Carcinogen-induced S-Phase Arrest Is Chk1 Mediated and Caffeine Sensitive[1]

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Abstract

We have investigated the mechanism of S-phase arrest elicited by the carcinogen benzo(*a*)pyrene dihydrodiol epoxide (BPDE) in p53-deficient cells. Inhibition of DNA synthesis after BPDE treatment was rapid and dose dependent (~50% inhibition after 2 h with 50 nM BPDE). Cells treated with low doses (50–100 nM) of BPDE resumed DNA synthesis after a delay of ~4–8 h, whereas cells that received high doses of BPDE (600 nM) failed to recover from S-phase arrest. The checkpoint kinase Chk1 (but not Chk2) was phosphorylated after treatment with low doses of BPDE. High concentrations of BPDE elicited phosphorylation of both Chk1 and Chk2. Adenovirus-mediated expression of "dominant-negative" Chk1 (but not dominant-negative Chk2) and the Chk1 inhibitor UCN-01 abrogated the S-phase delay elicited by low doses of BPDE. Consistent with a role for the caffeine-sensitive ATM or ATR protein kinase in low-dose BPDE-induced S-phase arrest, both Chk1 phosphorylation and S-phase arrest were abrogated by caffeine. However, low doses of BPDE elicited Chk1 phosphorylation and S-phase arrest in AT cells (from ataxia telangiectasia patients), demonstrating that ATM is dispensable for S-phase checkpoint responses to this genotoxin. BPDE-induced Chk1 phosphorylation and S-phase arrest were abrogated by caffeine treatment in AT cells, suggesting that a caffeine-sensitive kinase other than ATM is an important mediator of responses to BPDE-adducted DNA. Overall, our data demonstrate the existence of a caffeine-sensitive, Chk1-mediated, S-phase checkpoint that is operational in response to BPDE.

Introduction

PAHs,[3] typified by the common pollutant B(*a*)P, are widespread and ubiquitous environmental pollutants with known carcinogenic properties (1, 2). Therefore, PAHs are likely to be contributory and risk factors for cancer and pose a potentially serious threat to public health.

Many PAHs, including B(*a*)P, are substrates for the oxidative reactions carried out by cytochrome P-450 proteins (3, 4). The hydroxyl and epoxide species resulting from PAH metabolism are highly electrophilic and bind covalently to cellular macromolecules to form bulky hydrophobic adducts (5–7). BPDEs are the major genotoxic species resulting from B(*a*)P metabolism and bind covalently to the exocyclic amino group of deoxyguanosine (N2 position) in genomic DNA (5–7). PAH-induced mutagenesis and carcinogenesis result from carcinogen-adducted DNA. Potentially, PAH-induced mutations may arise during DNA misreplication at damaged sites of electrophilic attack or during error-prone DNA repair (5–7). Indeed, substitution mutations as well as frameshifts can result from replication of BPDE-adducted DNA (5–7). Therefore, chemical carcinogenesis appears to be attributable to carcinogen-induced mutations in proto-oncogenes and/or tumor suppressor genes. High levels of PAH-adducted DNA have been found in neoplastic tissues from cancer patients (8), suggesting that environmental PAHs may indeed contribute to neoplasia in humans.

A crucial requirement for PAH-induced carcinogenesis is that mutations become "fixed" by misreplication of damaged genes. Because cells are exposed to intrinsic and extrinsic DNA-damaging agents, they have evolved mechanisms to prevent mutagenic misreplication of damaged DNA. When the integrity of the genome is compromised, "checkpoint" mechanisms exert negative controls on cell cycle progression (resulting in growth arrest or apoptosis). These controls prevent potentially error-prone DNA synthesis and therefore protect against mutagenesis and genetic instability (9–12).

Retardation of cell cycle progression in response to genotoxic agents is widely hypothesized to provide cells extra time for DNA repair, thereby reducing the potential for mutagenic misreplication of damaged gene (9–12). Individuals with inherited defects in known checkpoint genes (such as Li-Fraumeni patients with defects in the *p53* gene) are prone to cancer, highlighting the importance of checkpoint control mechanisms. In recent years, p53 has been shown to be an important component of G_1 and G_2 checkpoint mechanisms (13). The p53 tumor suppressor protein is a transcription factor that is stabilized and activated after acquisition of DNA damage. When activated, p53 regulates the expression of several DNA damage-induced genes that contain p53-responsive elements. The p53-responsive *p21* gene encodes a CDK inhibitor that binds to and inhibits CDKs that drive cell cycle progression (13–15). p53-dependent p21 induction is

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[3] The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; B(*a*)P, benzo(*a*)pyrene; BPDE, benzo(*a*)pyrene diol epoxide; IR, ionizing radiation; AT, ataxia telangiectasia; ATM, AT mutated; DSB, double strand break; GFP, green fluorescent protein.

believed to mediate G_1 and some G_2 cell cycle blocks in response to DNA damage (16, 17).

The DNA damage-activated kinases Chk1 and Chk2 have been implicated in regulation of G_2 checkpoints in eukaryotic organisms (18–20). The mitotic Cdc25C protein phosphatase is a substrate for both Chk1 and Chk2. Chk1/Chk2-mediated phosphorylation of Cdc25C on serine 216 results in export of Cdc25 from the nucleus. Cytosolic sequestration of Cdc25 from its physiological substrate (Cdc2/Cdk1) is thought to prevent full activation of the mitotic cyclin B/Cdk complex, thereby eliciting cell cycle arrest in G_2 (18–20). In addition to Cdc25C (and other Cdc25 family members), p53 is a known substrate of both Chk1 and Chk2 (21, 22). Both checkpoint kinases can phosphorylate p53 on serine 20. Therefore, it is likely that these kinases may also contribute to establishment or maintenance of p53-dependent cell cycle checkpoints. Individuals with a variant form of Li-Fraumeni syndrome do express "wild-type" p53 but exhibit defects in Chk2 (23). These data suggest that Chk2 and p53 function in the same pathway and are consistent with an upstream role for Chk2 in p53 activation. Additionally, checkpoint kinases are implicated in control of a DNA replication checkpoint that prevents the onset of mitosis in cells containing unreplicated DNA (24). Elegant experiments using reconstituted *Xenopus* egg extracts and sperm nuclei have demonstrated the existence of a Chk1-mediated mechanism for preventing inappropriate entry into mitosis (25, 26). Results from these studies have shown that Chk1 acts downstream of the caffeine-sensitive protein kinase ATR in the DNA replication checkpoint (25–27).

We have documented previously PAH-induced cell cycle arrest in G_1 and S-phases of the cell cycle in both p53-expressing and p53-null cells (28, 29). The BPDE-induced checkpoint that is the focus of the present report retards cell cycle progression in S-phase in a p53-independent manner. DNA damage-induced S-phase delay has been studied extensively by many laboratories, but mainly in the context of γ-irradiated cells. Studies by Painter and Young (30) first suggested a role for ATM in S-phase responses to γ-irradiation. Lim *et al.* (31) have shown recently that S-phase arrest in response to ionizing radiation-induced DNA double strand breaks is mediated via the ATM protein kinase and the p95/NBS protein. In contrast, little is known regarding molecular mechanisms of S-phase checkpoints elicited in mammalian cells by other forms of DNA damage, such as bulky adducts or base modifications. Our previous results implicated a role for cyclin A down-regulation in establishment and maintenance of B(*a*)P-induced G_1-S-phase arrest in p53 and p21-proficient rodent cells (28). However, cells lacking p53 also undergo B(*a*)P/BPDE-induced S-phase arrest in the absence of cyclin A down-regulation (28, 29), thereby demonstrating the existence of additional p53/p21-independent checkpoint mechanisms. In experiments presented here, we have investigated the mechanism of BPDE-induced, p53-independent, S-phase arrest in the p53-deficient lung cancer cell line H1299 (32). These cell lines were chosen instead of rodent fibroblasts (which we used in our previous studies) for several reasons: (*a*) cell cycle parameters and checkpoint responses (to other genotoxins) have been studied extensively in H1299 cell lines; (*b*) these epithelial-derived carcinoma cells are amenable to adenovirus-mediated expression of ectopic genes. As such, these cells are appropriate for transient expression of cell cycle regulatory genes that we have been unable to express stably using conventional expression plasmid and retroviral vectors (*e.g.,* wild-type and mutant forms of checkpoint kinases); and (*c*) results from studies in these (human) cells are more relevant to mechanisms of chemical carcinogenesis in humans. Our data demonstrate an important role for the checkpoint kinase Chk1 in mediating p53-independent S-phase arrest in response to BPDE and most probably other genotoxins.

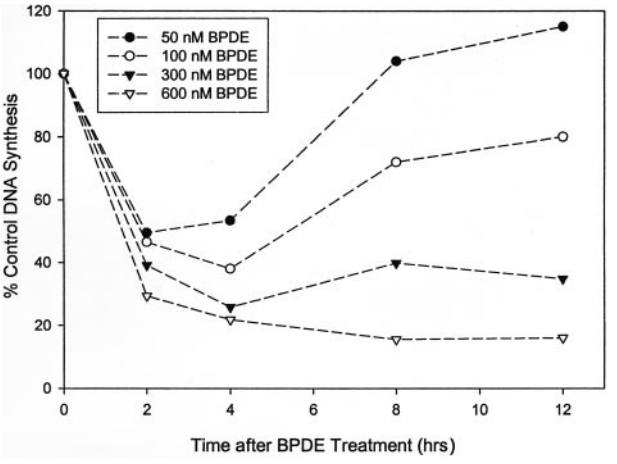

Fig. 1. Kinetics of inhibition and recovery of DNA synthesis in BPDE-treated H1299 cells. H1299 cells were cultured in the presence of [^{14}C]thymidine for 48 h as described in "Materials and Methods." The resulting cultures were given different doses of BPDE for 1 h. Then, the BPDE-containing medium was replaced with fresh growth medium. At different times after BPDE treatment, the cells were pulsed for 1 h with [^{3}H]thymidine. The ratios of DNA-incorporated ^{3}H:^{14}C (which provide an index of rates of DNA synthesis at each time point) were calculated and expressed as a percentage of the rate of DNA synthesis immediately before BPDE addition. Although not shown in the figure, the rate of DNA synthesis in untreated cells remained constant for at least 3 h after the time when BPDE was added to other cultures. That is, rates of DNA synthesis in exponentially growing cultures did not change significantly within a period of ~3 h. Therefore, in all subsequent experiments, rates of DNA synthesis in control cultures (not receiving BPDE) were measured during the second hour after BPDE treatment. All data points in Fig. 1 represent means of duplicate determinations that differed by no more than 4%. Error bars in the above figure are omitted for clarity.

Results

Duration of BPDE-induced S-Phase Arrest Is Dependent on the Dose of Genotoxin. We performed experiments to determine the kinetics of onset and the duration of BPDE-induced inhibition of DNA synthesis in H1299 cells. In the experiment shown in Fig. 1, rates of DNA synthesis in control and BPDE-treated cells were measured at various times after treatment with various doses of BPDE. These experiments were performed by determining rates of incorporation of [^{3}H]thymidine during 1-h pulse periods at different times after BPDE treatment, as described in "Materials and Methods." As shown in Fig. 1, treatment with a low dose (50 nM) of BPDE resulted in a transient inhibition of DNA synthesis (50% inhibition 2 h after receiving BPDE). However, DNA

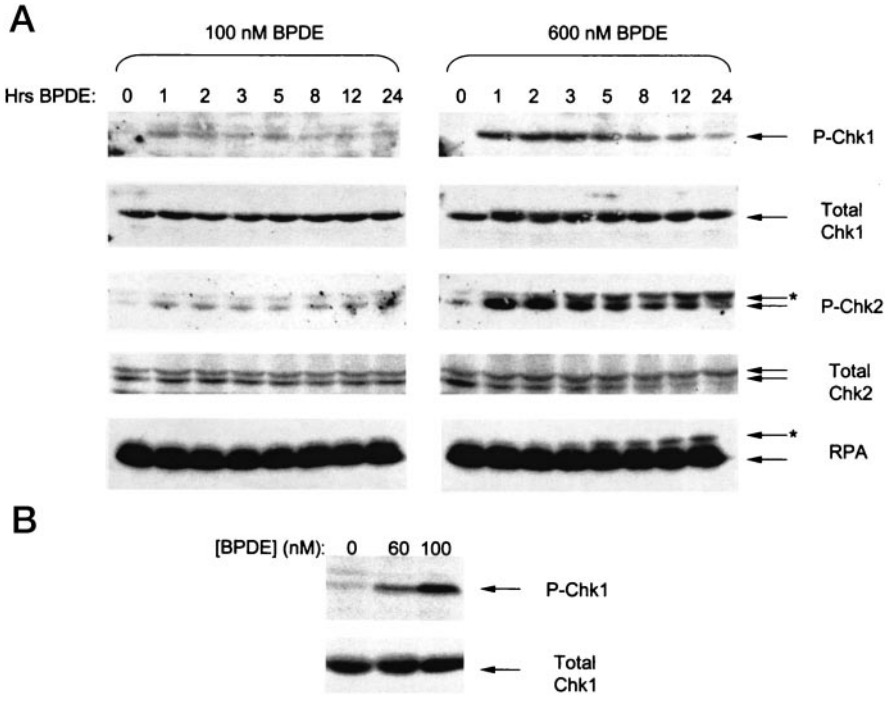

Fig. 2. Immunoblots showing effect of low and high doses of BPDE on Chk1, Chk2, and RPA. *A,* H1299 cells were treated with 100 or 600 nM BPDE for different times, after which cell lysates were prepared and analyzed by SDS-PAGE and immunoblotting. Blots were probed with antisera against Phospho-Chk1 (phospho-Ser-345), Chk1, Phospho-Chk2 (phsopho-Thr-68), Chk2, and RPA. *, electrophoretically retarded forms of Chk1, Chk2, and RPA corresponding to phosphorylated forms of these proteins. For the Chk1 and Chk2 immunoblots, filters were first probed with antisera against the phosphorylated forms of the checkpoint kinases. After visualizing the blots, the filters were stripped and reprobed with antisera that detect the total cellular pools of these proteins. For RPA blots, the lower portions of the same filters used to detect Chk1 were probed with anti-RPA antibody. *B,* H1299 cells were given 0, 60, or 100 nM BPDE for 2 h. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phospho Chk1 antisera (*upper panel*) or total Chk1(*lower panel*).

synthesis resumed later and recovered to control rates by 8 h after BPDE treatment. Higher doses of BPDE led to more rapid and greater inhibition of DNA synthesis. However, cells displayed progressively reduced ability to recover from S-phase arrest after receiving higher doses of BPDE. The S-phase arrest (after low dose BPDE treatment) displayed the hallmarks of a "checkpoint," *i.e.,* a transient inhibition of cell cycle progression after genotoxic insult. Therefore, we performed experiments (described below) to identify components of the putative checkpoint pathway that mediates the BPDE-induced S-phase checkpoint.

BPDE-induced S-Phase Arrest Correlates with Chk1 Phosphorylation. In both budding and fission yeast, checkpoint kinases Chk1 and Cds1 (*Schizosaccharomyces pombe*)/Rad53 (*Saccharomyces cerevisiae*) have been shown to mediate the DNA damage-induced cell cycle checkpoints including checkpoints during S-phase (12, 20). In recent years, mammalian homologues of these checkpoint kinases (Chk1 and Chk2) have been identified (18, 19). Mammalian checkpoint kinases have been implicated in mediating cell cycle arrest in G_1 (via p53 activation) and G_2-M (via negative regulation of Cdc25C). However, roles in S-phase checkpoint responses to DNA damage have not been described for the mammalian checkpoint kinases. Therefore, we tested potential roles for Chk1 and Chk2 in BPDE-induced S-phase arrest.

Mammalian Chk1 and Chk2 are activated in response to genotoxic damage as a result of phosphorylation by the upstream kinases ATR and ATM, respectively (12). Activated forms of both checkpoint kinases can be detected by mobility shifts on SDS-PAGE (18, 19). Additionally, phospho-specific antisera that recognize DNA damage-induced phosphorylation sites have been developed that specifically recognize the active forms of the checkpoint kinases. We used both mobility shifts and phospho-specific antibodies to test for Chk1/Chk2 modifications induced by BPDE.

Chk1, Chk2, and RPA Are Differentially Phosphorylated in Response to High and Low Doses of BPDE. In the experiment shown in Fig. 2*A,* H1299 cells were treated with 100 nM (*left-hand panels*) or 600 nM BPDE (*right-hand panels*) for various times. Protein extracts from the BPDE-treated cultures were analyzed by SDS-PAGE and immunoblotting with antisera against phosphorylated forms of the checkpoint kinases as well as with antibodies that detect total cellular Chk1 and Chk2. The commercially available phospho-specific antisera we used recognize phosphoserine 345 of Chk1 and phosphothreonine 68 of Chk2. These are sites that other investigators have shown are phosphorylated by the upstream activating kinases ATR and ATM, respectively (18, 19, 33, 34). In the immunoblots shown in Fig. 2, we investigated the kinetics of phosphorylation of Chk1 and Chk2. These experiments were performed using extracts

from cells that received 100 and 600 nM BPDE (doses that elicited transient and sustained S-phase arrest, respectively).

Chk1 was phosphorylated within 1 h of treatment with BPDE (100 and 600 nM), as evidenced by the presence of a band recognized by anti-phospho Chk1 in extracts from BPDE-treated cells. The higher dose of BPDE elicited a more pronounced and sustained phosphorylation of Chk1. A slight shift in Chk1 mobility was detected in response to 600 nM BPDE (but not in response to 100 nM BPDE). This mobility shift was most evident when the extracts were separated on a lower percentage acrylamide gel than the 10% gel used to resolve the protein samples in Fig. 2 (data not shown). Chk1 phosphorylation was maximal within the first 3 h of BPDE treatment and declined progressively at later time points. However, levels of phosphorylated Chk1 from BPDE-treated cells were still slightly elevated relative to control cultures 24 h after BPDE treatment.

In contrast with Chk1, Chk2 displayed a relatively modest increase in phosphorylation in response to the low dose of BPDE (100 nM). However, the higher dose (600 nM) elicited a very robust and sustained increase in the levels of phosphorylated Chk2. Both commercial Chk2 antibodies used in this study (as well as another antipeptide Chk2 antibody that we generated) detected a Chk2 doublet, indicating the presence of at least two forms of this kinase that differ with respect to posttranslational modifications. A BPDE-induced Chk2 mobility shift (resulting in a relative increase in levels of the slowly migrating species) was detectable only with the higher doses of BPDE and at the latest (24-h) time point.

As an additional control for protein loading, we probed the same protein samples (from BPDE-treated H1299 cells) with anti-RPA antisera. Similar to Chk2, RPA is known to be a substrate of the ATM kinase (35). Interestingly, RPA in extracts from 600 nM BPDE-treated cells was phosphorylated at late time points (5–24 h), as evidenced by BPDE-induced mobility shifts in those samples. We did not detect an RPA mobility shift in protein samples from cells that received 100 nM BPDE (even with more prolonged exposure of the blots shown in Fig. 2).

Therefore, our immunoblot data show that Chk1 (an ATR substrate) is phosphorylated transiently in response to low doses of BPDE (100 nM) that activate the S-phase checkpoint, as well as in cells that undergo irreversible inhibition of DNA synthesis resulting from higher doses of BPDE (600 nM). In contrast, Chk2 and RPA (two ATM substrates) are phosphorylated to a significant extent only in cells treated with higher doses of BPDE (600 nM). Moreover, phosphorylation of Chk2 and RPA is more sustained relative to that of Chk1. Overall, these data suggest a correlation between phosphorylation of Chk1 (but not of Chk2) during the S-phase checkpoint elicited by low doses of BPDE. Importantly, BPDE-induced Chk1 phosphorylation occurred at the low doses of BPDE that transiently inhibited DNA synthesis (Fig. 2*B*). These data are consistent with a role for Chk1 in mediating the BPDE-induced S-phase checkpoint.

Dominant-negative Chk1 and the Kinase Inhibitor UCN-01 Abrogate BPDE-induced S-Phase Arrest. The experiments described above suggested a potentially important role for Chk1 in mediating BPDE-induced S-phase arrest. To test this hypothesis, we performed loss-of-function experiments to abrogate Chk1 activity and test for consequent effects (of Chk1 perturbation) on BPDE-induced S-phase checkpoint. We used two strategies to interfere with Chk1 activation: (*a*) adenovirus-mediated overexpression of "dominant-negative" Chk1; and (*b*) use of the Chk1 inhibitor UCN-01. These experiments are described below.

We mutated sequences encoding the catalytic lysine of Chk1 cDNA (to arginine), thereby generating a kinase-inactive mutant protein (Chk1 K-R). We and other groups have shown that this mutation results in a dominant-negative protein that interferes with Chk1 signaling when overexpressed (22, 27). We expressed the mutant Chk1 in H1299 cells using a recombinant adenovirus (AdChk1 K-R). We estimate that the levels of mutant Chk1 protein in AdChk1 K-R-infected cells exceeded endogenous Chk1 levels by at least 10-fold (Fig. 3, *lower left panel*).

H1299 cells infected with AdChk1K-R or with AdGFP (for control) were treated with 60 nM BPDE (or DMSO for controls) for 1 h and then pulsed with [^{3}H]thymidine for another 60 min. Immediately after the pulse with [^{3}H]thymidine, we determined rates of DNA synthesis in the control and BPDE-treated cultures (as described for Fig. 1 above and in "Materials and Methods"). In the experiment shown in Fig. 3 (*upper left panel*), 60 nM BPDE elicited 38% inhibition of DNA synthesis in the control (AdGFP-expressing) cells. In contrast, this concentration of BPDE only inhibited DNA synthesis by 5% in AdChk1 K-R-infected cultures. Therefore, BPDE-induced S-phase arrest was largely inhibited as a result of dominant-negative Chk1 expression. This result suggests that Chk1 is an important mediator of BPDE-induced arrest in S-phase.

In parallel experiments, we pretreated H1299 cells with the kinase inhibitor UCN-01 (100 nM), which inhibits Chk1 kinase *in vitro* (36). Control or UCN-01-treated cells was given 60 nM BPDE for 1 h and then pulsed with [^{3}H]thymidine to determine relative rates of DNA synthesis (exactly as described above). As also shown in Fig. 3, whereas BPDE inhibited DNA synthesis by 38% in control cultures, rates of [^{3}H]thymidine incorporation were only inhibited by 12% in UCN-01 pretreated cultures. Experiments with UCN-01 should be interpreted with caution because this compound may not be entirely specific for Chk1 and could potentially inhibit other enzymatic activities (36). However, our demonstration that "dominant-negative" Chk1 as well as a known Chk1 inhibitor (UCN-01) can inhibit BPDE-induced S-phase arrest provides strong evidence that Chk1 is an important mediator of the BPDE-induced intra-S-phase checkpoint.

In other experiments, we infected cells with a virus encoding kinase-inactive Chk2 (AdChk2 K-R) and tested the effects of kinase-dead Chk2 on BPDE-induced S-phase arrest. Chehab *et al.* (21) have shown that this mutant protein acts in a dominant-negative manner. The levels of mutant Chk2 protein in AdChk2 K-R-infected cells exceeded endogenous Chk2 levels by at least 10-fold, as shown in the immunoblot in Fig. 3 (*lower right panel*). In contrast with AdChk1 K-R-infected cells, Chk2 K-R overexpressing cells retained sensitivity to BPDE-induced S-phase arrest (Fig. 3, *upper right panel*). The lack of effect of dominant-negative Chk2 on the

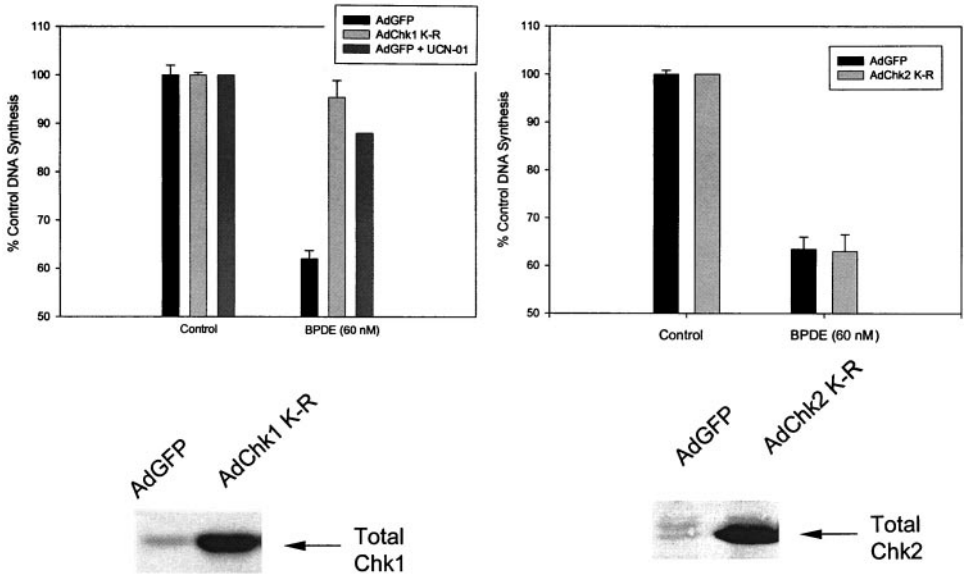

Fig. 3. Perturbation of Chk1 signaling inhibits BPDE-induced S-phase arrest. H1299 cells were infected with adenovirus encoding GFP (*AdGFP*) or kinase-dead Chk1 (*AdChk1 K-R*) for 16 h. Some AdGFP-infected cultures received 100 nM UCN-01 during the last hour of infection (*left panel*). In a similar experiment, cells were infected with AdGFP or with a virus encoding kinase-dead Chk2 (AdChk2 K-R; *right panel*). The resulting cultures (of AdGFP, AdGFP+UCN-01, AdChk1K-R, and AdChk2 K-R-infected cells) were given 60 nM BPDE (or DMSO for controls) for 1 h before receiving a 1-h pulse of [^{3}H]thymidine. Immediately afterward, rates of DNA synthesis were determined as described previously and in "Materials and Methods." Rates of DNA synthesis are expressed as a percentage of thymidine incorporation in control cultures that did not receive BPDE. However, it should be noted that the actual values for [^{3}H]thymidine incorporation (before normalization) were essentially identical in AdGFP-, AdGFP+UCN-01-, AdChk1 K-R-, and AdChk2 K-R-treated cells in the absence of BPDE. That is, rates of normal DNA synthesis were unaffected by expression of kinase-inactive checkpoint kinases or UCN-01 during the course of this experiment. The *lower panels* show immunoblot analysis of Chk1 and Chk2 in AdGFP-, AdChk1 K-R-, and AdChk1K-R-expressing cells, thereby confirming high-level expression of adenovirus-encoded mutant Chk1. *Bars,* SE.

BPDE-induced S-phase checkpoint, together with the modest effect of low doses of BPDE on Chk2 phosphorylation (Fig. 2), suggests little (if any) role for Chk2 in the S-phase checkpoint elicited by low doses of BPDE.

To further characterize the effect of kinase-inactive Chk1 on BPDE-induced S-phase arrest, we performed dose-response analyses of BPDE-induced S-phase arrest in control and kinase-inactive Chk1-expressing H1299 cells. As shown in Fig. 4, expression of Chk1 K-R perturbed the DNA damage-induced arrest S-phase at all doses of BPDE tested. However, the inhibitory effect of Chk1 K/R on the S-phase arrest was most pronounced at lower doses of BPDE that likely affect initiation of DNA replication and not elongation (37–39).

Chk1K-R-expressing cells maintained high levels of DNA synthesis after low dose BPDE treatment for at least 6 h (the latest time point examined) after receiving DNA damage. In the experiment shown in the lower panel of Fig. 4, we measured rates of DNA synthesis in control and Chk1 K-R-expressing 6 h after treatment with 100 nM BPDE. This experiment was performed in parallel with the dose-response analysis presented in the upper panel of the figure.

Six h after receiving BPDE, DNA replication in the control cultures had recovered to 88 ± 4% of the rate of DNA synthesis before BPDE treatment. Six h after BPDE treatment, the rate of DNA synthesis in the Chk1 K-R-expressing cells had actually exceeded the DNA replication rate at the earlier time point (113.5 ± 2.5%). This slight increase, which we consistently observe, appears to result from refeeding of the cells with fresh growth medium after removal of the BPDE-containing culture medium. Nevertheless, this result shows that abrogation of the Chk1-mediated S-phase checkpoint in the Chk1 K-R-expressing cells did not have delayed inhibitory effects on DNA replication.

BPDE-induced Chk1 Phosphorylation and S-Phase Arrest Are Abrogated by Caffeine. As noted earlier, mammalian Chk1 is thought to act downstream of a phosphatidylinositol 3-kinase family member protein kinase, most likely ATR, as suggested by the results of the Liu *et al.* (33) and Zhao and Piwnica-Worms (34). Both ATR and the related ATM kinase are sensitive to inhibition by caffeine (40). In the experiment shown in Fig. 5, we tested the effect of caffeine on BPDE-induced Chk1 phosphorylation. H1299 cells were pretreated with 5 mM caffeine (or were left untreated for controls) for 1 h before stimulation with various doses of BPDE. The control and caffeine ± BPDE-treated cultures were harvested, and lysates from the cells were separated by SDS-PAGE and probed with antisera against phospho-Chk1. As shown in the *upper panel* of Fig. 5, Chk1 phosphorylation was easily detected in BPDE-treated cells that did not receive caffeine but was significantly ablated by caffeine pretreatment.

We also tested the effects of caffeine on BPDE-induced S-phase delay. Exponentially growing cultures of control and 5 mM caffeine-pretreated H1299 cells were given 60 nM BPDE (or DMSO for controls) for 1 h. The resulting cultures were pulsed with [^{3}H]thymidine for determination of relative rates of DNA synthesis. In the experiment presented in the

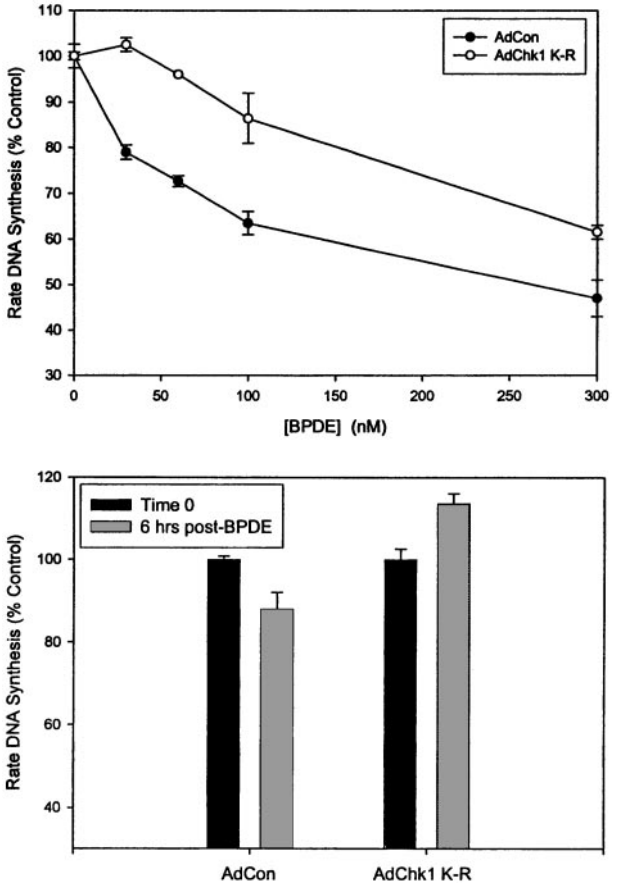

Fig. 4. Dose-dependent inhibition of DNA synthesis by BPDE in control and kinase-inactive Chk1-expressing H1299 cells. H1299 cells were infected with control adenovirus (*AdCon*) or kinase-dead Chk1 (*AdChk1 K-R*) for 16 h. The resulting cultures were given 0, 60, 100, or 300 nM BPDE for 1 h. After replacing the culture medium with fresh drug-free DMEM, the cells received a 1-h pulse of [^{3}H]thymidine immediately after the medium change (*upper panel*) or 6 h later (*lower panel*). Then, rates of DNA synthesis were determined exactly as described previously and in "Materials and Methods." Rates of DNA synthesis are expressed as a percentage of [^{3}H]thymidine incorporation in control cultures that did not receive BPDE.

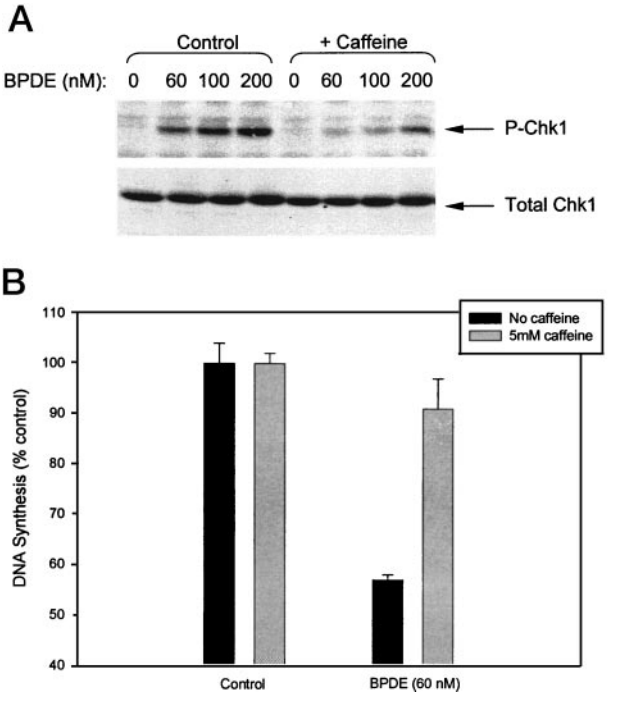

Fig. 5. BPDE-induced Chk1 phosphorylation and S-phase arrest are abrogated by caffeine. Exponentially growing H1299 cells were given fresh medium with or without 5 mM caffeine for 1 h. The control and caffeine-treated cells then received the indicated doses of BPDE for 1 h. After normalizing for protein content, lysates from the cells were analyzed by SDS-PAGE and immunoblotting with phospho-Chk1 antisera (*upper immunoblot*) or with antisera against total Chk1(*lower immunoblot*). In a parallel experiment, cells were given 60 nM BPDE (or received no BPDE for controls) for 1 h. Rates of DNA synthesis in the control and caffeine ± BPDE-treated cells were determined by measuring [^{3}H]thymidine incorporation during a 1-h period immediately after BPDE treatment.

lower panel of Fig. 5, BPDE elicited a 44% inhibition of DNA synthesis in control cells. However, caffeine-treated cells displayed near-normal (90% of control) rates of DNA synthesis. The acute treatment with 5 mM caffeine in these experiments did not affect rates of DNA synthesis in cells that did not receive DNA damage (data not shown). Overall, our data show that a caffeine-sensitive and Chk1-mediated pathway elicits S-phase arrest in response to BPDE-adducted DNA in p53-deficient cells.

BPDE-induced Chk1 Phosphorylation and S-Phase Arrest Are Caffeine Sensitive in AT Cells. Although the mechanism of the BPDE-induced S-phase checkpoint has not been reported previously, numerous laboratories have documented a transient S-phase arrest that is elicited by IR in normal cells. Importantly, the IR-induced S-phase arrest is absent in ATM-deficient cells from AT patients and transgenic mice (a phenomenon known as radioresistant DNA synthesis) but is restored after ectopic expression of the ATM protein (30, 31, 41, 42).

It was of interest to us to determine the role, if any, of ATM in the S-phase arrest elicited by BPDE. We obtained cultures of ET163 fibroblasts originally derived from AT patients and tested these for sensitivity to BPDE-induced S-phase arrest. It should be noted that unlike H1299 cells, the ET163 line does express p53. However, ET163 cells are SV40 transformed, and p53 function is perturbed as a result of SV40 large T antigen expression in this line. These cells are also likely to have defects in G_1 checkpoint control that result from SV40 T antigen-induced perturbation of the Rb signaling axis. Therefore, the G_1 checkpoint-defective ET163 cells provide an appropriate model system for studying p53-independent checkpoint events.

Exponentially growing cultures of ET163 cells were treated with 50 nM BPDE for 1 h and then pulsed with [^{3}H]thymidine for determinations of rates of DNA synthesis. As shown in the *lower panel* of Fig. 6, BPDE elicited a 46% inhibition of DNA synthesis in ET163 cells, demonstrating that ATM-deficient cells retain the BPDE-induced S-phase checkpoint. In the same experiments, some plates of ET163 cells were preincubated with 5 mM caffeine before treatment with BPDE. As also shown in the *lower panel* of Fig. 6, caffeine abrogated

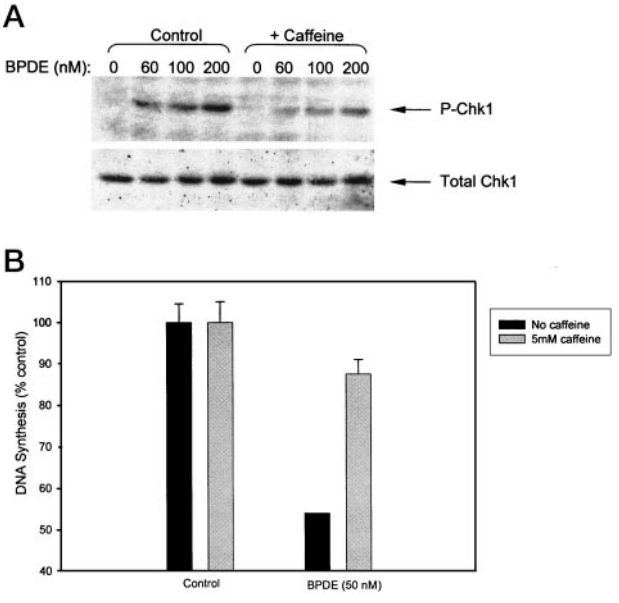

Fig. 6. Caffeine sensitivity of BPDE-induced Chk1 phosphorylation and S-phase arrest in AT cells. Exponentially growing ET163 cells were given fresh medium with or without 5 mM caffeine for 1 h. The control and caffeine-treated cells then received the indicated doses of BPDE for 1 h. Lysates from the cells were subject to SDS-PAGE and immunoblot analysis with phospho-Chk1 antisera (*upper immunoblot*) or antisera against total Chk1 (*lower immunoblot*). In a parallel experiment, control and caffeine-treated ET163 cells were given 50 nM BPDE (or no BPDE for controls) for 1 h. Rates of DNA synthesis in the control and caffeine ± BPDE-treated cells were determined exactly as described in the legend to Fig. 5. *Bars,* SE.

the inhibitory effects of BPDE on DNA synthesis. This result shows that AT cells retain a caffeine-sensitive checkpoint (most likely mediated via the caffeine-sensitive ATR kinase), and that the BPDE-induced S-phase checkpoint is distinct from IR-induced delay.

We also performed immunoblot analysis of Chk1 (using phospho-Chk1 antisera) in BPDE-treated ET163 cells. As shown in the *upper panel* of Fig. 6, BPDE elicited Chk1 phosphorylation in the ATM-deficient cells. As in the H1299 cells, Chk1 phosphorylation was reduced by caffeine treatment.

Liu *et al.* (33) and Zhao and Piwnica-Worms (34) have demonstrated that the ATR kinase can phosphorylate Chk1 on multiple sites (33, 34). Therefore, we consider it highly likely that ATR mediates both Chk1 phosphorylation and S-phase arrest in response to BPDE-adducted DNA. ATR-deficient cell lines are not yet widely available, and we are unable to test this hypothesis at present. However, a long-term goal is to formally test a role for ATR in S-phase responses to BPDE.

Discussion

In this study, we have investigated the mechanism of p53-independent BPDE-induced S-phase arrest. Our results show that low doses of BPDE elicit phosphorylation of the checkpoint kinase Chk1 concomitant with transient inhibition of DNA synthesis. Furthermore, perturbation of Chk1 signaling (using a dominant-negative Chk1 mutant or the Chk1 inhibitor UCN-01) abrogates the BPDE-induced S-phase checkpoint. In other unpublished experiments, we have found that ectopically expressed wild-type Chk1 augments BPDE-induced inhibition of bromodeoxyuridine or [^{3}H]thymidine incorporation, and that very high-level overexpression of WT Chk1 elicits arrest in S-phase (as well as in G_2-M) in H1299 cells, even in the absence of DNA damage. Collectively, these data suggest an important role for Chk1 in the S-phase checkpoint resulting from BPDE-induced DNA damage. Further consistent with a role for Chk1 in the S-phase checkpoint, both Chk1 phosphorylation and S-phase arrest elicited by BPDE are prevented by caffeine.

Previous workers have documented differential effects of low and high doses of genotoxic carcinogens (including BPDE) on DNA replication (37–39). Reversible inhibitory effects of DNA damage on initiation of DNA replication (DNA synthesis within individual replicons) have been observed in response to low doses of carcinogen. However, higher doses also affect elongation (the joining of replicon-sized units of nascent DNA). The inhibition of elongation is presumed to result from interference of the damaged DNA with replicative DNA polymerases (37–39). Our data, which demonstrate abrogation of BPDE-induced S-phase arrest after Chk1 inhibition, suggest that the carcinogen-induced inhibition of initiation documented by previous workers is Chk1 mediated.

The caffeine-sensitive ATM and ATR kinases are considered to mediate checkpoint responses to DNA damage in mammalian cells (12, 33, 42). ATM is believed to mediate responses to DNA DSBs, as evidenced by defective p53 signaling and cell cycle arrest in AT cells after irradiation (42). However, p53 responses to base-modifying genotoxic agents are generally unimpaired in AT cells (42). Such results have led to the suggestion that the ATM-related ATR protein kinase mediates responses to non-DSB forms of DNA damage (12). Indeed, in one study expression of dominant-negative forms of ATR was reported to perturb G_1 and G_2-M checkpoints in mammalian cells (43).

PAHs such as BPDE directly form adducts with DNA (4–8). However, BPDE-adducted DNA can elicit DSBs indirectly via single-stranded DNA nucleotide excision repair intermediates that are prone to breakage. Thus, it is likely that PAH-adducted DNA may lead to activation of both ATM and ATR, but with distinct kinetics. This possibility is also suggested by our finding that known substrates of ATR and ATM (Chk1, an ATR substrate; Chk2 and RPA, ATM substrates) are phosphorylated with different kinetics after treatment with high doses of BPDE. Alternatively, stalling of DNA polymerases resulting from high levels of adducted DNA (inhibition of elongation) may elicit activation of ATM and phosphorylation of ATM substrates.

Our results showing BPDE-induced phosphorylation of Chk1 and S-phase arrest in AT cells, and reversal by caffeine, demonstrate that ATM is not required for responses to low doses of BPDE and suggest that an alternative upstream activator, probably ATR, is the important mediator of the S-phase checkpoint. Indeed, Chk1 is a known downstream target of ATR (33, 34). However, it has been suggested that ATM and ATR might serve partially redundant or overlapping

roles (12). At present, we cannot exclude the formal possibility that ATM normally plays a role in Chk1 phosphorylation and S-phase arrest in response to BPDE-treatment of ATM-proficient cells.

Chk1 and ATR have been implicated in regulation of the DNA replication checkpoint. In elegant experiments using *Xenopus* extracts, the Kumagai *et al.* (25) and Hekmat-Nejad *et al.* (26) have shown that ATR and Chk1 are necessary for prevention of mitotic entry in the presence of unreplicated DNA (25, 26). In collaboration with Nghiem *et al.* (27), we have also shown a role for ATR and Chk1 in the replication checkpoint in U2OS cells (27). It remains to be seen whether the upstream events resulting in BPDE-induced Chk1 activation are the same as those involved in maintenance of the DNA replication checkpoint.

We have not yet determined the downstream effectors of Chk1 signaling that mediate arrest in S-phase. The Cdc25C protein is an important physiological substrate for Chk1 (18–20). However, Cdc25C is believed to regulate the G_2-M transition (by virtue of its ability to dephosphorylate and activate Cdc2/Cdk1). Thus, it is unlikely that Cdc25C plays a role in S-phase regulation. The other Cdc25 family members, Cdc25A and Cdc25B, are thought to regulate the G_1 and S-phase transitions (44). Moreover, both phosphatases are substrates for checkpoint kinases *in vitro* (19). It is possible, therefore, that Chk1-mediated inactivation of Cdc25A and B is responsible for BPDE-induced S-phase arrest.

Alternatively, it is possible that Chk1 directly affects the activity of components of the DNA replication machinery. The Cdc7/Dbf4 kinase has been hypothesized to be a target of the S-phase checkpoint (45). Additionally, Pelizon *et al.* (46) have speculated that the Cdc6 replication factor is involved in S-phase checkpoint control. However, no links with S-phase arrest or Chk1 have yet been demonstrated for Cdc6 or Cdc7/Dbf4 (46). Additional experiments are necessary to identify the targets of Chk1 signaling that mediate S-phase arrest in BPDE-treated cells.

In conclusion, we have identified a new mechanism for carcinogen-induced S-phase arrest in p53-null cells. This mechanism is distinct from S-phase checkpoint mechanisms that are responsive to other forms of DNA damage (such as IR-induced DSBs) and also differ from checkpoint responses to BPDE that we have defined previously in p53-proficient cells. In fact, previously described p53/p21-mediated checkpoints that arrest genotoxin-treated cells at the G_1-S boundary are likely to "mask" the Chk1-mediated S-phase arrest that predominates in p53-null cell lines. Additional experiments are necessary to characterize the proximal activators of Chk1 as well as the downstream targets of Chk1 signaling. These events undoubtedly play an important role in protecting against the cancer-causing effects of arylhydrocarbons and probably other carcinogens.

Materials and Methods

Chemicals. (±)*r*-7,*t*-8-Dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (anti) (BPDE) was purchased from the National Cancer Institute carcinogen repository. BPDE was dissolved in anhydrous DMSO (10 mM). BPDE stocks were protected from light and were stored at −70°C for no longer than 1 month. UCN-01 was a gift from Dr. Robert J. Schultz (Drug Synthesis and Chemistry Branch, National Cancer Institute). All other chemicals were from previously specified sources (28, 29).

Cells and Culture. H1299 cells were obtained from Dr. Sam Thiagalingnam (Boston University School of Medicine) and were grown in DMEM (supplemented with penicillin and streptomycin) containing 10% heat-inactivated fetal bovine serum. SV40-transformed ATM-deficient ET163 cells [derived from AT patients (37)] were obtained from Dr. Xiaohua Wu (Dana-Farber Cancer Institute, Boston, MA) and were cultured in DMEM (supplemented with penicillin and streptomycin) containing 15% fetal bovine serum.

BPDE Treatment. Cells were grown to ~50–60% confluence in 60-mm culture dishes containing 3 ml of appropriate growth medium. BPDE was added directly to the serum-containing medium as a 1000× stock solution in DMSO. After 1 h, the BPDE-containing medium was aspirated, and the cells were washed twice with 3 ml of fresh prewarmed medium before being returned to the incubator.

Adenovirus and Infections. cDNAs for Chk1 and Chk2 were PCR amplified from cDNA libraries, and the resulting clones were verified by sequencing. Site-directed mutagenesis was performed according to standard protocols, and mutations were verified by sequencing. Recombinant adenovirus encoding GFP, and WT or mutant Chk1 and Chk2, were constructed and purified as described previously (26, 27). Adenovirus infections were performed by addition of purified virus to the tissue culture medium. Expression of adenovirus-encoded proteins was confirmed by fluorescence microscopy (GFP) and immunoblotting (Chk1 and Chk2).

Assay of BPDE-induced S-Phase Arrest. Determination of rates of DNA synthesis were performed essentially as described by numerous other investigators (30, 31, 39, 40). In brief, cells were plated in 60-mm dishes and cultured for 2 days in the presence of 15 nCi/ml [^{14}C]thymidine (Amersham) to label total cellular DNA. The cells were fed with fresh [^{14}C]thymidine-containing medium 24 h after initial plating. After the 48-h labeling period, the cells were 60–70% confluent. The [^{14}C]thymidine prelabeled cells received different concentrations of BPDE (added as a 1000× stock solution in DMSO) or 0.1% DMSO as a negative control. Each treatment with BPDE (and each control experimental condition) was performed in duplicate. After 1 h of BPDE treatment, the cells were given fresh medium and pulsed with 6 μCi/ml of [^{3}H]-methyl thymidine (New England Nuclear) for 1 h. In some experiments, cells were also pulsed with [^{3}H]methyl thymidine at later time points (to test for recovery from S-phase arrest). Immediately after incubation with [^{3}H]methyl thymidine, the tritium-containing medium was aspirated, and the cells were washed twice with 4 ml of ice-cold 5% trichloroacetic acid. The trichloroacetic acid-fixed cells were solubilized in 1 ml of 0.3 N NaOH for 20 min. 0.3 ml of the NaOH-solubilized material was added to a scintillation vial and neutralized with 0.1 ml of glacial acetic acid. After addition of scintillant (5 ml) the ^{3}H and ^{14}C radioactivity present in each sample was determined by dual-channel liquid scintillation counting. The relative rates of DNA synthesis for the duration

of each [^{3}H]thymidine pulse were determined by calculating the ratio of ^{3}H cpm:^{14}C cpm. The ^{3}H:^{14}C ratio at each time point after BPDE treatment was expressed as a percentage of the ^{3}H:^{14}C ratio in cells that received no BPDE at time zero. In some experiments in which rates of recovery from S-phase arrest were not studied, the prelabeling step with [^{14}C]thymidine was omitted.

Protein Extracts and Immunoblotting. Monolayers of cells in 60-mm culture dishes were washed twice with 4 ml of PBS and then lysed by direct addition of 0.5 ml of ice cold detergent-containing lysis buffer supplemented with protease inhibitors, as described previously (27). The lysate was scraped into a microcentrifuge tube and incubated on ice for 5 min with repeated vortexing. Nuclear material and cell debris were removed by centrifugation (10,000 $\times$ *g* for 5 min at 4°C). The resulting supernatant was frozen in liquid N_2 and stored at −70°C before immunoblot analysis. For immunoblotting, cell extracts were normalized for protein content, boiled in SDS-reducing buffer, then separated on 7.5% SDS-polyacrylamide gels (50–75 μg protein/lane). After transfer to nitrocellulose, membranes were probed using commercially available polyclonal antisera against phosphorylated forms of Chk1 and Chk2 (Cell Signaling), RPA (Oncogene Sciences), and Chk1 or Chk2 (Santa Cruz Biotechnology). Bound primary antibody was detected using horseradish peroxidase-conjugated goat-antirabbit IgG. Protein bands were visualized using an ECL kit (Amersham).

Reproducibility. All data are representative of experiments that were performed at least three times. Qualitatively similar results were obtained on each occasion.

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