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Advances in Brief

Persistent Inhibition of DNA Synthesis in Irradiated Rat Embryo Fibroblasts Expressing the Oncogenes H-*ras* plus v-*myc* Derives from Inhibition of Replicon Initiation and Is Mitigated by Staurosporine[1]

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Abstract

We have previously shown that rat embryo fibroblasts expressing the oncogenes H-*ras* plus v-*myc* experience a prolonged inhibition of DNA replication after exposure to ionizing radiation as compared to normal rat embryo fibroblasts, or rat embryo fibroblasts expressing H-*ras* or v-*myc* alone. Here we show that this enhanced inhibition of DNA replication in cells expressing H-*ras* plus v-*myc* is due to inhibition of the main controlling event of DNA replication, *i.e.*, replicon initiation, that this inhibition is reversible, and that the expression of this phenotype is reverted by staurosporine, a protein kinase inhibitor. These findings implicate genetic influences in the processes that control DNA replication in irradiated cells and identify events in the regulation of DNA replication that become apparent several hours after irradiation. The products of the oncogenes H-*ras* and v-*myc* appear to be members of, or exert influence on, this controlling pathway.

Introduction

We have recently shown (1) a pronounced inhibition by ionizing radiation of DNA synthesis in REF[3] transfected with the oncogenes H-*ras* plus v-*myc* as compared to normal REF, or REF expressing H-*ras* or v-*myc* alone. Specifically, inhibition of DNA synthesis in the former cells was only slightly higher than in the latter cells when measured immediately after irradiation but much larger when measured 3–5 h later. This difference in the levels of inhibition several hours after irradiation was the combined result of an enhanced (compared to zero time levels) and persistent inhibition of DNA synthesis in cells expressing H-*ras* plus v-*myc* and of a gradual recovery in normal REF, or REF expressing H-*ras* or v-*myc* alone. These observations suggested genetic influences in the processes that control onset and recovery of inhibition of DNA synthesis in irradiated cells and implicated the products of the oncogenes H-*ras* and v-*myc* in the controlling pathway. In the above-described experiments total DNA synthesis was measured and, therefore, it was impossible to conclusively establish whether the persistent inhibition observed in cells expressing H-*ras* plus v-*myc* reflected inhibition of replicon initiation or chain elongation, although the shape of the dose-response curves obtained suggested a rather specific effect on replicon initiation (2, 3). In addition, radiation-induced perturbations in the distribution of cells throughout the cell cycle did not allow us to establish, using total DNA synthesis measurements, recovery of DNA replication inhibition in cells expressing H-*ras* plus v-*myc*. Here we show that persistent inhibition of DNA synthesis in cells expressing H-*ras* plus v-*myc* derives from persistent inhibition of replicon initiation and demonstrate that this inhibition is reversible. Furthermore, we show that the inhibition of DNA replication in cells expressing H-*ras* plus v-*myc* is mitigated by staurosporine (4, 5), thus implicating protein kinases in the controlling pathway.

Materials and Methods

Cell Culture and Irradiation. For experiments 3.7 cells were used as a representative cell line expressing H-*ras* plus v-*myc*, and MR cells were used as a control cell line expressing only c-*myc* (6, 7). Both cell lines were kindly provided by Dr. W. G. McKenna (University of Pennsylvania) and were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum, at 37°C in an atmosphere of 5% CO_2 and 95% air. In experiments involving measurement of total DNA synthesis, or centrifugation in alkaline sucrose gradients, 1×10^5 cells from a growing culture were seeded in 60-mm tissue culture dishes and were allowed to grow for 2 days in the presence of 0.37 kBq/ml [^{14}C]thymidine plus 2.5 μM cold thymidine (see Ref. 1 for more details). Staurosporine (Sigma) was generally added 1 h before irradiation from a 1 μM working solution prepared in phosphate-buffered saline by dilution of a 100 μM stock solution prepared in dimethyl sulfoxide. Cells were exposed to X-rays (250 kV, 15 mA, 2-mm Al filter) at room temperature at a dose rate of 8.5 Gy/min. Dosimetry was carried out with a Victoreen dosimeter.

Cell Synchronization. Cells synchronized at the beginning of S phase were obtained by a combination of centrifugal elutriation and chemical resynchronization with aphidicolin (8). Cells grown in 100-mm tissue culture dishes ($5–10 \times 10^5$ cells/dish) for 30–40 h were trypsinized and pooled. Cells ($1.5–2.0 \times 10^8$) were elutriated at 18°C using a Beckman JE-6 elutriation rotor and a Beckman J2-21 M high-speed centrifuge. Fractions highly enriched in G_1 cells (typically more than 90%) were collected; $3–5 \times 10^5$ cells were distributed in 60-mm dishes and incubated in the presence of 1 μg/ml aphidicolin (Sigma) for 17 or 20 h, to achieve an accumulation of 3.7 and MR cells, respectively, at the G_1/S border. The cells were irradiated 2 h after release of aphidicolin block as they progressed through S. Progression of cells through the cycle was followed by flow cytometry carried out with a mercury arc lamp flow cytometer (PAS II; Partec). Cells were stained by direct suspension in a solution (1 ml) containing 0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, 0.05% Triton X-100, and 2 μg/ml 4′,6-diamidino-2-phenylindole. The rate of progression through the cycle was estimated by the relative increase of the modal channel of the DNA distribution histograms as a function of time.

Measurement of Total DNA Synthesis. Following radiation exposure, cells were supplied with fresh growth medium and returned to 37°C for 3 h. Subsequently, [^{3}H]thymidine at 3.7 kBq/ml was added for 1 h, and cells were trypsinized, loaded onto glass microfiber filters (Whatman GF/A), washed with 10% solution of trichloroacetic acid and deionized water, and incubated for 20 h in 0.5 ml of 1 N NaOH at 60°C. Filters were neutralized with HCl, scintillation fluid (Scintiverse, Fisher) was added, and samples were counted for ^{3}H and ^{14}C (present during the growth period) activity in a liquid scintillation counter (TriCarb 2200CA; Packard). The rate of DNA synthesis for each sample was calculated as ^{3}H dpm/^{14}C dpm and is presented as a percentage of the values obtained in sham-irradiated controls.

Alkaline Sucrose Gradient Sedimentation. This method was used to determine the molecular weight distribution of newly synthesized DNA in MR and 3.7 cells, in order to examine the effect of H-*ras* plus v-*myc* on radiation-induced inhibition of replicon initiation (9, 10). Gradients were calibrated

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[3] The abbreviations used are: REF, rat embryo fibroblasts; PK, protein kinase; SV40, simian virus 40.

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using DNA isolated from T2 (kindly provided by Dr. M. Showe, The Wistar Institute) and T7 (obtained from the American Type Culture Collection) phages. Linear sucrose gradients (5–20%) were prepared in a buffer containing 0.1 M NaOH, 0.9 M NaCl, and 0.01 M EDTA (pH 12.5) in polyallomer tubes. Cells were irradiated at room temperature and returned, after medium change, to 37°C for various periods of time as indicated; then [^{3}H]thymidine at 185 kBq/ml was added for 15 min. Cultures were trypsinized, and 50 µl of cell suspension (2×10^5 cells) were gently layered on 0.3 ml of lytic solution [0.5 M NaOH; 0.02 M EDTA, pH 12.5; and 0.1% Nonidet P-40 (Sigma)] in a cut 1-ml syringe, and incubated, after sealing to prevent evaporation, at room temperature for 3 h (see Ref. 11 for a more detailed description of this lysis method). Subsequently, lysate was gently layered on the top of a gradient, and centrifugation was carried out for 90 min using an AH 627 rotor in a Sorval RC60 centrifuge, at 27,000 rpm at 25°C. After centrifugation gradients were fractionated in 1-ml fractions, DNA was precipitated with 10% trichloroacetic acid (containing 25 µg carrier DNA) and loaded onto glass fiber filters. Radioactivity was measured as described above. Results are presented as a percentage of total activity per fraction. Values obtained with irradiated cells were normalized to those of nonirradiated cells to account for radiation-induced inhibition of DNA synthesis. The effect of radiation on chain elongation was measured by chasing the activity incorporated during the 15-min pulse, given 3 h after irradiation, for various periods of time. After this chasing period cells were lysed and DNA was applied to sucrose gradients as described above.

Results

Persistent Inhibition of Replicon Initiation in Irradiated 3.7 Cells. We used alkaline sucrose density gradient centrifugation to test whether the enhanced inhibition of DNA synthesis observed in H-*ras* plus v-*myc* transfected REF derives from sustained inhibition of replicon initiation. The sedimentation profiles obtained after centrifugation of exponentially growing MR (Fig. 1, *top*) and 3.7 (Fig. 1, *bottom*) cells exposed to 0 or 30 Gy X-rays and returned to 37°C for various periods of time are shown in Fig. 1. The percentage of ^{3}H radioactivity recovered in each fraction (after correction for radiation-induced inhibition of DNA replication, where applicable) is plotted as a function of fraction number. Shown for comparison in the figure are also the sedimentation distances of T2 (167 kilobases) and T7 (37 kilobases) DNA. In nonirradiated cells (MR or 3.7) the size distribution of newly synthesized DNA was between 15 and 300 kilobases, as expected from the replicon size of mammalian DNA. A general reduction in the levels of radioactivity was observed in irradiated 3.7 cells (Fig. 1, *bottom*) at all times tested (1–6 h after irradiation), including a depletion of activity from fractions 3–7. In controls, this part of the profile reflects replicon initiation processes that occur during the period of incubation with [^{3}H]thymidine. Thus, these data indicate a strong suppression of replicon initiation in 3.7 cells, evident already 1 h after irradiation and persistent for up to 6 h thereafter. Although reduced levels of radioactivity were also observed in irradiated MR cells, the distribution around fractions 3–7, when measured 1–2 h after irradiation, suggests a weaker suppression of replicon initiation as compared to 3.7 cells. More importantly, measurements 3–6 h after irradiation demonstrate a gradual recovery of replicon initiation. These results are in agreement with previous observations showing a slightly stronger inhibition of DNA replication in 3.7 as compared to MR cells when measured 1 h after irradiation, and a partial recovery of DNA replication in MR, but not in 3.7 cells, 3 h (and longer) after irradiation (1). Clearly, sustained inhibition of replicon initiation is one component of the enhanced inhibition of DNA replication in irradiated 3.7 cells as compared to MR cells.

We also carried out pulse-chase experiments to test the effect of radiation on chain elongation in MR and 3.7 cells. In these experiments cells were irradiated, returned to the incubator for 3 h, pulsed with [^{3}H]thymidine for 15 min, and analyzed 1–4 h thereafter (results not shown). We did not observe any measurable differences in the inhibition of chain elongation between MR and 3.7 cells, and radioactivity was mainly present in the high-molecular-weight fractions 1 h after pulse labeling in both cell lines. These results are consistent with the notion that the persistent inhibition of DNA replication in 3.7 cells derives mainly from a sustained inhibition of replicon initiation.

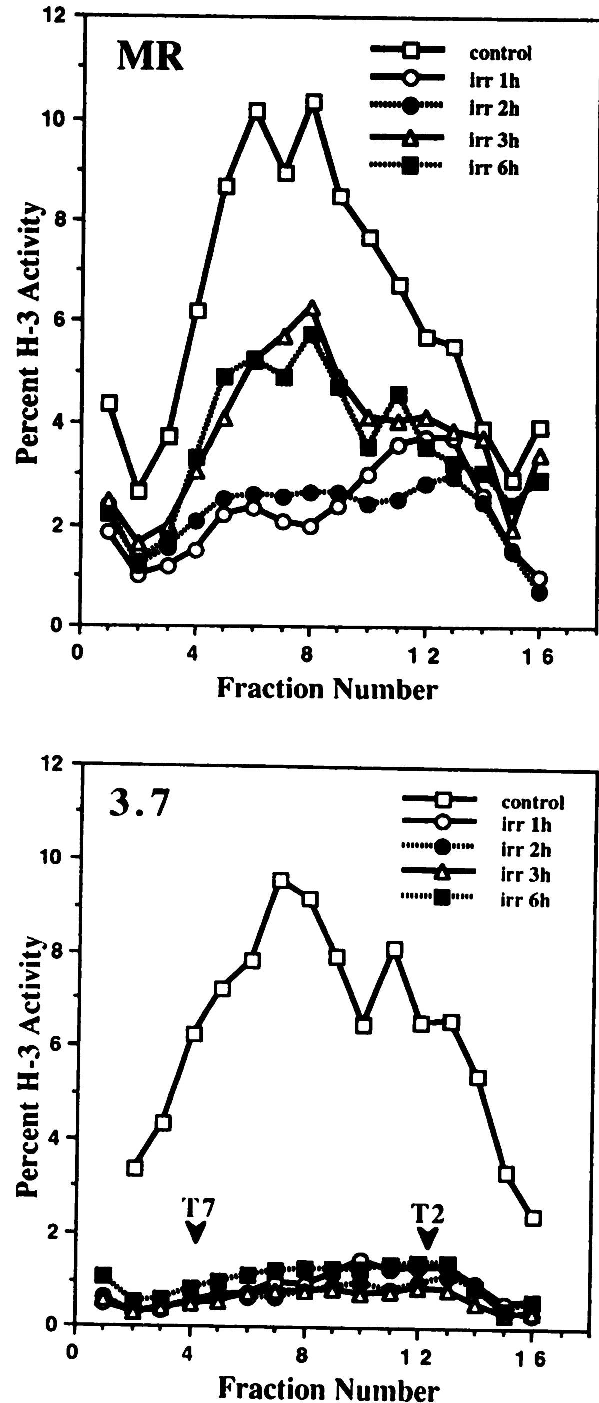

Fig. 1. Alkaline sedimentation profiles of nascent DNA from exponentially growing MR (*top*) and 3.7 (*bottom*) cells exposed to 30 Gy X-rays and returned to 37°C for various times as indicated before analysis. Plotted is the percentage ^{3}H activity as a function of fraction number after correction for radiation-induced inhibition of replication. Shown in the figure for comparison are also the sedimentation distances of T2 (167 kilobases) and T7 (37 kilobases) phage DNA. Similar results were obtained in 7 additional experiments.

Inhibition of DNA Replication Is Reversible in Irradiated 3.7 Cells. The above results, as well as those published previously (1), raise the question as to whether inhibition of DNA replication recovers in 3.7 cells. It was not possible to conclusively establish recovery in exponentially growing 3.7 cell populations because radiation-induced

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division and cell cycle-progression delays modified the distribution of cells throughout the cell cycle as a function of time after irradiation, and made interpretation of the results difficult. Because radiation-induced inhibition of DNA replication causes delays in the progression of cells through S, we followed progression through the cycle in synchronized cell populations as an alternative and evaluated S-phase-specific radiation-induced delays and recovery from these delays. The rate of progression through the cycle was measured in cells synchronized and irradiated at the beginning of S (2 h after release from aphidicolin block) and was compared to that of nonirradiated cells. The results obtained with MR and 3.7 cells exposed to 10, 20, and 50 Gy of X-rays are shown in Fig. 2 (*top* and *bottom*, respectively), together with those of nonirradiated controls. Plotted in the figure is the modal channel ratio, calculated as the quotient between the modal channel number of the DNA distribution of the progressing wave and that of G_1 cells, as a function of time after release from the aphidicolin block. Both cell lines had synthesized approximately 25% of their

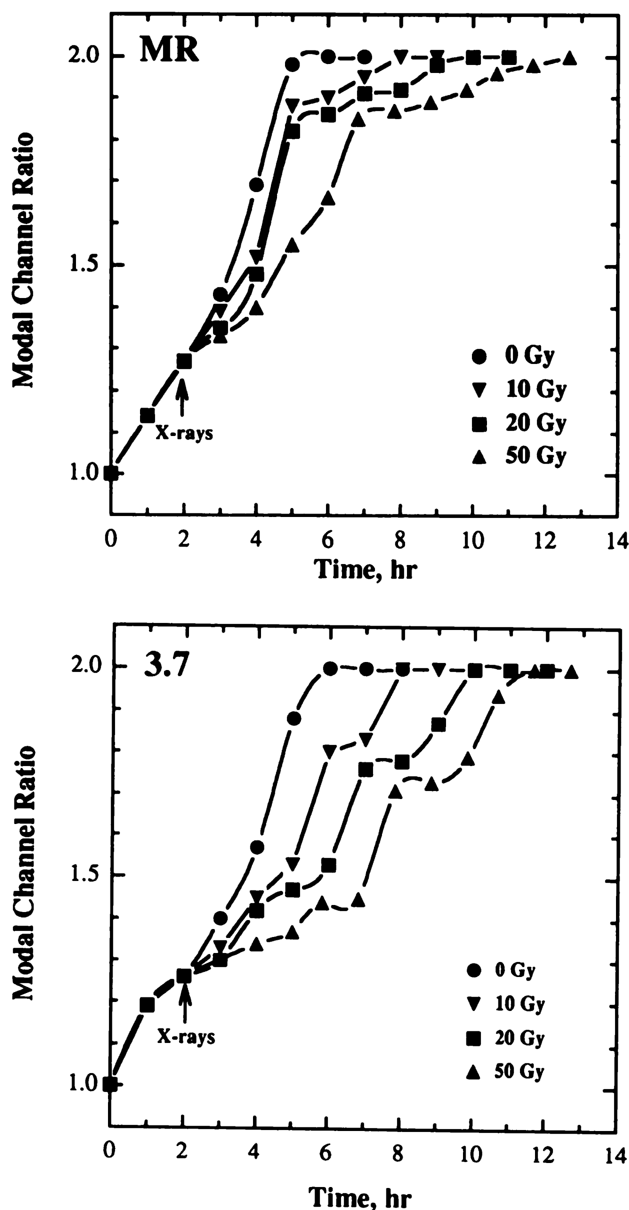

Fig. 2. Progression through the cycle, expressed as relative increase in the modal channel ratio as a function of time, of synchronized MR (*top*) and 3.7 (*bottom*) cells exposed to 0, 10, 20, and 50 Gy X-rays 2 h after release from an aphidicolin block of G_1 cells obtained by centrifugal elutriation.

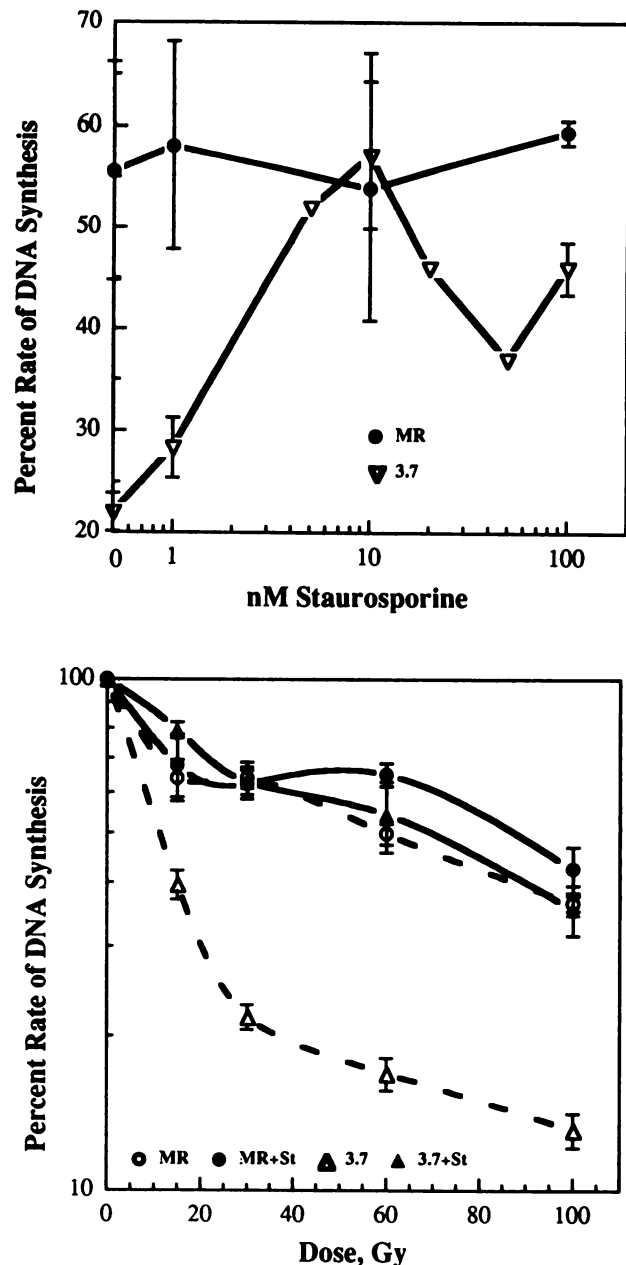

Fig. 3. *Top*, DNA synthesis inhibition in MR and 3.7 cells exposed to 30 Gy X-rays and incubated in the presence of various concentrations of staurosporine as indicated. The mean and SE from three independent experiments (when available) are shown. Staurosporine had no significant effect on the replication of nonirradiated cells at concentrations lower than 10 nM. At 10 nM there was a reduction of DNA synthesis to 0.87 ± 0.12 and 0.76 ± 0.15 (averaged from 11 independent determinations) for nonirradiated MR and 3.7 cells, respectively. At 100 nM a reduction to 0.58 ± 0.2 and 0.61 ± 0.07 (averaged from three independent determinations) was observed for MR and 3.7 cells, respectively. *Bottom*, inhibition by radiation of DNA synthesis in MR and 3.7 cells in the presence or absence of 10 nM staurosporine. Cells were irradiated and incubated for 3 h in the presence of the inhibitor before analysis. The mean and SE from three independent experiments is shown.

DNA at the time of radiation exposure (2 h). It is evident that radiation produced in both cell lines a delay in the progression through S, observable at doses as low as 10 Gy. Yet, the delay produced in 3.7 cells was 2–3 times longer than that in MR cells, at all doses examined. Delays measured halfway between modal channel ratios of 1.25 and 2.0 were 0.5, 0.6, and 1.9 h for MR cells and 1.2, 2.2, and 3.3 h for 3.7 cells exposed to 10, 20, and 50 Gy, respectively. These results confirm the observation of longer radiation-induced inhibition of DNA replication in 3.7 *versus* MR cells and show that DNA replication recovers in both cell lines; cells complete replication of their

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DNA even after exposure to 50 Gy. Qualitatively similar results were obtained in four other experiments. Quantitative differences were due to some variations in the degree of synchrony and the rate of cell progression through the cycle.

Staurosporine Reduces Radiation-induced Inhibition of DNA Replication in 3.7 Cells. The frequent activation of protein kinases by *ras* (12–15) led us to examine the effect of protein kinase inhibitors on radiation-induced inhibition of DNA replication in H-*ras* plus v-*myc* transfected cells. We examined the inhibition of DNA replication in irradiated MR and 3.7 cells after treatment with staurosporine, a potent, nonspecific inhibitor of PK (4, 5). Staurosporine, at various concentrations known to produce inhibition of PK, was added 1 h before irradiation in exponentially growing cells and was allowed to act for 3 h thereafter. Fig. 3 (*top*) shows the results obtained after exposure to 30 Gy. There was no significant difference in the degree of inhibition of DNA replication in MR cells at all staurosporine concentrations used. In 3.7 cells, however, incubation with 10 nM staurosporine reduced the inhibition of DNA replication to the extent that it reached levels similar to those observed in MR cells. A reduction in inhibition of DNA replication, as compared to untreated controls, was also observed at higher staurosporine concentrations, but the levels reached were lower than those at 10 nM, suggesting secondary effects of staurosporine or drug toxicity.

We then examined alterations in the dose-response relationship in cells incubated in the presence of 10 nM staurosporine using treatment protocols identical to those previously described (1). The results obtained are shown in Fig. 3 (*bottom*). Here again, incubation with staurosporine reduced the inhibition of DNA replication typically observed in 3.7 cells to levels similar to those measured in MR cells at all radiation doses examined. In agreement with the results shown in Fig. 3 (*top*), staurosporine had no significant effect in MR cells at all radiation doses examined.

As a further test for the effects of staurosporine on MR and 3.7 cells we measured the size of newly synthesized DNA using exponentially growing cultures, alkaline sucrose gradient centrifugation, and protocols similar to those described above. The results obtained with cells treated for 3 h with 15 nM staurosporine after exposure to 30 Gy X-rays are shown in Fig. 4. Results of control cultures exposed to radiation and incubated under the same conditions in the absence of staurosporine were presented in Fig. 1. It is obvious that in the presence of staurosporine replicon initiation recovers in 3.7 cells and reaches levels similar to those observed in MR cells. These results indicate that development of the phenotype of enhanced inhibition of DNA replication observed in REF transfected with the oncogenes H-*ras* plus v-*myc* requires the action of unknown protein kinases that are inhibited by staurosporine.

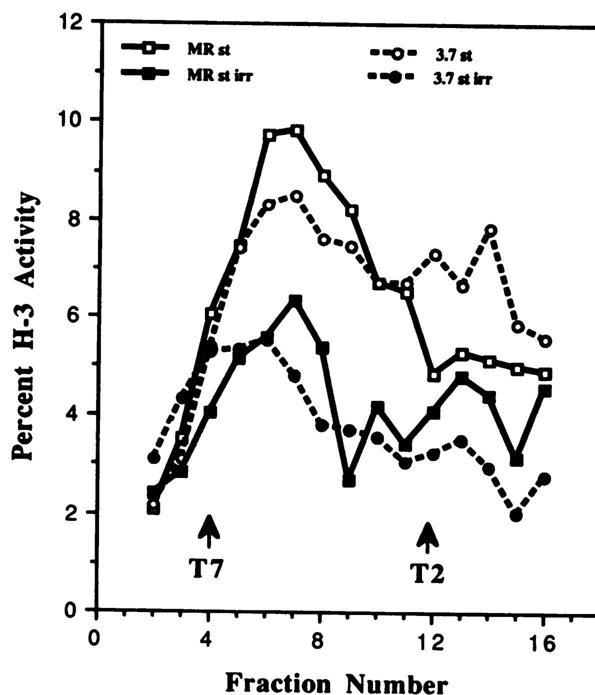

Fig. 4. Results similar to those shown in Fig. 1 with cells treated with 15 nM staurosporine for 3 h after irradiation. Similar results were obtained in two additional experiments.

Discussion

The above results indicate that the prolonged inhibition of DNA replication in irradiated REF expressing the oncogenes H-*ras* plus v-*myc*, as compared to normal REF or REF expressing H-*ras* or v-*myc* alone, derives from a persistent inhibition of replicon initiation. Replicon initiation is a step in DNA replication where most genetic controls are expected to act. In agreement with this notion, two of the proteins required for replication in an SV40-based model for eukaryotic DNA replication, the SV40 large T-antigen and RP-A (16–18) have been implicated in events controlling initiation and were shown to be activated in a cell cycle-dependent manner. It is likely, therefore, that the products of the oncogenes H-*ras* and v-*myc* cooperate in a transduction pathway that controls DNA replication in irradiated cells or that they exert indirect influences in such a controlling pathway.

The observation that staurosporine, an indole carbazole known to inhibit widely divergent members of the protein kinase (PK) family including protein kinase C, $p34^{cdc2}$ and $p34^{cdc2}$-like kinases, cAMP-dependent PK (PKA), epidermal growth factor receptor tyrosine PK, $pp60^{v\text{-}src}$ tyrosine PK, insulin receptor tyrosine PK, etc. (4, 5, 19), reverts the replication response of irradiated 3.7 cells to levels similar to those observed in MR cells, suggests the involvement of protein kinases in the controlling pathway. Since staurosporine specifically modulated replication in irradiated cells expressing H-*ras* plus v-*myc*, it is likely that the products of these oncogenes activate pathways not involved in the regulation of DNA replication in nontransfected cells. Thus, H-*ras* and v-*myc* may propagate (or receive) signals induced in irradiated cells by activation of diverse protein kinases. In line with this notion are reports indicating that Ras transduces signals through a direct regulation of protein kinases (12), that it activates a mitogen-activated protein kinase (13), and that it is a converging point for numerous signal transduction pathways (14, 15). We tested the effect of the isoquinoline H-7, another protein kinase inhibitor acting at the ATP binding site of the enzyme, and found no modulation of DNA replication in irradiated 3.7 or MR cells. This result suggests a specific effect of staurosporine on PKs involved in the regulation of DNA replication in irradiated 3.7 cells.

We have previously speculated that the prolonged inhibition of DNA replication in cells expressing the oncogenes H-*ras* plus v-*myc* derives from the activation, or synthesis, of a factor termed synthesis inhibiting factor (1). The nature of this factor is not known, but the results presented here allow some speculation regarding possible sites of action. Experiments investigating the control of DNA replication in *in vitro* model systems have shown the requirement for phosphorylation of large T-antigen and PR-A by $p34^{cdc2}$ kinase for SV40 DNA replication (20, 21). A similar involvement for $p34^{cdc2}$ kinase in events regulating DNA replication was indicated by experiments using cell extracts to study SV40 replication (22). Thus, activation of $p34^{cdc2}$ kinase appears to be a key event in the regulation of DNA replication. Regulation of the kinase activity of $p34^{cdc2}$ may require, among others, upstream protein kinases and phosphatases (synthesis inhibiting factor?) that respond to signals transmitted from Ras, and the downstream action of the activated complex might be by phos-

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phorylation of RP-A and large T-like proteins. It is likely that inhibition of DNA replication in irradiated cells expressing H-*ras* plus v-*myc* relies on the inactivation of the kinase activity of $p34^{cdc2}$ and that this process is affected by staurosporine. Experiments to test this hypothesis are under way.

The observation that the progression of irradiated, synchronized 3.7 cells through S was 2–3 times slower than that of MR cells confirms, in an independent manner, that DNA replication is inhibited in 3.7 cells to a larger extent than in MR cells. It also shows that this inhibition is reversible and that irradiated cells complete DNA replication even after exposure to doses as high as 50 Gy.

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References

1. Wang, Y., and Iliakis, G. Prolonged inhibition by X-rays of DNA synthesis in cells obtained by transformation of primary rat embryo fibroblasts with oncogenes H-*ras* and v-*myc*. Cancer Res., *52:* 508–514, 1992.
2. Painter, R. B. Inhibition of mammalian cell DNA synthesis by ionizing radiation. Int. J. Radiat. Biol., *49:* 771–781, 1986.
3. Lavin, M. F., and Schroeder, A. L. Damage-resistant DNA synthesis in eukaryotes. Mutation Res., *193:* 193–206, 1988.
4. Ruegg, U. T., and Burgess, G. M. Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. Trends Pharmacol. Sci., *10:* 218–220, 1989.
5. O'Brian, C. A., and Ward, N. E. Staurosporine: A prototype of novel class of inhibitors of tumor cell invasion? J. Natl. Cancer Inst., *82:* 1734–1735, 1990.
6. Ling, C. C., and Endlich, B. Radioresistance induced by oncogenic transformation. Radiat. Res., *120:* 267–269, 1989.
7. McKenna, W. G., Weiss, M. C., Endlich, B., Ling, C. C., Bakanauskas, V. J., Kelsten, M. L., and Muschel, R. J. Synergistic effect of v-*myc* and H-*ras* on radioresistance. Cancer Res., *50:* 97–102, 1990.
8. Iliakis, G., and Okayasu, R. Radiosensitivity throughout the cell cycle and repair of potentially lethal damage and DNA double-strand breaks in an X-ray sensitive CHO mutant. Int. J. Radiat. Biol., *57:* 1195–1211, 1990.
9. Studier, F. W. Sedimentation studies of the size and shape of DNA. J. Mol. Biol., *11:* 373–390, 1965.
10. Painter, R. B. Are lesions induced by ionization radiation direct blocks to DNA chain elongation? Radiat. Res., *95:* 421–426, 1983.
11. Bloecher, D. DNA double strand breaks in ehrlich ascites tumor cells at low doses of x-rays. I. Determination of induced breaks by centrifugation at reduced speed. Int. J. Radiat. Biol., *42:* 317–328, 1982.
12. Marshall, C. J. How does p21*ras* transform cells? Trends Genet., *7:* 91–95, 1991.
13. Shibuya, E. K., Polverino, A. J., Chang, E., Wigler, E., and Ruderman, J. V. Oncogenic Ras triggers the activation of 42-kDa mitogen-activated protein kinase in extracts of quiescent *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA, *89:* 9831–9835, 1992.
14. Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S., and Brugge, J. S. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. Cell, *68:* 1031–1040, 1992.
15. Wood, K. W., Sarnecki, C., Roberts, T. M., and Blenis, J. *ras* mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell, *68:* 1041–1050, 1992.
16. Wold, M. S., and Kelly, T. Purification and characterization of replication protein A, a cellular protein required for *in vitro* replication of simian virus 40 DNA. Proc. Natl. Acad. Sci., *85:* 2523–2527, 1988.
17. Tsurimoto, T., and Stillman, B. Multiple replication factors augment DNA synthesis by the two eukaryotic DNA polymerases, α and γ. EMBO J., *8:* 3883–3889, 1989.
18. Brill, S. J., and Stillman, B. Replication factor-A from *Saccharomyces cerevisiae* is encoded by three essential genes coordinately expressed at S phase. Genes Dev., *5:* 1589–1600, 1991.
19. Gadbois, D. M., Hamaguchi, J. R., Richard, A. S., and Bradbury, E. M. Staurosporine is a potent inhibitor of $p34^{cdc2}$ and $p34^{cdc2}$-like kinases. Biochem. Biophys. Res. Commun., *184:* 80–85, 1992.
20. McVey, D., Brizuela, L., Mohr, I., Marshak, D. R., Gluzman, Y. U., and Beach, D. Phosphorylation of large tumour antigen by cdc2 stimulates SV40 DNA replication. Nature (Lond.), *341:* 503–507, 1989.
21. Dutta, A., and Stillman, B. cdc2 family kinases phosphorylate a human cell DNA replication factor, RPA, and activate DNA replication. EMBO J., *11:* 2189–2199, 1992.
22. D'Urso, G., Marraccino, R. L., Marshak, D. R., and Roberts, J. M. Cell cycle control of DNA replication by a homologue from human cells of the $p34^{cdc2}$ protein kinase. Science (Washington DC), *250:* 786–791, 1990.

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