Regulation of Cellular and SV40 Virus Origins of Replication by Chk1-dependent Intrinsic and UVC Radiation-induced Checkpoints*

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DNA replication is inhibited by DNA damage through cis effects on replication fork progression and trans effects associated with checkpoints. In this study, we employed a combined pulse labeling and neutral-neutral two-dimensional gel-based approach to compare the effects of a DNA damaging agent frequently employed to invoke checkpoints, UVC radiation, on the replication of cellular and simian virus 40 (SV40) chromosomes in intact cells. UVC radiation induced similar inhibitory effects on the initiation and elongation phases of cellular and SV40 DNA replication. The initiation-inhibitory effects occurred independently of p53 and were abrogated by the ATM and ATR kinase inhibitor caffeine, or the Chk1 kinase inhibitor UCN-01. Inhibition of cellular origins was also abrogated by the expression of a dominant-negative Chk1 mutant. These results indicate that UVC induces a Chk1- and ATR or ATM-dependent checkpoint that targets both cellular and SV40 viral replication origins. Loss of Chk1 and ATR or ATM function also stimulated initiation of cellular and viral DNA replication in the absence of UVC radiation, revealing the existence of a novel intrinsic checkpoint that targets both cellular and SV40 viral origins of replication in the absence of DNA damage or stalled DNA replication forks. This checkpoint inhibits the replication in early S phase cells of a region of the repetitive rDNA locus that replicates in late S phase. The ability to detect these checkpoints using the well characterized SV40 model system should facilitate analysis of the molecular basis for these effects.

DNA replication is rapidly inhibited when cells are subjected to DNA damage during the S phase of the cell cycle. In mammalian cells, this inhibitory effect is generally detected as a decrease in incorporation of radioactive DNA precursors into newly synthesized DNA. The dose-dependent magnitude of the inhibitory effect induced by ionizing radiation and UVC radiation is biphasic in nature—an initial steep component occurs at low levels of damage, followed by a shallower component at higher levels. Analysis of cellular DNA pulse-labeled shortly after inducing DNA damage with ionizing radiation (1), UVC radiation (2, 3), topoisomerase inhibitors (4), and DNA reactive compounds (5) suggest these two components correspond to effects on two fundamentally different processes involved in DNA replication, initiation of DNA replication at origins of replication, and subsequent elongation of nascent chains at replication forks.

Early studies of these effects suggested that inhibition of initiation of DNA replication induced by ionizing radiation might occur in cis as a result of alterations in chromatin structure produced by radiation-induced strand breaks (6). The elongation arrest observed at high doses of UVC radiation also was thought to occur in cis when lesions formed a physical block to replication fork progression (7). However, more recent experiments indicate that the inhibitory effects of ionizing radiation (8–10) and mebolization damage (11) on episomal DNA replication can occur in trans. Furthermore, the inhibitory effect of ionizing radiation on DNA replication is reduced in cells from ataxia telangectasia and Nijmegen breakage syndrome patients containing mutations in the ATM and NBS1 genes, indicating that the products of these genes mediate this effect in trans as part of an intra-S phase checkpoint response to DNA damage (12, 13). Similarly, in both budding and fission yeast, the DNA replication-inhibitory effect of DNA damage corresponds in part to a trans-acting intra-S phase DNA damage checkpoint mediated by the products of a number of different genes, including structural and functional homologues of ATM and the related checkpoint kinase ATR (14, 15). In budding yeast, this and a similar checkpoint that responds to stalled replication forks repress late S phase origins of replication in early S phase cells (16–18). In both fission (19) and budding (17) yeast, this latter checkpoint also requires homologues of ATR and ATM, in addition to other checkpoint proteins. A similar checkpoint in mammals requires the checkpoint kinase Chk1 (20) and is abrogated by the ATM and ATR inhibitor caffeine (21). Although this latter checkpoint can be induced by the ribonucleotide reductase inhibitor hydroxyurea in p53-defective cells, a p53-dependent intra-S phase checkpoint that responds to hydroxyurea treatment has also been described (22).

Although most of the DNA damage and DNA replication intra-S phase checkpoints described above have been shown to inhibit initiation of DNA replication, the molecular targets of these checkpoints remain unknown. Furthermore, whether these checkpoints can also inhibit elongation of nascent chains remains unclear. In fact, recent evidence suggests that UVC radiation also induces a checkpoint that contributes to the elongation-inhibitory effects of this DNA damaging agent (23). In addition, the initiation-inhibitory effect of the intra-S phase checkpoint that responds to stalled forks could be caused by checkpoint stabilization of protein complexes at stalled replication forks in early S phase cells (21, 24).

Thus, a detailed understanding of checkpoints and their molecular targets requires the ability to distinguish initiation
from elongation-inhibitory effects, as well as information about the specific conditions under which these effects occur. Most efforts to distinguish between these effects in cellular replicons have employed an indirect approach involving velocity sedimentation of pulse-labeled nascent DNA. Experiments that rely completely on indirect labeling techniques can be problematic in their interpretation, however, because of differences in rate of replication fork movement, radiolabel incorporation at sites of DNA repair, and DNA damage in nascent radiolabeled DNA molecules. Further characterization of checkpoint effects also has been hindered by the difficulty with which the velocity sedimentation technique can be applied to the analysis of DNA replication in the experimental systems that have proved most useful as model eukaryotic replicons, those of yeast and small viral DNA replicons such as simian virus 40 (SV40).

SV40 is a particularly useful model system for studying different aspects of the regulation of DNA replication because of the small size of its genome, its replication to high copy numbers, and except for the viral initiator protein and helicase large T antigen, its dependence on cellular proteins for DNA synthesis. Cell-free systems that replicate SV40 DNA have been extensively employed to analyze the effects of DNA damage on SV40 DNA replication (25). However, most of these studies do not distinguish between initiation versus elongation-inhibitory effects. Consequently, the relationship between the biochemical events detected in these experiments and cellular responses to DNA damage that inhibit DNA replication in intact cells remains unclear.

We previously employed SV40 to study the effects of drugs on SV40 DNA replication in intact cells using an assay that clearly and simply distinguishes initiation-specific inhibitory effects from even subtle effects on the elongation phase of SV40 DNA replication (26–28). This assay employs radiolabeling of replicating SV40 DNA combined with a neutral-neutral (N/N) two-dimensional gel electrophoresis technique that separates replicating from nonreplicating molecules of DNA. In this study, we employed the velocity sedimentation technique and the neutral-neutral two-dimensional gel-based assay to analyze the initiation and elongation-inhibitory effects on cellular and SV40 DNA replication induced by UVC radiation, which is frequently employed to study DNA damage and checkpoint effects on DNA replication in other systems. We also asked whether the effects of UVC radiation on cellular and viral DNA replication require the checkpoint kinase Chk1. Our results confirm the interpretation of velocity sedimentation experiments in uninfected cells and show that UVC radiation induces a Chk1-dependent and caffeine-sensitive checkpoint that does not require p53 and targets the initiation, but not elongation, phase of cellular and viral DNA replication. They also reveal the existence of a novel intrinsic Chk1-dependent checkpoint pathway that regulates both cellular and viral origins of replication in the absence of DNA damage or stalled DNA replication forks.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—Mouse embryo fibroblasts (MEF) were a gift of Dr. Tyler Jacks, M.I.T., Boston, MA. MDA041 and TR-7 cells were a gift of Dr. George Stark, Lerner Foundation, Cleveland, OH. Cell culture reagents were purchased from Invitrogen. Aphiicolin was obtained from Sigma. It was diluted in 100% Me2SO, and stock solutions were stored at −20 °C. Proteinase K, restriction endonucleases, and Sephadex G-50 spin columns were obtained from Roche Molecular Biochemicals (Indianapolis, IN.). High strength analytical grade agarose was used for two-dimensional gels obtained from Bio-Rad. [α-32P]dATP (10 mCi/ml, 3000 Ci/mmol) and [3H]thymidine (1 mCi/ml, 63–67 Ci/mmol) was from Moravek Biochemicals, Inc. (Brea, CA). [α-3H]dATP (10 mCi/ml, 3000 Ci/mmol) and Hybond N* nylon were purchased from Amersham Biosciences, Inc. Prime-It DNA labeling kit was from Stratagene (La Jolla, CA). Adenovirus vectors expressing GFP or dn-Chk1 were a gift of Cyrus Vaziri (Boston University School of Medicine). UCN-01 was a gift of the National Cancer Institute, Bethesda, MD. All other chemicals were purchased from Sigma and were reagent grade or better.

SV40 Virus and Adenovirus Infections—For SV40 virus infections, BSC-1 monkey cells were seeded at 5 × 105 cells per 100-mm plate and grown for 24 h until 70–80% confluent in Dulbecco’s minimum essential media containing Earle’s salts supplemented with 10% fetal bovine calf serum and 1% nonessential amino acids. Cells were infected with SV40 virus (multiplicity of infection >1) diluted 1:10 in Dulbecco’s modified essential media containing Earle’s salts supplemented with 10% fetal bovine calf serum at 37 °C. Virus was then removed and cells were incubated an additional 23 h in fresh medium. MDA041 cells were infected with 5 × 106 infectious units/ml of recombinant adenovirus expressing either GFP or the dominant negative Chk1 (Lys-Arg) mutation (Ref. 47; gift of C. Vaziri) and analysis of UVC and UCN-01 effects on DNA replication was performed 22 h later. Expression of adenovirus-encoded proteins was confirmed by fluorescence microscopy (GFP) or immunoblotting (dn-Chk1).

UV Irradiation and Treatment with Caffeine or UCN-01—Culture medium was briefly removed from infected and uninfected cells just before irradiation to a sterile bottle in a 37 °C water bath, and monolayers were irradiated with 254 nm wavelength UVC radiation using a Stratagene Stratalinker. Medium was then returned to the plates and cells were cultured for an additional period of time until labeling and/or harvest of DNA. Caffeine or UCN-01 were added to medium to final concentrations of 2 mM or 100 mM, respectively, 2 min before UVC irradiation.

Labeling and Velocity Sedimentation Analysis of Cellular DNA—Sucrose gradients were prepared from equal volumes of solutions containing 0.1 M NaOH, 0.9 M NaCl, and 0.02 mM EDTA and either 5 or 20% sucrose. Measurements of UVC effects on cellular DNA replication were performed by labeling DNA in UVC-irradiated or sham-irradiated cells with 10 μCi/ml [3H]thymidine for 15 min beginning at 50 min after irradiation. Medium was then removed, and monolayers were washed once with phosphate-buffered saline. They were then trypsinized at room temperature and cells were suspended in 500 μl of 1× SSC. Aliquots of suspensions containing 3 × 106 cells were then diluted into an equal volume of cell lysis buffer containing 0.2 M NaOH, 0.02 mM EDTA, and 0.1% (w/v) Nonidet P-40 that had been gently layered on top of each sucrose gradient. Cells were allowed to lyse for 3 h at room temperature. The gradients were then centrifuged in a Beckman SW28 rotor at 20 °C for 2.5 h at 27,000 rpm. They were then distributed into 1-ml fractions and the DNA in each fraction was precipitated on ice for 15 min after adding 100 μg of herring sperm DNA and perchloric acid to a final concentration of 4%. The precipitated DNA was collected on GF/C glass fiber filters prefilled with 1.6 × HCl containing 6% sodium perchlorate. Each filter was washed twice with 0.4% perchloric acid, and then twice with 5 ml of 70% ethanol, and finally 5 ml of 100% ethanol. The dried filters were counted in 3 ml of scintillation mixture.

Labeling and Isolation of SV40 Viral DNA—SV40 viral DNA replicating in infected cells was labeled for 20 min with [3H]thymidine (10 μCi/ml medium) at times after irradiation as indicated in the figure legend. Viral DNA was harvested by washing 3 times with phosphate-buffered saline followed by incubation for 1 h at 37 °C with 3 ml/100-mm culture plate of 100 mM EDTA, 0.6% SDS containing 0.2 mg/ml proteinase K. Cell lysates were scraped into Falcon round-bottomed polypropylene tubes on ice, and 1 ml of 4 M NaCl was added to each tube with gentle mixing prior to refrigeration for a minimum of 1 h. Hirt supernatants were prepared by centrifugation of the precipitated containing high molecular weight cellular DNA for 30 min at 10,200 × g at 4 °C in a Beckman J-13.1 centrifuge rotor. Supernatants were extracted twice with 10 mM Tris, 1 mM EDTA (TE)-buffered phenol (pH 7.6) and once with chloroform:isoamyl alcohol (24:1). DNA was ethanol precipitated, recovered by centrifugation as above, washed with 70% ethanol, re-ethanol precipitated, and resuspended in restriction enzyme containing 0.1 μg/ml ethidium bromide (EtBr) for 25 h at 0.7 V/cm. The lanes were excised from the first dimension gel, inserted into enlarged preparative wells in second dimension gels of 1% agarose gel in 0.5× TBE containing 0.5 μg/ml EtBr, and sealed in place with excess agarose.

1 The abbreviations used are: MEF, mouse embryo fibroblasts; GFP, green fluorescent protein; RI, replication intermediates.
Inhibitory Effects of UVC Radiation on Cellular DNA Replication—To compare the effects of UVC radiation on SV40 DNA replication detected by two-dimensional gel methodology to its effects on cellular DNA replication detected by velocity sedimentation of pulse-labeled DNA, we first established the precise conditions under which the cellular effects could be observed in our laboratory using the velocity sedimentation technique. In this approach, nascent molecules are pulse-labeled with [³H]thymidine for a short time in control cells or cells subjected to DNA damaging agents, and then size-fractionated on alkaline sucrose gradients. A selective decrease in numbers of small, [³H]thymidine-labeled origin-proximal nascent molecules compared with larger molecules that had been extended further from origins before the pulse label indicates an initiation-inhibitory effect. A decrease in label associated with nascent molecules of all sizes indicates an elongation-inhibitory effect.

Exponentially proliferating cultures of MEFs were subjected to various doses of 254-nm wavelength UVC radiation, pulse-labeled with [³H]thymidine for 15 min beginning 50 min after irradiation, and then size-fractionated on alkaline sucrose gradients. Nascent DNA molecules pulse-labeled in sham-irradiated control cells had a broad distribution of sizes from short, origin-proximal molecules near the top of the gradient to longer molecules that had been extended further to regions distal to origins, which migrated closer to the bottom of the gradient (Fig. 1A, 0 J/m²). Incorporation of label into both populations was reduced in cells irradiated with 10 J/m² UVC (Fig. 1A, 10 J/m²), indicating inhibitory effects on both initiation and elongation, although the greater decrease in numbers of short, origin-proximal nascent molecules compared with large nascent molecules indicated that the predominant inhibitory effect at this dose of UVC was on initiation of DNA replication. Similar effects were observed at 25 J/m² UVC radiation (Fig. 1A, 25 J/m²) except that the labeling of long nascent DNA molecules was somewhat reduced, suggesting a slightly more pronounced elongation-inhibitory effect at this higher fluence. These results are similar to those described earlier except that levels of UVC radiation reported previously to induce these effects were somewhat lower compared with our experiments (3). At the highest dose of UVC radiation (Fig. 1A, 50 J/m²), the size distribution of nascent molecules was shifted significantly toward shorter molecules, and the pulse label associated with large molecules was reduced even further. This pattern also has been observed previously at higher fluences of UVC, and has been attributed to a pronounced inhibitory effect on elongation coupled to reinitiation of DNA synthesis beyond a lesion that blocks DNA
synthesis on the leading strand template, or discontinuous synthesis beyond a lesion on the lagging strand template (2).

Previous studies have reported that caffeine abrogates the initiation-inhibitory effects of ionizing radiation (31) and UVC radiation (32), similar to the effects of mutations in the ATM gene (12). We next sought to determine whether caffeine would have a similar effect on DNA synthesis in the UVC-irradiated MEFs employed in our experiments (Fig. 1B). Irradiation of caffeine-treated cells with 10 and 25 J/m² UVC only slightly reduced the amount of incorporation into short molecules (Fig. 1B). Therefore, caffeine abolishes the initiation-inhibitory effect on cellular DNA replication induced by these fluences of UVC radiation in MEFs. Caffeine also slightly reduced the inhibitory effect of 10 J/m², but not 25 J/m² UVC radiation on the labeling of long nascent chains (Fig. 1B, 25 J/m²). These results are consistent with earlier studies demonstrating an initiation-inhibitory effect of UVC radiation by a checkpoint that is inhibited by caffeine (32), and suggest that the elongation-inhibitory effect at higher doses of UVC is not part of this checkpoint. The initiation-inhibitory checkpoint does not depend on p53, because UVC radiation had similar effects on DNA replication in p53+/−MEFs (Fig. 1C).

Interestingly, the overall amount of incorporation of [³H]thymidine in these experiments was somewhat greater in the sham-irradiated control cells treated with caffeine (Fig. 1B) compared with control cells that were not treated with this compound (Fig. 1A, note change of scale for the ordinate, B compared with A). Furthermore, the size distribution of nascent DNA molecules in sham-irradiated control cells treated with caffeine was shifted to somewhat shorter lengths (Fig. 1B, 0 J/m²) compared with the distribution observed in unirradiated control cells in the absence of caffeine (Fig. 1A, 0 J/m²). The increased incorporation of [³H]thymidine and shorter lengths of nascent molecules suggest that caffeine activates origins of replication independently of its effects on a UVC-induced checkpoint. Stimulatory effects of caffeine on cellular DNA replication have been reported previously (32, 33).

**Dose- and Time-dependent Effects of UVC Radiation on SV40 DNA Replication**—Application of the velocity sedimentation technique for distinguishing between initiation- and elongation-inhibitory effects on DNA replication is limited to relatively large nascent molecules. To compare the dose-dependent effects of UVC radiation observed in cellular replicons with those induced on the initiation and elongation phases of DNA replication in the much smaller SV40 genome, we analyzed replicating SV40 genomes isolated from UVC-irradiated cells using an assay based on the neutral-neutral two-dimensional gel electrophoresis technique developed by Brewer and Fangman (30). This method separates branched replicating molecules from linear, nonreplicating molecules on the basis of size in the first dimension, and size and shape in the second dimension. Specific restriction fragments of replicating DNA can be detected as arcs that arise from nonreplicating DNA fragments by probing blots of two-dimensional gels with radiolabeled probes.

24 h after viral infection, SV40-infected BSC-1 monkey cells were subjected to the same fluences of UVC radiation employed in experiments involving uninfected cells. They were then incubated for 50 min before replicating SV40 DNA was pulse-labeled with [³H]thymidine for an additional 15 min. These conditions were identical to those used in the analysis of UVC effects on cellular DNA replication as described in the legend to Fig. 1. SV40 DNA was then isolated from cells and digested with BamHI. Equal aliquots of DNA were fractionated on two neutral-neutral two-dimensional gels run in parallel. One gel was blotted to a nitrocellulose membrane and probed with SV40 sequences to measure the number of SV40 replication intermediates, and the second gel was fluorographed to detect incorporation of [³H]thymidine into SV40 RIs as a measure of replication activity. Signals from the probed blot were measured by PhosphorImager analysis. [³H]Thymidine incorporation was measured by densitometric tracing of multiple exposures of the fluor-impregnated gel to x-ray film within the linear response range of the film. In both cases, measurements of signals from RIs in the bubble arc were normalized to signals from fully replicated (1n) DNA to account for any differences in overall yields of DNA.

PhosphorImager analysis of probed blots indicated a dose-dependent decrease in signals from SV40 RIs compared with signals from completely replicated DNA at UVC fluences up to 50 J/m² (Fig. 2A). Quantitation of the signals from the bubble arc and 1n DNA revealed that the number of SV40 RIs decreased in a dose-dependent fashion to ~10% of the number observed in sham-irradiated control samples (Fig. 2B, number RIs). This decrease suggested that RIs were maturing in the absence of new initiation events. The ability of RIs to mature implies the absence of a significant inhibitory effect on elongation of nascent DNA chains. This expectation was confirmed when replication activity was measured by quantitatively measuring signals from the fluorograph. Fig. 2B shows that at UVC fluences up to and including 50 J/m², incorporation of [³H]thymidine into SV40 RIs decreased essentially in parallel with decreases in the number of RIs. Thus, the decrease in incorporation could be completely accounted for by a decrease in the number of these RIs rather than an inhibitory effect on elongation.

These results indicate that the predominant inhibitory effect of 10–25 J/m² UVC radiation on SV40 DNA replication occurred at the level of initiation, similar to cellular replicons (Fig. 1). Although elongation was significantly inhibited in cellular replicons at 50 J/m² (Fig. 1), this effect was absent at this dose in the SV40 experiments (Fig. 2). This may reflect fewer lesions that block replication forks in cis within each SV40 replicon because of their smaller size (see “Discussion”). However, a higher dose of UVC radiation (500 J/m²) induced elongation-inhibitory effects in SV40 replicons as well. This was indicated by an increase in the number of SV40 RIs detected in Southern blots in conjunction with a continued decrease in their replication activity measured in fluorographs (Fig. 2, A and B, 500 J/m²). A similar pattern of large numbers of RIs with reduced replication activity was observed in parallel control experiments that analyzed SV40 RIs recovered from infected, but unirradiated cells exposed to the elongation-inhibitor aphidicolin (Fig. 2B, aphid). Thus, the larger numbers of SV40 RIs observed at 500 J/m² UVC radiation compared with lower doses appeared to reflect the failure of RIs to mature because of an elongation-inhibitory effect of UVC radiation, similar to the effects of aphidicolin treatment.

It was also possible, however, that the decrease in number of RIs in UVC-irradiated cells could reflect their destabilization. To unambiguously distinguish between these possibilities, we performed an additional control experiment in which SV40-infected cells were treated with the elongation-inhibitor aphidicolin during and after UVC irradiation. If UVC radiation destabilizes RIs, the reduced number of RIs in UVC-irradiated cells should also be observed in cells exposed to both UV and aphidicolin. However, if these decreases are caused by an initiation-inhibitory effect, aphidicolin treatment of UVC-irradiated cells should also be observed in cells exposed to both UVC and aphidicolin. Therefore, if these decreases are caused by an initiation-inhibitory effect, aphidicolin treatment of UVC-irradiated cells should block these decreases by inhibiting the maturation of RIs. As in Fig. 2A, UVC irradiation of virus-infected cells with 25 J/m² UVC radiation reduced the number of SV40 RIs to ~20% of the number observed in sham-irradiated con-
FIG. 2. Dose- and time-dependent inhibition of SV40 viral DNA replication by UVC and its abrogation by caffeine. SV40-infected BSC-1 monkey cells were subjected to various doses of UVC radiation and incubated for an additional 50 min before pulse labeling with [3H]thymidine for 15 min (A and B) or were subjected to 25 J/m² of 254-nm wavelength UVC radiation and incubated for the indicated periods of time before pulse labeling with [3H]thymidine for 15 min (C–F). SV40 replication intermediates recovered from Hirt extracts were separated on two-dimensional agarose gels and visualized by Southern blotting and hybridization to 32P-labeled SV40 DNA. The number of SV40 replication intermediates was quantitated by PhosphorImager analysis. Replication activity was assessed by measuring incorporation of [3H]thymidine directly into replication intermediates or into fully replicated SV40 DNA by densitometric analysis of fluor-impregnated gels run in parallel with the blotted agarose gels and exposed to x-ray film. C, SV40-infected cells were sham-irradiated (sham control), or subjected to UVC radiation alone (UVC 25 J/m²), UVC radiation after addition of aphidicolin (aphid/UVC) or with aphidicolin alone (aphid). G, SV40-infected monkey cells were treated or not treated with 2 mM caffeine beginning 2 min before irradiation with 10 J/m² of 254 nm wavelength UVC radiation. All values represent the averages of three independent experiments.
controls (Fig. 2, C and D). In contrast, addition of aphidicolin to the medium just before irradiation occurred (Fig. 2, C and D, UVC/aphid) reduced the magnitude of the decrease in SV40 RIs induced by UVC radiation to levels similar to those observed in cells treated with aphidicolin alone. The decrease in number of SV40 RIs observed in association with aphidicolin treatment alone may reflect their partial destabilization by aphidicolin treatment (26). However, the similar numbers of RIs observed in aphidicolin-treated cells that were also subjected to UVC irradiation indicates that UVC radiation did not cause a further destabilization of these RIs. Therefore, the decrease in SV40 RIs induced by UVC radiation is, in fact, related to the maturation of these RIs in the absence of new initiation events.

The inhibitory effects of UVC radiation on cellular DNA replication are rapid, occurring maximally over a period of 1 h followed by recovery of DNA replication a few hours later (3, 7). For comparison, we analyzed the time-dependent inhibitory effects on SV40 DNA replication induced by 25 J/m² UVC radiation. Fig. 2E shows that signals from SV40 RIs obtained from blots probed with SV40 sequences gradually decreased during the first hour after irradiation. Quantitation of these signals by PhosphorImager analysis revealed that the number of SV40 RIs observed 1 h after irradiation amounted to ~20% of those observed in the sham-irradiated controls (Fig. 2F) similar to the number observed at this fluence after 50 min in the dose-response experiments. Densitometric quantitation of signals from x-ray films exposed to the fluor-impregnated two-dimensional gel run in parallel demonstrated that incorporation of [³H]thymidine into SV40 DNA decreased in parallel with the decrease in numbers of RIs in a similar time-dependent fashion (Fig. 2F, [³H]-TdR incorporation). Thus, the kinetics of the inhibitory effect of UVC on SV40 DNA replication are rapid, similar to its effect on cellular DNA replication. As in the dose-response experiments, the similar number of SV40 RIs compared with their replication activity (Fig. 2F) indicates the inhibitory effect is on initiation, and not elongation.

Four hours after irradiation, the number of RIs increased once again compared with the signals from RIs observed at the 1-h time point (Fig. 2, E and F). Incorporation of [³H]thymidine also increased at the 4-h time point, and the magnitude of this increase was similar to the increase in the number of RIs (Fig. 2, E and F, 240'). Therefore, the increase in number of RIs is related to restoration of DNA synthesis as SV40 genomes more frequently initiate DNA replication. The restoration of SV40 DNA synthesis is also similar to that observed in cellular replicons of uninfected cells several hours after UVC irradiation (3).

The similarity of the initiation-inhibitory effect of UVC radiation on the replication of SV40 DNA to its effect on cellular DNA mediated by a caffeine-sensitive checkpoint suggests that this checkpoint targets both cellular and SV40 viral origins of replication. To explore this possibility, we asked whether caffeine could abrogate the inhibitory effects of UVC radiation on initiation or elongation of SV40 viral DNA replication. Caffeine treatment of SV40-infected cells irradiated with 25 J/m² of UVC caused an increase in both the number and replication activity of SV40 RIs compared with those isolated from irradiated cells that were not treated with caffeine (Fig. 2G, 25 J/m²). Thus, caffeine at least partly abrogated the inhibitory effect of UVC radiation on initiation of SV40 DNA replication, similar to its effect on cellular DNA replication in UVC-irradiated uninfected cells (Fig. 1B). In addition, treatment of sham-irradiated control SV40-infected cells with caffeine also caused an increase in the numbers and replication activity of SV40 RIs compared with levels observed in the absence of caffeine (Fig. 2G, 0 J/m²). Thus, caffeine also stimulates initiation of SV40 DNA replication in the absence of DNA damage. These results confirm the interpretation of the velocity sedimentation experiments and suggest that both cellular and SV40 DNA replication are regulated by a caffeine-sensitive checkpoint induced by UVC, and an intrinsic checkpoint that regulates origins in the absence of DNA damage or other perturbations.

Chk1 Is Required for UVC-induced and Intrinsic Checkpoints That Target Cellular and SV40 Viral Origins—Although caffeine inhibits both of the checkpoint kinases ATR and ATM (1), UVC radiation activates ATR, rather than ATM (34). Furthermore, UVC radiation induces the phosphorylation of Chk1 by ATR (35), and Chk1 functions downstream of ATR in other checkpoint pathways induced by UVC or replication arrest (34). To determine whether Chk1 plays a role in the caffeine-sensitive pathway that inhibits initiation of DNA replication, we asked whether the Chk1 kinase inhibitor UCN-01 has effects on cellular DNA replication similar to those of caffeine. These experiments employed MDA041 human fibroblasts lacking functional p53. As in MEFS, 10 J/m² UVC radiation induced an initiation-specific inhibitory effect on DNA replication indicated by a significant reduction in the number of shorter nascent DNA molecules (Fig. 3A, 10 J/m²). Treatment of these cells with UCN-01 inhibited the decrease in [³H]thymidine-labeled origin-proximal nascent molecules induced by UVC radiation (Fig. 3A, 10 J/m² + UCN-01), similar to the effect of caffeine on UVC-irradiated MEF cells (Fig. 1B). Also similar to caffeine, UCN-01 treatment caused an increase in small nascent DNA strands in sham-irradiated control cells (Fig. 3A, 0 J/m² + UCN-01). These results are consistent with the possibility that Chk1 is required for the UVC-induced checkpoint that inhibits cellular origin function, as well as an intrinsic checkpoint that regulates cellular origins of replication in the absence of DNA damage.

To rule out the possibility these results were related to Chk1-independent effects of UCN-01, we repeated this analysis in cells infected with an adenovirus vector expressing a dominant-negative mutant of Chk1, or, as a control, a vector expressing green fluorescent protein (GFP). As expected, DNA replication in MDA041 cells expressing GFP was affected by UVC and UCN-01 treatments similarly to uninfected controls (Fig. 3B). However, a number of differences in these effects were observed in MDA041 cells expressing dn-Chk1 (Fig. 3C). In the absence of UCN-01 or UVC, these cells produced slightly more small nascent DNA molecules compared with parental cells or cells expressing ad-GFP (Fig. 3C, 0 J/m², compare with 0 J/m² in panels A and B). Furthermore, in contrast to its effects in other cell lines, UCN-01 treatment did not change the size distribution of nascent molecules in dn-Chk1-expressing cells (Fig. 3C). Thus, the intrinsic checkpoint abrogated by UCN-01 is missing from these cells. Similar to the combined effects of UCN-01 and UVC in control cells (Fig. 3, A and B), UVC radiation increased, rather than decreased, the number of small nascent DNA molecules in these cells compared with the sham-irradiated controls (Fig. 3D, 10 J/m²); the sham-irradiated control values (0 J/m²) from Fig. 3C are replotted as a solid line for comparison). Although it is not clear why UVC caused this increase in the absence of UCN-01, UCN-01 had little additional stimulatory effect on initiation (Fig. 3D, 10 J/m² + UCN-01). Thus, similar to MEF cells treated with caffeine (Fig. 1B), both the intrinsic checkpoint and the checkpoint induced by UVC that inhibit initiation are largely absent from cells expressing dn-Chk1, indicating these checkpoints require Chk1.

We next asked whether Chk1 is required for the similar, caffeine-sensitive inhibitory effects on SV40 viral DNA replication. Measurements of number of RIs (Fig. 3E) or of their
replication activity (data not shown) indicated that, like caffeine, UCN-01 partly abrogated the initiation-inhibitory effect of 10 J/m² UVC radiation on SV40 DNA replication. Thus, at lower doses of UVC radiation, initiation of SV40 DNA replication appears to be regulated at least in part by the same UVC-induced Chk1-dependent pathway that inhibits cellular origins. The inhibitory effect of higher doses of UVC radiation was not reversed by UCN-01 treatment (Fig. 3E), despite the fact that 25 J/m² UVC radiation exerts predominantly initiation-inhibitory effects that are partly abrogated by caffeine (Fig. 2G). The nature of this difference is not clear, but may be related to more complete abrogation of checkpoints by caffeine compared with UCN-01 in these experiments. UCN-01 treatment also caused an increase in the number and activity of SV40 replication intermediates in sham-irradiated controls (Fig. 3D), indicating that the intrinsic, Chk1-dependent pathway that regulates cellular DNA replication also regulates initiation of SV40 DNA replication.

The Chk1-dependent Intrinsic Checkpoint Targets Late S Cellular Origins of Replication—Both yeast (16–19, 36) and
mammalian (20, 21) cells harbor checkpoints that block the activation of late S phase origins of replication when DNA replication is blocked in early S phase. In mammals, this checkpoint is abrogated by both caffeine and UCN-01. The increased number of small nascent DNA molecules detected in UCN-01- and caffeine-treated uninfected cells in our experiments theoretically could be accounted for by the unscheduled activation of late S origins in early S phase cells. To test this possibility, we analyzed the effect of UCN-01 on DNA replication in a late S replicating fragment of the rDNA locus in the human fibroblast cell line TR9-7 cells. Populations of these cells (which are derived from MDA041 cells) can be easily synchronized in early S phase by the ectopic induction of p53, which blocks progression through the cell cycle mostly in G1 (29), and subsequent release of this block into medium containing aphidicolin. Because the stimulatory effect of caffeine and UCN-01 occurred in the absence of stalled replication forks, our analysis was performed beginning 2 h after release of the aphidicolin block, at which time cells have completely recovered their ability to replicate DNA (data not shown).

Control experiments first established that UCN-01 treatment caused an increase in incorporation of [3H]thymidine and in the number of small nascent strands in exponentially proliferating populations of TR9-7 cells (Fig. 4A), similar to the effect of UCN-01 on DNA replication in MDA041 cells described above (Fig. 3A). To analyze origin timing in the rDNA locus, we employed the neutral-neutral two-dimensional gel electrophoresis technique. The tandemly repeated human rDNA locus contains a polymorphism that produces a specific EcoRI fragment in some, but not all, repeats. Consistent with an earlier report that this fragment resides in rDNA repeats that replicate later in late S phase (37), replication intermediates were not detected in this fragment when replicating DNA was isolated from cells in early S phase, 2 h after release of the aphidicolin block (Fig. 4B, early S phase, (-)UCN-01). This was the case even after much longer exposures of blots to PhosphorImager screens (data not shown). However, replication intermediates were detected in these cells when they were also treated with UCN-01 (Fig. 4B, early S phase, (+)UCN-01). In contrast, equivalent numbers of replication intermediates were detected in DNA isolated from cells allowed to proceed through S phase for another several hours regardless of whether or not they were treated with UCN-01 (Fig. 4B, late S phase). Thus, this fragment of rDNA normally replicates in these cells in late S phase, and UCN-01 treatment accelerates its replication so that it now replicates in early S phase. These results establish that the Chk1-dependent intrinsic checkpoint inhibits late S origins of replication in early S phase cells.

![Image](http://www.jbc.org/)

**Fig. 4.** UCN-01 accelerates the replication of a region of the rDNA locus from late to early S phase. A, nascent DNA pulse-labeled in untreated control cultures of TR9-7 human fibroblasts or cultures treated with 100 nM UCN-01 for 50 min were analyzed by velocity sedimentation analysis. Direction of sedimentation was from left to right. B, neutral-neutral two-dimensional gel analysis of DNA replication intermediates from a region of the repetitive rDNA locus isolated from cells treated or not treated with 100 nM UCN-01 2 h ("early S") or 6 h ("late S") after release from an aphidicolin arrest in early S phase and 50 min before their isolation.

**DISCUSSION**

A complete understanding of how DNA damaging agents and intra-S phase checkpoints inhibit DNA replication requires the ability to analyze the molecular events involved in two fundamentally different aspects of replication, those involved in initiation, and those required for the elongation of nascent DNA chains. We previously developed an assay that clearly distinguishes between these effects induced by drugs that inhibit viral DNA replication in the well characterized SV40 model system (26–28). In this study, we asked whether this assay can distinguish these effects on SV40 DNA replication induced by a DNA damaging agent more frequently used to study checkpoint regulation of cellular DNA replication, UVC radiation. Our results show that lower fluences of UVC radiation induce initiation-inhibitory effects on SV40 DNA replication very similar in magnitude and kinetics to those induced by the same levels of UVC in cellular replicons of uninfected cells. The initiation-specific effects of UVC radiation on cellular DNA replication were indicated by a decrease in small, presumably origin-proximal strands of nascent DNA (Fig. 1), similar to those reported previously. UVC effects on SV40 viral DNA replication were detected as substantial dose- and time-dependent decreases in both the number and replication activity of SV40 RIs isolated from SV40 virus-infected cells subjected to the same conditions of irradiation and labeling employed in the analysis of cellular replicons (Fig. 2). At higher doses, similar to its effect on cellular replicons, UVC radiation also inhibited the elongation phase of SV40 DNA replication (Fig. 2, A and B). These similarities confirm the validity of both techniques for distinguishing initiation versus elongation-inhibitory effects and suggest that a common mechanism underlies these effects in cellular and viral replicons.

Inhibition of initiation of cellular and viral DNA replication by low doses of UVC radiation was at least partly abrogated by the checkpoint kinase inhibitors caffeine and UCN-01. The specific inhibition of Chk1 by UCN-01 at the concentration used in these experiments (38, 39) suggested that this corresponded to abrogation of a Chk1-dependent checkpoint. This was confirmed by the observation of reduced inhibitory effects on initiation in cells expressing a dominant-negative Chk1 mutant in uninfected cells (Fig. 3D). Similar results indicating a role for Chk1 in a UVC-induced checkpoint were recently
obtained by others as well (48). This checkpoint clearly does not depend on p53, because it is present in p53-null MEFs (Fig. 1C), and in MDA041 cells (Fig. 3, A–C), which are functionally p53-null (40), as well as in SV40-infected cells (Fig. 2), where p53 presumably is inactivated by SV40 large T antigen. This is consistent with the previously described roles of Chk1 in p53-independent checkpoints (41). The UVC-induced checkpoint may be related to S phase checkpoints induced by the topoisomerase I inhibitor camptothecin (42) or cisplatin (43), which are also abrogated by UCN-01. It may also be related to a recently described transient, p53-independent late G1 checkpoint that blocks entry into S phase in response to UVC radiation, which depends on ATR and is also abrogated by UCN-01 (44).

Failure of both caffeine and UCN-01 to abrogate inhibitory effects at higher doses of UVC radiation that block elongation indicates that the checkpoint detected in our experiments specifically targets the initiation, and not the elongation phase of DNA replication. Because elongation effects were observed only at higher doses of UVC radiation, they are likely to occur in cis as replication forks are blocked by a larger number of lesions in DNA. This could explain the elongation inhibitory effects induced by 50 J/m2 UVC radiation in cellular replicons (Fig. 1), but not in SV40 viral replicons (Fig. 2). Presumably, the larger cellular replicons contain more fork-arresting lesions compared with much smaller viral replicons.

The increased frequency of initiation of cellular (Fig. 1) and viral (Fig. 2) DNA replication observed in unirradiated cells treated with caffeine or UCN-01 is consistent with earlier reports that caffeine stimulates initiation of cellular DNA replication in the absence of DNA damage (32, 33, 45). Our findings indicate that this stimulation is caused by the abrogation of an intrinsic checkpoint requiring Chk1, and either ATR or ATM, which targets both cellular and viral origins of replication. The cellular origins targeted by the intrinsic checkpoint appear to be normally activated in late S phase (Fig. 4). In fact, previous studies demonstrated inhibition by a checkpoint of late S origins in mammalian cells, and this checkpoint was also abrogated by UCN-01 (20) and caffeine (21). However, the activation of late S origins by caffeine or UCN-01 observed in these earlier studies was only detected when replication forks were blocked in early S phase, and did not coincide with the accelerated activation of these origins in S phase (20, 21). In contrast, the intrinsic checkpoint detected by our experiments occurs in the absence of stalled forks or other perturbations and its abrogation occurs in the accelerated activation of cellular origins. These seemingly contradictory observations can be explained if the intrinsic pathway regulates only a subset of late S origins, the effects on which were not detected in the global analysis of replication timing performed previously. In fact, mutations in the budding yeast checkpoint genes RAD53 and ORC2 similarly abrogate both types of checkpoints, one that regulates late S origins in cells blocked in early S phase that, in checkpoint defective strains, are activated in these cells with normal kinetics (16, 18, 36), and an intrinsic checkpoint that targets only some late S origins, which are activated in an accelerated fashion in checkpoint defective strains in the absence of a replication block or other perturbations (16). Furthermore, mutations in Rad3, the fission yeast homologue of ATR, abrogate both a replication arrest-dependent checkpoint that targets late S origins (19) and an intrinsic checkpoint that regulates some, but not all of these origins in the absence of a replication block.2 The existence of a Chk1 and ATR-dependent intrinsic checkpoint is consistent with the observation in Xenopus of an increased association of ATR with chromatin during DNA replication unperturbed by drugs (46).

In summary, our study shows that initiation of cellular and SV40 viral DNA replication are both regulated by a caffeine-sensitive, p53-independent and Chk1-dependent checkpoint induced by UVC radiation, and by a novel Chk1-dependent intrinsic checkpoint that inhibits both cellular and viral origins of replication in the absence of DNA damage or other perturbations. The intrinsic checkpoint targets a late S replicating region of the rDNA locus in uninfected cells. The discovery of an intrinsic checkpoint that regulates DNA replication in the absence of stalled forks or other perturbations is important in the context of the frequency with which checkpoint pathways are defective in cancer cells. For instance, accumulating evidence suggests that some of the genetic instability in cancer cells arises at replication forks, which, in cells with defects in the intrinsic checkpoint identified here, are likely to be more numerous compared with cells with intact checkpoints.

The detection of similar effects of UVC radiation and checkpoint inhibitors on the initiation and elongation phases of cellular and SV40 DNA replication confirms the interpretation of earlier, less direct analyses of UVC radiation and caffeine-induced effects on cellular DNA replication detected by velocity sedimentation of pulse-labeled nascent DNA. Our results delineate the precise conditions required to observe these checkpoint effects in the well characterized SV40 experimental system, and identify proteins that regulate initiation, and not elongation, as potential targets of these checkpoints. This provides a framework for further investigating the underlying molecular mechanisms.

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Note Added in Proof—Recent experiments published after review of our manuscript was complete indicate that Chk1 phosphorylates a downstream checkpoint protein, Cdc25A, in the absence of DNA damage or other perturbations (Zhoa, H., Watkins, J. L., and Pijnwica-Worms, H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14705–14800). This is consistent with the role for Chk1 in an intrinsic checkpoint revealed by our experiments.

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Regulation of Cellular and SV40 Virus Origins of Replication by Chk1-dependent Intrinsic and UVC Radiation-induced Checkpoints
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