is due to effects on initiation, while the radioresistant portion is due to effects on chain elongation. Watanabe (12) has also reported that chain elongation is radioresistant in mouse leukemic cells.

The similar degree of depression 20-30 min after 800 and 1600 rads and the failure to completely inhibit incorporation into DNA $< 4 \times 10^7$ daltons suggest that only a limited number of initiation sites can be affected and that a relatively small dose can produce maximal initiation inhibition. This is consistent with the results reported earlier in which the bulk of replication in irradiated S-phase cells terminated at a time similar to that of control cells (2). It is also consistent with results from the sample taken 2 hr after irradiation. These data show that the relative distribution of radioactivity across the gradient of the 2-hr sample was very similar to the control (Fig. 2B) but that the depression of incorporation into DNA both less than and greater than 4×10^7 daltons was similar (Table 1), unlike that of the 20-min sample. This indicates that a new state was attained in which relatively fewer replicons were participating in replication (e.g., in the 2-hr post-irradiation period much of the replication already initiated at time of irradiation would be completed). While it may be premature to speculate on specific radiation effects on replicon initiation, it is interesting that both

the temporal relationship and the degree of inhibition of histone f1 phosphorylation in x-irradiated Chinese hamster cells are similar to the effects on DNA synthesis (13) and that both DNA synthesis and f1 phosphorylation can be affected to a similar degree by caffeine treatment (14). While histone f1 phosphorylation is a very complex phenomenon, there is an S-phase-specific phosphorylation (15) which may be related to the radiation response of replication reported here.

Although two reports have appeared recently indicating a paucity of small, newly replicated DNAs after x-irradiation of mouse leukemic cells (12, 16), the data presented in this communication are the only information available to date relating in a quantitative way the (1) relative size distribution of DNA replicated after irradiation, (2) radioactive incorporation into various size classes, and (3) changes associated with post-irradiation recovery of DNA replication.

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