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Induction of replicative DNA synthesis in quiescent human fibroblasts by DNA damaging agents

(ultraviolet radiation/*N*-methyl-*N*-nitrosourea/*N*-acetoxy-2-acetylaminofluorene/excision repair/chemical carcinogenesis)

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ABSTRACT **A marked induction of DNA replication was observed in confluent human diploid fibroblast cultures treated with low relatively nontoxic doses of UV radiation, *N*-methyl-*N*-nitrosourea (MNU), and *N*-acetoxy-2-acetylaminofluorene (AAAF). Isopycnic CsCl density gradient analysis of newly synthesized DNA labeled with BrdUrd indicated that most of the synthesis was semiconservative. The rate of semiconservative DNA synthesis was maximal 24 hr after damage. This induction of DNA replication was greatest at ≈3 J/m² UV, 0.5 mM MNU, or 1.0 μM AAAF; was inhibited by hydroxyurea and aphidicolin; and also occurred in repair-deficient xeroderma pigmentosum fibroblasts. Autoradiographic examination of both confluent cultures and serum-arrested cultures showed a large increase in the fraction of densely labeled (S phase) cells after UV treatment. These densely labeled cells retain the capacity for cell division and subsequent proliferation. We conclude that low doses of at least three different DNA damaging agents are capable of recruiting quiescent cells into a state of DNA replication similar to that observed in the normal cell cycle.**

DNA replication occurring soon after DNA damage is an important factor in mutagenesis, cell survival, and *in vitro* transformation (e.g., see refs. 1–5). Furthermore, DNA replication after carcinogen treatment *in vivo* appears to be a necessary step in the initial stages of carcinogenesis in many tissues (reviewed in refs. 6 and 7). Although most studies have indicated that DNA-damaging agents inhibit DNA replication in actively growing cultured cells (e.g., see refs. 8–10) and in regenerating tissues *in vivo* (reviewed in ref. 6), a few papers suggest that carcinogenic agents may also induce or stimulate DNA replication (11–13). We have investigated this possibility in detail and have found that at least three agents that damage DNA can also induce semiconservative DNA synthesis in quiescent human fibroblasts.

MATERIALS AND METHODS

Cell Culture. Normal human diploid fibroblasts (AG1518; Human Genetic Mutant Cell Repository, Camden, NJ), xeroderma pigmentosum group G (GM3021A; Human Genetic Mutant Cell Repository), and xeroderma pigmentosum group A (CRL1223; American Type Culture Collection) were cultured, labeled with [^{14}C]thymidine and grown to confluence as described (14). Cells were used between passages 12 and 17 within 7 days of reaching confluence. For some experiments, growth was arrested at low cell density by incubating cultures in medium with 0.05% fetal calf serum.

UV and Chemical Damage and Labeling of Damaged Cells. Thirty minutes prior to damage, 50 μM BrdUrd (final concentration) was added to the culture medium. Some cultures were irradiated with UV light as described (15) and, after addition of either 20 μCi of [^{3}H]thymidine per ml (40–60 Ci/mmol; 1 Ci = 37 GBq; Amersham) or 4 μCi of [^{3}H]deoxycytidine per ml (5 Ci/mmol; Moravek Biochemicals, City of Industry, CA) to the medium, were incubated for the times indicated. Other cultures were damaged for 20 min with either *N*-acetoxy-2-acetylaminofluorene (AAAF) or with *N*-methyl-*N*-nitrosourea (MNU) (Ashe-Stevens, Detroit, MI), as described (16). In experiments involving chemical damage, the final dimethyl sulfoxide (Me_2SO) concentration was 2% (vol/vol), and 2% Me_2SO was added to control cultures. After chemical damage, the culture medium was replaced with conditioned medium containing 50 μM BrdUrd and 20 μCi of [^{3}H]thymidine per ml, and the cultures were incubated for 36 hr.

Isopycnic Centrifugation in CsCl. DNA was prepared by the method of Davis *et al.* (17) and ethanol-precipitated. For alkaline CsCl gradients, the precipitated DNA was dissolved in 4.5 ml of CsCl, pH 13 (0.1 M KOH; ρ = 1.799) and centrifuged at 50,000 rpm for 8 hr at 20°C in a Sorvall TV865 rotor. For neutral CsCl gradients, the precipitated DNA was dissolved in 4.5 ml of CsCl, pH 7.5 (ρ = 1.725), and centrifuged at 45,000 rpm in a Sorvall TV865 rotor for 8 hr at 20°C. Gradients were fractionated, and the radioactivity in each fraction was determined as described (16). Replicative incorporation was quantitated from CsCl gradients by determining the ratio of total ^{3}H cpm in DNA of greater than parental density to the sum of ^{14}C cpm in parental density DNA. Incorporation due to excision repair was calculated by determining the ratio of ^{3}H cpm to ^{14}C cpm in DNA of parental density (14).

Autoradiography. For autoradiography, fibroblasts not labeled with [^{14}C]thymidine were grown on sterile glass slides. After damage and incubation in medium containing the indicated concentrations of [^{3}H]thymidine or [^{14}C]thymidine, autoradiography was carried out as described (16). Fields were chosen in a previously determined manner for quantitation of S-phase nuclei to eliminate observer bias.

RESULTS

Induction of Replicative DNA Synthesis by UV or Chemical Damage. Confluent AG1518 human diploid fibroblasts were irradiated with UV light (6 J/m²), incubated for 36 hr with BrdUrd and [^{3}H]thymidine, and DNA isolated from these cells was separated by isopycnic centrifugation in alkaline CsCl into a more dense peak, containing DNA newly synthesized by replication, and a less dense peak, composed of parental DNA and containing any patches of DNA newly synthesized by excision repair (18). Irradiation with this dose of UV light stimulated a large amount of incorporation of labeled nucleotides into fully BrdUrd-substituted replicative

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Abbreviations: AAAF, *N*-acetoxy-2-acetylaminofluorene; MNU, *N*-methyl-*N*-nitrosourea; Me_2SO, dimethyl sulfoxide.

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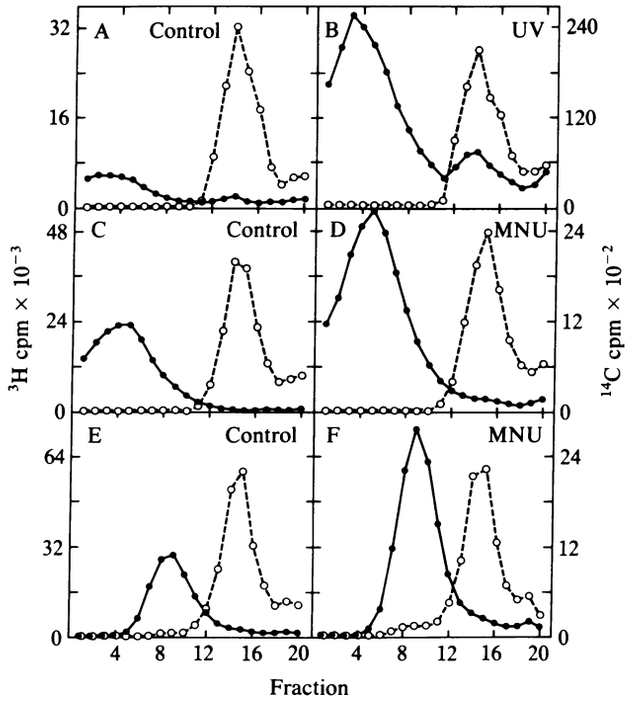

FIG. 1. Isopycnic centrifugation of DNA synthesized after exposure to UV irradiation or MNU. Confluent normal human fibroblasts, labeled with [^{14}C]thymidine, were treated as follows and incubated in 50 μM BrdUrd and [^{3}H]thymidine for 36 hr. DNA was analyzed by isopycnic centrifugation in alkaline (*A–D*) or neutral (*E* and *F*) CsCl. *A*, no treatment; *B*, UV light at 6 J/m^2; C and E, 2% (vol/vol) Me_2SO; *D* and *F*, 2% (vol/vol) Me_2SO + 0.5 mM MNU. ●, ^{3}H; ○, ^{14}C. The increased level of ^{14}C cpm in *A* and *B* is due to a higher specific activity of [^{14}C]thymidine present during the labeling period.

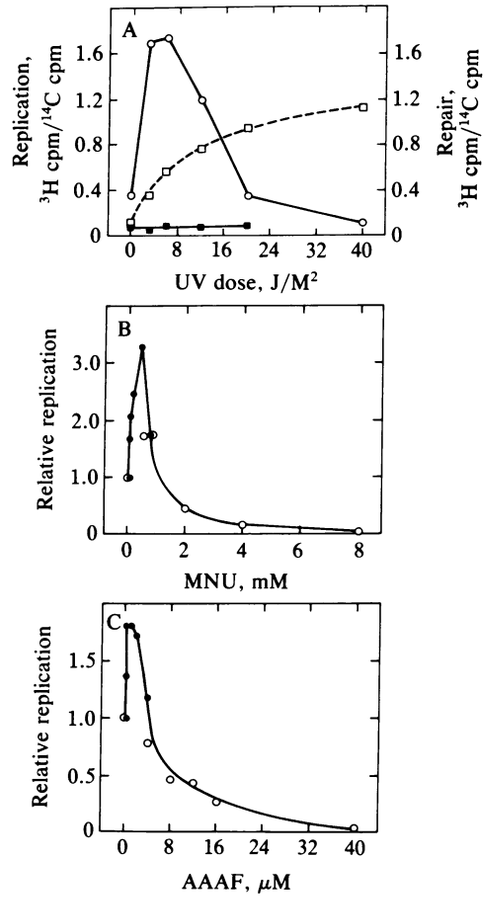

FIG. 2. DNA synthesis as a function of dose of DNA-damaging agent. (*A*) Confluent normal human fibroblasts, labeled with [^{14}C]thymidine, were irradiated with various doses of UV light and incubated for 36 hr in 50 μM BrdUrd and [^{3}H]thymidine in the presence (■) or absence (○, □) of 10 mM hydroxyurea. Replicative incorporation (○, ■) and repair incorporation (□) were determined as described in *Materials and Methods*. (*B*) Confluent fibroblasts, labeled with [^{14}C]thymidine, were damaged with various doses of MNU. Replicative incorporation was determined as described in *Materials and Methods*. Open and closed symbols represent data from two experiments, which have been normalized to the undamaged Me_2SO control. (*C*) Confluent fibroblasts were damaged with various doses of AAAF and otherwise treated as in *B*.

density DNA and a smaller amount of incorporation, due to excision repair, into parental density DNA (Fig. 1 *A* and *B*). Cells damaged with 0.5 mM MNU and labeled for 36 hr had a similar large stimulation of incorporation of nucleotides into replicative density DNA and a very small amount of repair synthesis (Fig. 1 *C* and *D*). When DNA from MNU-treated cells was examined on neutral CsCl gradients (Fig. 1 *E* and *F*), the newly synthesized DNA banded at hybrid density (ρ = 1.73 g/cm^3). Similarly, newly synthesized DNA from UV-treated cells banded at hybrid density on neutral CsCl gradients (data not shown). Thus, under these conditions, MNU and UV primarily stimulate semiconservative DNA replication.

The stimulation of DNA replication by UV and chemicals varied with the dose. In cells labeled for 36 hr after damage, incorporation of nucleotides into replicative density DNA was maximal at UV doses of 3–6 J/m^2 and decreased rapidly at higher doses (Fig. 2*A*). In contrast, UV-stimulated excision repair synthesis measured over 36 hr continued to increase up to UV doses of 20 J/m^2 and reached a plateau between 20 and 40 J/m^2. In cells damaged with MNU (Fig. 2*B*) or with AAAF (Fig. 2*C*), the variation of replicative DNA synthesis with dose was qualitatively similar to that observed for UV irradiation. The stimulation of replication was maximal at ≈0.5 mM MNU and between 0.5 and 2.0 μM AAAF; however, the magnitude of stimulation of replication was considerably less with AAAF treatment than with UV irradiation or MNU treatment. Replication decreased sharply with further increases in the dose of all of these agents and, at high doses, replication was inhibited to a level below that seen in undamaged control cells.

We also investigated the effect of hydroxyurea and aphidicolin, agents known to inhibit normal DNA replication (19, 20), on the replicative synthesis occurring after UV damage. At all UV doses examined, hydroxyurea completely inhibited incorporation of nucleotides into replicative density DNA (Fig. 2*A*) but had no effect on incorporation by repair synthesis into DNA of parental density (data not shown). After UV irradiation (5 J/m^2), aphidicolin (40 μg/ml) totally inhibited incorporation of labeled nucleotides into replicative density DNA and only partially inhibited incorporation of nucleotides due to excision repair (data not shown).

Induction of DNA replication by UV also occurs in confluent cultures of excision repair-deficient xeroderma pigmentosum fibroblasts (14) (Table 1). Thus, a functional excision repair system is not necessary for induction of DNA replication. The somewhat smaller increase in replicative DNA synthesis in the xeroderma pigmentosum cells may result from competing inhibitory effects of unrepaired UV-induced lesions in these cells.

Time Course of Induction of Replicative Synthesis. The time course of the induction of DNA replication was examined using cultures pulse-labeled at various times after damage with UV light at 5 J/m^2. The rate of replicative synthesis was

Table 1. DNA replicative synthesis after treatment of normal human fibroblasts or xeroderma pigmentosum fibroblasts with UV irradiation

UV dose, J/m²	Relative replicative synthesis: Normal human fibroblasts	Xeroderma pigmentosum group A	Xeroderma pigmentosum group G
0	1.0	1.0	1.0
0.5	5.1	2.6	3.4
2.0	13.0	7.4	6.4

Confluent cultures of fibroblasts were damaged with the indicated dose of UV light and pulse-labeled with [^{3}H]deoxycytidine and BrdUrd for 7 hr beginning 23 hr after damage. DNA replicative synthesis was calculated from alkaline CsCl gradients and is expressed as the ratio of replicative synthesis in UV-damaged cells to replicative synthesis in unirradiated cells.

initially the same as that in undamaged cells; however, after ≈12 hr, the rate of DNA replication began to increase, reaching a maximum at 24 hr after irradiation, and then declining (Fig. 3).

Cellular Distribution of DNA Synthesis After UV Irradiation. Autoradiography of irradiated confluent cultures revealed a marked increase in the number of nuclei densely labeled, as those of cells in S phase of the normal cell cycle (Fig. 4*A*). The number of S-phase nuclei increased from 0.4% in unirradiated cultures to 6% at 3 J/m² of UV light, and decreased at higher doses (Fig. 4*A*), producing a dose curve similar to that for the stimulation of nucleotide incorporation into replicative density DNA (Fig. 2). The UV-induced increase in S-phase nuclei was also suppressed by 10 mM hydroxyurea (data not shown). In cells labeled from 12 to 36 hr after irradiation (Fig. 4*B*), the fraction of S-phase cells is nearly the same as that seen when cells were labeled for the entire 36-hr period. This finding is consistent with the time course (Fig. 3), which indicates that the UV-stimulated DNA replication begins about 12 hr after damage.

Induction of DNA replication in confluent cultures is probably not the result of cell death or focal cell loss. The total number of nuclei per microscopic field was not decreased by UV irradiation, even at doses of 20 J/m² (Fig. 4), and inspection of the monolayers revealed no lacunae or foci

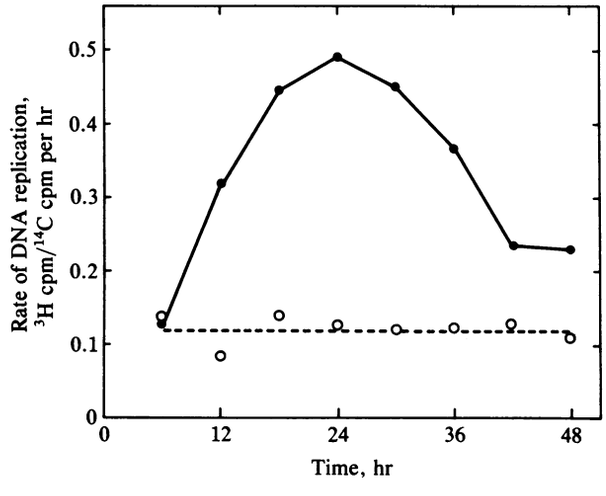

FIG. 3. Time course of replicative synthesis after UV irradiation. Confluent normal human fibroblasts were treated with UV light at 5 J/m² (●) or no irradiation (○). At each time point, the rate of replicative synthesis was measured during the preceding 6-hr pulse with [^{3}H]thymidine and BrdUrd and normalized to the amount of [^{14}C]thymidine-labeled parental density DNA in each alkaline CsCl gradient.

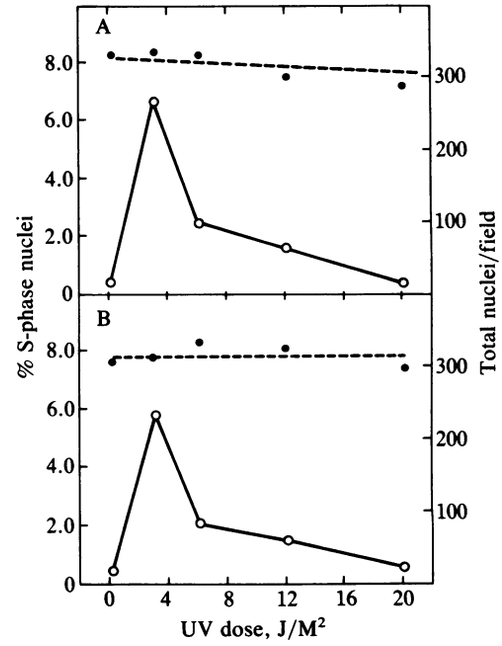

FIG. 4. The percentage of densely labeled S-phase nuclei as a function of UV dose. Confluent normal human fibroblasts were incubated (*A*) from 0 to 36 hr or (*B*) from 12 to 36 hr after UV irradiation in 50 μM BrdUrd and 50 μCi of [^{3}H]thymidine per ml, coated with NTB-3 emulsion and exposed for 1 week at 4°C. Nuclei were stained with Harris hematoxylin and the percentage of S-phase nuclei was determined. ○, S-phase nuclei; ●, total nuclei.

of damaged cells adjacent to densely labeled nuclei. Furthermore, in cells labeled from 12 to 36 hr after damage with UV light at 20 J/m², all non-S-phase nuclei showed the expected light labeling ("unscheduled DNA synthesis") due to excision repair, indicating that these cells are still metabolically active (data not shown). These data suggest that under our experimental conditions loss of cell viability was not significant even at doses of UV light much higher than those that maximally stimulate semiconservative replication.

We also studied induction of DNA replication in cultures of fibroblasts that were growth-arrested at low cell density by serum deprivation. In these cultures, only 4.5% of cells had microscopically detectable contact with adjacent cells (data not shown). In serum-arrested cells treated with various doses of UV light and pulse-labeled with [^{14}C]thymidine 23 hr after irradiation, the dose response of DNA replication was similar to that observed in confluent cultures (Fig. 5*A*). Induction of replication was maximal at 4 J/m² of UV light, at which ≈18% of the cells were labeled during the pulse period. Because these cells were pulse-labeled for only 6 hr, while UV-induced replication continues for 24 hr (Fig. 3), 18% represents a minimal estimate of the fraction of the cells in which replication is induced. As with confluent cultures, no cell loss was detectable after UV treatment. When analyzed by isopycnic centrifugation in CsCl, the time course of induction of semiconservative DNA synthesis by UV light in serum arrested cultures was similar to that in confluent cultures (data not shown).

To determine whether cells in which DNA replication had been induced retain the capacity for further proliferation, we damaged serum-deprived cultures with UV light as described above, pulse-labeled them with [^{14}C]thymidine, and then incubated the cultures with fresh medium containing 10% fetal calf serum for an additional 4 days (Fig. 5*B*). Cells pulse-labeled after UV irradiation at 1–2 J/m², which induced a 2- to 3-fold increase in the number of cells densely labeled during the pulse, proliferated to the same extent as

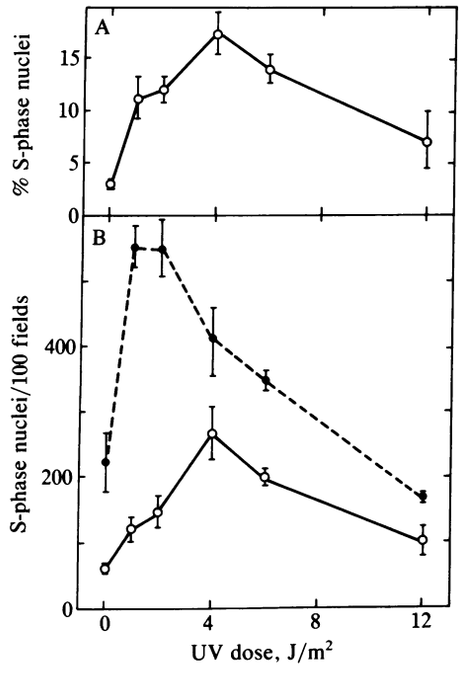

FIG. 5. Induction of DNA replication in serum-arrested cultures. Normal human fibroblasts were growth-arrested at low density by incubating cultures in medium containing 0.05% fetal calf serum. Cultures were treated with the indicated dose of UV light and after 23 hr, 0.025 μCi of [^{14}C]thymidine per ml was added for an additional 6 hr. Slides were then fixed immediately (○) or cultures were chased with fresh medium containing 10% fetal calf serum for an additional 4 days prior to autoradiography (●). Each point represents the mean ± SEM.

cells labeled in nonirradiated cultures during the subsequent chase period. In cultures treated with higher doses of UV light, labeled cells continued to proliferate during the chase period but to a somewhat lower level. This finding could result from the survival of only a fraction of cells undergoing UV-induced DNA replication at these higher doses or from a lengthening of the cell cycle in these cells.

DISCUSSION

We have shown that low doses of three DNA-damaging agents, each of which produces a different spectrum of DNA damage, induce replicative DNA synthesis in confluent human diploid fibroblasts. As in normal DNA replication, replication induced by DNA-damaging agents is semiconservative, is inhibited by both hydroxyurea and aphidicolin (19, 20), and is indistinguishable autoradiographically from S phase of the normal cell cycle. DNA replication induced by these agents is clearly different from excision repair in both its dose and time dependences and by the fact that it occurs in xeroderma pigmentosum fibroblasts, which are deficient in excision repair. These data suggest that low doses of DNA-damaging agents cause a fraction of the quiescent cells to enter a state similar to the S phase of the normal cell cycle. Because we have demonstrated damage-induced DNA replication in cells growth-arrested in two different ways, it is unlikely that the phenomenon is an artifact related to the cell culture system used (e.g., loss of contact inhibition due to death of small numbers of cells). We favor the hypothesis that this phenomenon represents a cellular response to injury. Although all three agents used in these studies damage DNA, they also affect other cellular constituents (e.g., proteins, membranes, RNA), and our data do not indicate which of these interactions is the proximate cause of induction of DNA replication.

A few previous studies have suggested that DNA damaging agents can induce DNA replication in non-growing cell populations. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine and benzo[*a*]pyrene have been found to stimulate hydroxyurea-sensitive thymidine incorporation in hamster embryo cells (11–13). Sidik and Smerdon (22) recently found that methyl methanesulfonate stimulates confluent human fibroblasts to incorporate nucleotides into replicative density DNA with a time course similar to that described above. These findings, taken together with our data, suggest that stimulation of DNA replication can result from exposure of quiescent cells to low doses of many different DNA-damaging agents. Many previous studies have suggested that DNA replication is inhibited transiently after DNA damage (e.g., see refs. 8–10). This observation is most likely due to the fact that these studies examined the effects of higher doses of DNA-damaging agents in asynchronous rapidly proliferating cultures so that stimulation of replication would be difficult to observe.

At least 6% of cells in confluent cultures and 18% of cells in serum-arrested cultures can be induced by these DNA-damaging agents to undergo DNA replication. Because the cells that undergo damage-induced DNA replication have the same potential for further proliferation as undamaged cells, the induction of DNA replication by DNA-damaging agents may have important biological consequences. Previous studies have shown that cultured cells that are passed shortly after damage have decreased colony-forming abilities and increased mutation frequency (1–3) compared to cells that are held at confluence prior to passage. DNA replication after damage has also been implicated in fixation of changes leading to cellular transformation induced by chemical agents (4) and radiation (5). Cells undergoing DNA replication induced by DNA-damaging agents might be expected to show similar effects, and, in fact, it has been suggested that carcinogen-induced hydroxyurea-sensitive nucleotide incorporation in density-inhibited hamster embryo cells is correlated with morphological transformation (12, 21). Furthermore, in some animal systems, DNA synthesis and cell division have been shown to be essential steps in the initial stages of chemical carcinogenesis (reviewed in refs. 6 and 7). Thus, carcinogens may have two effects of importance for the initiation of transformation *in vitro* and carcinogenesis *in vivo*: (*i*) production of DNA damage and (*ii*) fixation of damage through the direct induction of semiconservative DNA replication soon after damage. It is important to investigate the biological effects of carcinogens and mutagens over a wide range of doses because the effects of these agents, such as induction of DNA replication, which are evident at very low doses, may be inapparent or even reversed at higher doses of these agents.

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