Down-Regulation of Histone H2B by DNA-Dependent Protein Kinase in Response to DNA Damage through Modulation of Octamer Transcription Factor 1

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INTRODUCTION

DSBs induced by IR or other DNA damaging agents activate a signal transduction cascade leading to the assembly of DNA repair complexes, cell cycle arrest and changes in specific gene transcription. The DNA-PK complex is an essential component of the DBS repair pathway. DNA-PK is a Ser/Thr kinase composed of Ku, a heterodimer of two subunits (Ku70/Ku80) and a large catalytic subunit, DNA-PKcs, member of the phosphatidylinositol-3-related kinase family (1–3). Ku is the DNA-binding subunit of the complex that recruits DNA-PKcs to DNA ends and some DNA sequences to activate the catalytic activity of the kinase (3–6).

DNA-PK is required for nonhomologous end-joining, which is the predominant repair process in cycling mammalian cells (7–9). Mice deficient in any component of this complex are radiosensitive, immune-deficient, and develop tumors (10, 11). Cells derived from these mice show an increased number of chromosomal aberrations, including chromosome fusions and telomeric fusions, which have suggested a "genome caretaker" role for the DNA-PK complex (9–12). Ku depends on ATM for its activation potential of Oct-1 in a manner that paradoxically also activates the mediator of apoptosis (23, 24). Several studies have also implicated DNA-PK in the modification of RPA2, nuclear factor-κB, and H2AX after IR (25–27). In contrast, DNA-PK appears to be important for the effect of p53 in mediating apoptosis (23, 24). Several studies have also implicated DNA-PK in the expression of several small nuclear RNA genes, including the RNA polymerase III-dependent gene U6 (43–47). Oct-1 has also been implicated in the regulation of DNA replication from viral and from mammalian origins (48, 49).

One of the transcriptional responses to DSBs that has recently been reported is the down-regulation of histone H2B expression (50). In the present study we show that U2 expression is also down-regulated in response to DSBs and that this down-regulation of histone H2B and U2 expression is dependent on DNA-PK. This effect appeared to be mediated through a DNA-PK-dependent down-regulation of the trans-activation potential of Oct-1 in a manner that paradoxically also results in stabilization of the protein. These results establish a role for catalytic activity is required for DNA repair (3, 9, 13–15). DNA-PK has been shown to phosphorylate in vitro other participants of the nonhomologous end-joining complex, including XRCC4 and Artemis (14, 16, 17), but the roles of these phosphorylations remain unclear.

In addition to participating directly in DNA joining, DNA-PK may also regulate DNA-damage-response-signaling pathways. Establishing a role for DNA-PK in DNA-damage response signaling has been complicated by the overlapping specificity of DNA-PK and ATM, a closely related kinase (1, 18–20). After a flurry of reports ascribing DNA damage-dependent signaling to one or the other kinase, it is now established that ATM is a key regulator of checkpoint responses and an activator of the homologous recombination repair pathway (1, 21, 22). In contrast, DNA-PK appears to be important for the effect of p53 in mediating apoptosis (23, 24). Several studies have also implicated DNA-PK in the modification of RPA2, nuclear factor-κB, and H2AX after IR (25–27). Recently, we also provided evidence that auto-inactivation of DNA-PK is essential for cell survival at a step after DNA repair (28).

IR-induced DNA damage results in profound changes in gene expression as the cells suspend progression through the cell cycle and repair their DNA. Histone gene transcription, for example, has been reported to be down-regulated (29–31). There are many reports in the literature of DNA-PK acting to regulate specific gene transcription (32–37). For example, we have demonstrated that recruitment of DNA-PK to the mouse mammary tumor virus promoter represses the induction of viral expression in response to steroid hormones (5). However, whether DNA-PK directly regulates specific gene transcription in response to DNA damage has not been established.

Recently, we showed that Ku antigen exhibits a specific interaction with the homeodomain of Oct-1 in a manner that strongly promotes phosphorylation by DNA-PK from DNA ends (38). Ku-dependent phosphorylation of Oct-1 by DNA-PK may be representative of a broader effect because Ku was also determined to interact similarly with several other homeodomain proteins, including HOXC4 and Dlx2.

Oct-1, a member of the POU homeodomain family, is a ubiquitous and essential transcription factor that regulates the expression of several genes essential to cell viability (39–41). For example, Oct-1 has been shown to be required for the S-phase-dependent activation of transcription of histone H2B (42) and has been shown to regulate the expression of several small nuclear RNA genes, including the RNA polymerase II-dependent gene U2 and the RNA polymerase III-dependent gene U6 (43–47). Oct-1 has also been implicated in the regulation of DNA replication from viral and from mammalian origins of replication (48, 49).

One of the transcriptional responses to DSBs that has recently been reported is the down-regulation of histone H2B transcription (50). In the present study we show that U2 expression is also down-regulated in response to DSBs and that this down-regulation of histone H2B and U2 expression is dependent on DNA-PK. This effect appeared to be mediated through a DNA-PK-dependent down-regulation of the trans-activation potential of Oct-1 in a manner that paradoxically also results in stabilization of the protein. These results establish a role for...
DNA-PK in regulating specific gene transcription in response to DSBs.

MATERIALS AND METHODS

Plasmids. pGEX 3X-Oct-1 POU was described previously (38). pGEX 3X-Oct-1 was generated by cloning the full-length Oct-1 cDNA isolated from pCGN-Oct-1 (51) in the EcoRI site of pGEX 3X. GST-Oct-1 NH2-terminal was produced by inserting an Oct-1 fragment (aa 1–268) in pGEX 2T, whereas the GST-Oct-1 COOH terminus resulted from the cloning of Oct-1 aa 414–743 in pGEX 3X. Deletions mutants of Oct-1 were generated from the pGEX-Oct-1 construct. pGEX-Oct-1 ΔC was obtained by removing the COOH-terminal fragment (aa 441–743), pGEX-Oct-1 ΔN was generated by deletion of aa 1–269, and internal deletion in the POU homeodomain (aa 397–441) yielded pGEX-Oct-1 ΔHD. Reporter plasmids pGSElBICAT, pG5 4xOct E1BICAT, and pG5 4xG/C/EIP E1BICAT have been described previously (52–54).

Cell Culture, Treatments, and Irradiation. Human HeLa and MCF-7 cells and Chinese hamster fibroblast cell lines V79 and XR-V15B were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO2. Human glioma derivatives M059K and -J were maintained cultured in high-glucose DMEM supplemented with 10% fetal bovine serum. For irradiation experiments, cells were plated the night before irradiation at 50,000 cells/cm2. Irradiations were performed with a Pantak irradiator at a dose rate of 1.68 Gy/min. Irradiations were performed with a Pantak irradiator at a dose rate of 1.68 Gy/min. Radiation at 2 Gy and above (e.g., 6 and 12 Gy) yielded equivalent results. Zeocin treatment was performed by incubating cells in medium containing 50 μg/ml Zeocin (Invitrogen) for 1 h. The medium was then replaced by regular medium for further incubation. Wortmannin treatment was done at 5 μM Wortmannin (Sigma) for 1 h before irradiation. Caffeine treatment was performed as described previously (55), with use of 3 mM caffeine (Sigma) for 1 h before irradiation. All experiments were repeated a minimum of three times, and quantification was performed as described for individual experiments.

Transfection Assays. Cells were transfected with ExGen 500 (MBI Fermentas, Burlington, ON, Canada) according to the manufacturer’s conditions. For Oct-1 protein-expression studies, 100-mm plates were transfected with 0.5 μg of pGEX-Oct-1 constructs, except in the experiments described in Fig. 4C, where 0.3 μg of pCGN-Oct-1 ΔN were transfected. After transfection, cells were incubated for 24 h, and then were trypsinized and divided into three 60-mm plates, two of which were subjected to irradiation, with the third used as the unirradiated control. For CAT assays in MCF-7 cells, 60-mm plates were transfected with 1 μg of CAT reporter construct (pG5E1BICAT or pG5 4xOct E1BICAT) and 0.6 μg of a CMV promoter β-Gal expression vector. After transfection, cells were irradiated and returned to the incubator for 18 and 26 h, at which time they were harvested for CAT and β-Gal assays, according to standard procedures. V79 and XR-V15B were transfected with 0.8 μg of CAT reporter construct and 0.1 μg of CMV-β-Gal plasmid and incubated for 16 h after irradiation before harvesting. Variations in transfection efficiencies were normalized with use of the values obtained from the β-Gal assay. Experiments were performed in duplicate or triplicate and repeated a minimum of three times. Error bars indicate 1 SD. A t test assessing unequal variance was used to test the statistical difference between control and irradiated samples. The level of significance was set at P = 0.05.

Generation of XR-V15B cells expressing human Ku80 (XR-V15B + Ku80) was accomplished by cotransfection of a human Ku80 cDNA expression plasmid (pBJ5 Ku80; Ref. 56) and pRSV-neo at an 8:1 ratio. Forty-eight h after transfection, G418 (400 μg/ml; Invitrogen) was added to the medium, and cells were selected for 10 days. Stable transfectants were maintained as a pool in medium containing 200 μg/ml G418. Extracts from these cells were analyzed by Western blot to confirm the expression of Ku80 and the restoration of the Ku70 protein levels, which were found to be equivalent to the levels observed in the wild-type V79 cells (data not shown).

Western Blot Analyses. Whole-cell extracts were prepared as described previously (38) in whole-cell extract buffer supplemented with 1 mM sodium vanadate and 20 mM NaF. For Western analysis, extracts were resolved by 8% or 10% SDS-PAGE. Gels were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and hybridized with either of the following antibodies: Oct-1 (C-21), p53 (FL-393; both from Santa Cruz Biotechnology, Santa Cruz, CA), Ku70 (N3H10; NeoMarkers, Fremont, CA), β-actin (AC-15; Sigma Chemical Co., St. Louis, MO), or HA (HA11; Covance, Princeton, NJ). The blots were developed with Enhanced Luminol Reagent (Weston Lightening; NEN Perkin-Elmer Life Sciences, Boston, MA). Protein levels were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Values obtained with HeLa cells were from eight samples analyzed in two separate experiments. Oct-1 protein levels measured in V79/V15B and M059 KJ were obtained in a minimum of three separate experiments. Oct-1 levels were analyzed in extracts of unirradiated cells (control) or cells taken at various time points between 1 and 8 h after irradiation and calculated relative to their respective endogenous actin levels (±SE).

Northern Blots. RNA was extracted with the RNeasy purification kit (Qiagen, Santa Clarita, CA). Five to 10 μg of total RNA/sample were run on a 1.2% agarose–formaldehyde gel. RNA was stained in the gel with SYBR Gold (Molecular Probes) and then transferred to a membrane according to the standard capillary procedure. The membrane was hybridized with a H2B cDNA probe as described previously (57). γ-Actin and U2 probes were obtained by PCR from a human cDNA library. RNA levels were quantified by phosphorimager analysis (Typhoon; Molecular Dynamics) and corrected for loading with the 18S RNA values, with use of ImageQuant software. All experiments included a minimum of three repetitions, and representative phosphoimages are displayed.

Phosphorylation of Oct-1 in Vivo. V79 and XR-V15B were transfected with pCGN-Oct-1 and incubated for 24 h. Cells were then incubated in phosphate-free medium for 3 h, at which time Zeocin (50 μg/ml) and 0.3 mM [32P]P, were added. After a 30-min incubation, cells were harvested, and whole-cell extracts were prepared. Oct-1 was immunoprecipitated by use of an Oct-1 antibody (C-21; Santa Cruz Biotechnology).

Immunoprecipitates were resolved by 10% SDS-PAGE and transferred to a membrane. After phosphotyramide analysis to detect 32P incorporation, the membrane was hybridized with an HA antibody (HA11; Covance) to determine Oct-1 levels. Quantification of Oct-1 phosphorylation was done with use of ImageQuant software. Phosphorylation levels were calculated as the ratio of the [32P] values and the Oct-1 levels measured by Western analysis.

Phosphorylation of Oct-1 by DNA-PK in Vitro. GST fusion proteins were expressed according to standard protocols (Amersham Biosciences Corp., Piscataway, NJ). In vitro DNA-PK assays were performed essentially as described previously (38). 0.1–0.2 μg of protein substrate was incubated for 20 min at 30°C in kinase buffer [50 mM HEPES (pH 7.5), 100 mM KCl, 10 mM MgCl2, 0.2 mM di sodium EGTA] in the presence of 20 ng of DNA, 0.5 units of holo-DNA-PK (Ku + DNA-PKcs; Promega), and 5 μCi of [γ-32P]ATP (6000 Ci/mmol; Amersham). Reactions were stopped with SDS loading buffer and loaded on SDS-polyacrylamide gels. 32P incorporation was visualized by autoradiography or phosphorimager analysis.

RESULTS

DNA-PK Is Required for Repression of Histone H2B Expression in Response to IR. To investigate whether the DNA-PK status of cells affected changes in Oct-1-dependent transcription after the formation of DSBs, we monitored the responses to IR of two Oct-1-dependent genes, histone H2B and U2, in several cell lines (Fig. 1). Exposure of MCF-7 cells, which retain p53-dependent cell cycle checkpoints in response to IR (58), to 6 Gy of radiation resulted in a 90% decrease in H2B mRNA levels over the 24 h after irradiation (Fig. 1A), confirming the IR sensitivity of H2B mRNA reported previously (50). Over the same period, U2 RNA levels declined >60%. The effects observed were not attributable to a general down-regulation of mRNA levels, because γ-actin mRNA levels in the same samples remained unchanged throughout the trial.

Experiments with the hamster fibroblast cell line V79 and clonal derivative XR-V15B, which lacks Ku80 and in which DNA-PKcs is inactive (56, 59), demonstrated that H2B and U2 down-regulation was dependent on an intact DNA-PK kinase complex (Fig. 1B). As in MCF-7 cells, H2B and U2 RNA levels declined in the V79 cells over the first 6 h after IR, whereas γ-actin levels were unaffected over this time. However, the levels of these RNAs were unaffected by IR in the
Fig. 1. Down-regulation of histone H2B mRNA after DNA damage is dependent on Ku and DNA-PKcs. A, MCF-7 cells were irradiated with 6 Gy and harvested after irradiation at the times indicated. Total RNA (5µg) was analyzed by Northern blot with H2B cDNA, U2, and γ-actin probes as indicated. 18S RNA visualized by SYBR gold staining was used to assess the loading. H2B, U2, and γ-actin signals were quantified by phosphorimager and the values normalized to the 18S RNA levels. Values below each lane indicate the amount of H2B, γ-actin, and U2 mRNA relative to the amount obtained for unirradiated cells (Lane C), which was set at 1. B, Northern analysis of H2B, U2, and γ-actin RNA levels in V79 cells (wild-type; wt), XR-V15B cells (Ku80-), and a pool of XR-V15B cells stably expressing human Ku80 (V15B + Ku80), prepared as described in the “Materials and Methods;” in response to IR. C, Northern analysis of H2B mRNA levels after irradiation (6 Gy) in M059K (wild-type) and M059J (DNA-PKcs mutant) cells performed as described in the legend for A.


A direct requirement for DNA-PKcs for the down-regulation in histone H2B mRNA was also observed in a pair of human glioma cell lines, M059K and M059J (Fig. 1C). M059J cells are a clonal derivative of M059K specifically lacking DNA-PKcs (60). H2B mRNA levels were refractory to IR in the DNA-PKcs- M059J derivative but repressed in the parental M059K cells, which were wild type for DNA-PK.

Accumulation of Oct-1 in Response to DSBs Is Dependent on DNA-PK. To determine whether Oct-1 was directly affected by DSBs, we monitored the response of Oct-1 to IR and the radiomimetic compound Zeocin (Fig. 2). In contrast to expectations based on the depression of H2B and U2 expression observed subsequent to IR, Oct-1 levels increased 2.7 ± 0.6-fold in HeLa cells within 1 h after irradiation at 12 Gy (Fig. 2A). The elevation in Oct-1 was sustained for at least 8 h, but subsequently returned to control levels by 24 h. Accumulation of Ku70 also increased in response to irradiation, as reported previously (61); β-actin protein levels, however, were unaffected throughout. Oct-1 was exquisitely sensitive to IR, with a full increase in protein level in response to a dose of only 2 Gy (Fig. 2B). The response was not specific to HeLa cells; we observed a similar rapid and reproducible accumulation of Oct-1 within 1 h of Zeocin treatment of MCF-7 cells (Fig. 2C). Notably, in MCF-7 cells the stabilization of Oct-1 preceded the stabilization of p53. A similar response was observed after treatment with IR (Fig. 3, and data not shown).

A second series of experiments indicated that accumulation of Oct-1 in response to the DNA-damage-inducing agents also was dependent on DNA-PK (Ku and DNA-PKcs; Fig. 3). Addition of the broad phosphatidylinositol-3-kinase inhibitor Wortmannin blocked the response of Oct-1 to IR in HeLa cells (Fig. 3A). In contrast, caffeine, which is a specific inhibitor of ATM and ATR (ATM and Rad3-related protein) but does not affect DNA-PK at the dose used (3 mM; Ref. 55), failed to block the accumulation of Oct-1 in response to IR in MCF-7 cells (Fig. 3B). Caffeine treatment did, however, severely blunt the p53 response in MCF-7 cells, as expected for an inhibitor of ATM.

Oct-1 levels also failed to respond to irradiation in DNA-PKcs- and Ku-deficient cells that are devoid of DNA-PK activity (Fig. 3, C and D). Thus the irradiation-induced accumulation of DNA-PK in wild-type M059K glioma cells was not observed in the DNA-PKc-M059J derivative (Fig. 3C), although steady-state Oct-1 levels were depressed in these cells. Quantification indicated a similar increase in Oct-1 levels at all time points taken between 1 and 8 h after irradiation in wild-type M059K samples (2.5 ± 0.3-fold), whereas no change was detected in the mutant M059J cells (0.97 ± 0.21-fold). Similarly, the up-regulation of Oct-1 in response to irradiation of V79 cells (2.4 ± 0.2-fold) was absent from the Ku80- XR-V15B cells.
Fig. 3. Response of Oct-1 to IR is dependent on Ku and DNA-PKcs. A, HeLa cells were incubated in the absence (Lanes 1–3) or presence of 5 μM Wortmannin (Lanes 4–6) and subjected to 2 or 6 Gy irradiation or left unirradiated (Lane 0). Whole-cell extracts were prepared 2 h after IR treatment, and 10 μg of extract were analyzed by Western blot with antibodies to Oct-1 and actin. B, MCF-7 cells were incubated with 3 mM caffeine for 1 h (Lanes 4–6) or left untreated (Lanes 1–3) before being subjected to 6 Gy irradiation or mock-treated (Lane C). Whole-cell extracts were prepared at the times indicated after irradiation and analyzed by Western blot with successive hybridization of the same membrane with antibodies to Oct-1, p53, and actin. C, M059K cells (DNA-PKcs wild-type) and M059J cells (DNA-PKcs mutant) were irradiated with a dose of 12 Gy and incubated for 1, 4, or 8 h after treatment. Whole-cell lysates were analyzed by Western blot with the antibodies indicated. Oct-1 proteins levels were quantified from samples in a minimum of three experiments, and the amount of Oct-1 in irradiated samples was calculated relative to the mock-irradiated control samples. M059K, 2.54 ± 0.34-fold (n = 11); M059J, 0.97 ± 0.21-fold (n = 10). D, V79 (wild-type), XR-V15B (Ku80 mutant; Ku−/−), and XR-V15B cells stably transfected with human Ku80 cDNA (V15B + Ku80) were harvested after 1 or 4 h of incubation after treatment with 6 Gy irradiation. Whole-cell extracts were analyzed as described in the legend for C. Quantification yielded the following levels of Oct-1 in irradiated samples relative to control samples: V79, 2.38 ± 0.23-fold (n = 9); V15B, 1.06 ± 0.15-fold (n = 9); V15B + Ku80, 2.3 ± 0.7-fold (n = 8).

(1.1 ± 0.1-fold; Fig. 3D). The IR-induced Oct-1 accumulation was confirmed to be mediated through a Ku-dependent pathway because reintroduction of wild-type Ku80 in XR-V15B cells restored the IR-induced up-regulation of Oct-1 close to levels observed in wild-type V79 cells (2.3 ± 0.7-fold).

An NH2-Terminal Domain of Oct-1 That Is Phosphorylated by DNA-PK Mediates Stabilization of Oct-1 in Response to IR. Previous results have shown that Oct-1 mRNA levels are unaffected by exposure to IR (62). To determine whether the change in Oct-1 protein levels we observed in response to IR resulted from changes in Oct-1 stability and to establish a platform for dissection of the effect on Oct-1, we expressed several recombinant HA-Oct-1 constructs in MCF-7 cells (Fig. 4B). The three forms of HA-Oct-1 expressing truncations maintained the Oct-1 nuclear localization signal and were confirmed by immunofluorescence to be fully localized to the nucleus of the transfected cells (data not shown). Interestingly, deletion of the NH2-terminus of Oct-1 to aa 270 resulted in a severalfold elevation in the level of accumulation of the HA-tagged protein. In contrast, HA-Oct-1 with a COOH-terminal truncation to aa 440 or containing a partial deletion of the homeodomain that abrogates the interaction of Oct-1 with Ku (38) accumulated to levels that were ~50% lower than those of full-length HA-Oct-1.

Deletion of the COOH-terminus of Oct-1 had no effect on the response to IR, with HA-Oct-1 ΔC being stabilized to the same extent as the endogenous Oct-1 in the same cells (Fig. 4C). In contrast, HA-Oct-1 ΔN was refractory to IR, suggesting that DNA-PK acted to stabilize Oct-1 through the NH2-terminus of the protein. Interestingly, deletion of the homeodomain of Oct-1 also abrogated Oct-1 stabilization in response to IR. The effect of this region, which we have previously shown to lack DNA-PK phosphorylation sites (38), suggested that the effect of DNA-PK on Oct-1 may be direct and dependent on the interaction of Oct-1 with Ku.

To determine whether phosphorylation of Oct-1 increased in response to DNA damage and whether this increase was attributable to DNA-PK, we analyzed 32P incorporation into HA-Oct-1 expressed in wild-type V79 cells and V15B Ku80− cells treated with Zeocin. A 30-min pulse of P, concomitant with Zeocin treatment showed a significant increase in the relative level of Oct-1 phosphorylation, at 1.51 ± 0.09-fold, when phosphorylation was corrected for changes in the absolute amount of Oct-1 (Fig. 5). In contrast, P labeling of HA-Oct-1 expressed in V15B cells was unaffected by IR (1.02 ± 0.17-fold), demonstrating that the change in Oct-1 phosphorylation was dependent upon the presence of holo-DNA-PK.

To determine whether the potential for phosphorylation of Oct-1 by DNA-PK correlated with the stabilization of Oct-1 in response to IR, we examined the phosphorylation of recombinant Oct-1 constructs by purified DNA-PK (Fig. 6). Full-length Oct-1, expressed as a GST fusion protein, was efficiently phosphorylated by DNA-PK with efficiency greater than a construct containing only the central region of Oct-1 including the homeodomain that we had used previously (Fig. 6B).

Analysis of constructs containing either the NH2- or COOH-terminal part of Oct-1 revealed that phosphorylation was localized to the NH2-terminal region because the Oct-1 COOH-terminal peptide was not appreciably phosphorylated by DNA-PK (Fig. 6C). Additionally, in this instance, the absence or truncation of the homeodomain from the NH2- and COOH-terminal constructs, respectively, prevented the interaction of these constructs with Ku, leading to a 40-fold reduction in total phosphate incorporation, as we have shown previously for other homeodomain truncations (38).

Addition of the homeodomain to the COOH-terminal region of Oct-1 did not enhance subsequent phosphorylation. However, attachment to the NH2-terminal region completely rescued full phosphorylation of this region (data not shown).
Transcriptional Activation by Oct-1 is Selectively Depressed in Response to IR.

To investigate how DNA-PK-dependent stabilization of Oct-1 in response to IR correlated with Oct-1-dependent transcription, we monitored Oct-1-dependent reporter gene transcription in transient transfection assays in MCF-7 cells (Fig. 7). To measure transcriptional activation by endogenous Oct-1, we evaluated reporter gene transcriptions from the minimal E1B promoter in response to IR. Fold activation of EIB expression by the octamer motifs lagged significantly 18 h after irradiation, despite the 2-fold increase in Oct-1 levels that were observed in these cells in response to IR (see Fig. 4). Fold activation of EIB expression by the octamer motifs was reduced to half of the level observed in control cells (Fig. 7B). This response was specific to the octamer motif because the C/EBP-dependent activation of transcription from a similar construct containing four copies of a C/EBP response element in lieu of the octamer motifs (52) was not repressed after irradiation (Fig. 7C).

At 26 h postirradiation, octamer motif-dependent CAT activity continued to lag the activity observed in mock-irradiated cells. However, the difference between the CAT activity in irradiated and control samples was reduced, consistent with gradual recovery in Oct-1 transactivation potential that correlated with the return of Oct-1 to normal levels in the cell.

**Fig. 4.** Oct-1 response to DNA damage is dependent on the NH2-terminal domain and the homeodomain. A, MCF-7 cells were transfected with pCGNOct-1 expressing a HA epitope-tagged Oct-1 cDNA. Each transfection was then split equally into three plates, of which one was used as control unirradiated (Lane 1) and the other two subjected to a 6-Gy dose of irradiation and harvested after 2 h (Lane 2) or 4 h (Lane 3). Ten μg of whole-cell extracts were analyzed by Western blot with antibodies to Oct-1 and HA-Oct-1, with a HA antibody to assess transfected HA-Oct-1 levels, and with an actin antibody to verify equal loading. B, schematic representation of the wild-type and deletion mutant HA-Oct-1 constructs. The numbering refers to the Oct-1 amino acids included in each construct. The darker gray boxes represent the POU domain (POU-specific and POU homeodomain). The HA epitope is indicated. Shown below is an analysis of the level of expression of each construct in MCF-7 cells after transfection with equal amounts of expression vector for each construct. Ten μg of whole-cell extracts were analyzed by Western blot with a HA antibody. C, cells were transfected separately with each construct (ΔC, ΔHD, and ΔN), as indicated and processed as described in the legend for A. Ten μg of whole-cell extracts were separated by SDS-PAGE, transferred to a membrane, and hybridized with antibodies to Oct-1 to analyze endogenous Oct-1 levels. The membrane was rehybridized with a HA antibody to reveal HA-Oct-1 mutants and with an actin antibody to verify equal loading. Nuclear localization of all constructs was verified by indirect immunofluorescence (data not shown). Quantification yielded the following levels of Oct-1 in irradiated samples relative to control samples. HA-Oct-1 wild-type (Lane C), 2.24 ± 0.2-fold (n = 6); HA-Oct-1ΔC, 1.91 ± 0.27-fold (n = 6); HA-Oct-1ΔHD, 1.01 ± 0.1-fold (n = 6); HA-Oct-1ΔN, 1.08 ± 0.13-fold (n = 6).

**Fig. 5.** In vivo phosphorylation of Oct-1 after DNA damage is dependent on DNA-PK activity. V79 and XR-V15B cells transfected with HA-Oct-1 were incubated for 30 min with 32P Pi in the presence (+) or absence (−) of 50 μg/ml Zeocin. Oct-1 was immunoprecipitated with an Oct-1 antibody, analyzed by phosphorimager to determine 32P phosphate incorporation (top) and subjected to Western blot analysis with a HA antibody to determine protein levels (bottom). Note that reduced amounts of Zeocin-treated V79 extracts were used for the immunoprecipitation to account for the increased 32P-phosphate incorporation in irradiated samples. α-HA: V79 cells, 1.51 ± 0.09-fold (n = 3), in V15B cells: 1.02 ± 0.17-fold (n = 3).

**Fig. 6.** DNA-PK phosphorylates residues present on the NH2-terminal domain of Oct-1. A, schematic representation of the GST-Oct-1 constructs used as substrates in DNA-PK phosphorylation assays. B, in vitro DNA-PK phosphorylation of purified GST-Oct-1 full-length and GST-Oct-1 POU. After separation of the products by 10% SDS-PAGE, gel was stained with Coomassie blue (Lanes 1 and 2) and phosphorylation was revealed by Phosphorimager analysis (Lanes 3 and 4). The arrows indicate the positions of the full-size GST-Oct-1 full-length and GST-Oct-1 POU proteins. C, Equal amount of GST-Oct-1 C-term (Lanes 1 and 3) on N-term (Lanes 2 and 4) proteins were in vitro phosphorylated by purified DNA-PK. Samples were separated by 10% SDS-PAGE and then stained with Coomassie blue (left) and autoradiographed to assess 32P incorporation (right).
To assess the role of DNA-PK in the repression of octamer-motif-dependent transcriptional activation, we repeated the transfection experiments in the V79 and XR-V15B cells. In the wild-type V79 cells, the octamer-dependent activation of transcription was again significantly reduced after IR treatment (Fig. 7D). In contrast, reporter gene expression was unaffected by irradiation in the Ku80⁺ XR-V15B cells.

We conclude that DNA-PK acts in response to IR to modulate expression of essential genes such as histone H2B and U2 through the promotion of a decrease in the transcriptional activation potential of Oct-1.

DISCUSSION

Our results identify a role for DNA-PK in modulating specific gene transcription in response to DSBs by regulating the levels and activity of Oct-1. These results suggest that the stimulation of DNA-PK activity from DSBs can rapidly and directly affect cellular transcription by targeting transcription factors for phosphorylation.

Elevation in the expression of the histone H2B gene is a primary requirement for cellular proliferation because new DNA synthesis must be accompanied by new histone deposition (42, 63). Thus, down-regulation of histone synthesis would seem a natural consequence of conditions in which cell cycle progression is suspended, such as in response to the DNA damage caused by IR. The absence of down-regulation of H2B in Ku⁻ and DNA-PK⁻⁻ cells provides compelling evidence for a direct role for DNA-PK in regulating this response.

Down-regulation of U2 small nuclear RNA, an essential component of the splicing machinery, was found to coincide with the H2B mRNA decrease. U2 expression has been shown to be dependent on Oct-1 binding to the octamer sequence present in the distal sequence element of the U2 promoter (47, 64). Thus, inhibition of Oct-1 transcriptional activity by DNA-PK after IR would be expected to have a direct effect on U2 gene transcription, as observed in our experiments. However, the regulation of U2 transcription is a complex process because full activation of U2 also requires transcription factor Sp1, shown to interact with Oct-1, as well as the assembly of a multisubunit complex recruited to the proximal sequence element (43, 47, 64, 65). Therefore, complete understanding of how U2 is down-regulated after DNA damage will require further investigation.

Several reports have indicated that the absence of Ku or DNA-PKcs does not affect checkpoint activation and has no effect on the initiation of DNA replication induced by IR (66–69). Indeed, the ATM-dependent S checkpoint activated in response to DNA damage is stronger in Ku and DNA-PKcs-deficient cells (70–72). However, the Ku- and DNA-PK-deficient cell lines (XR-V15B and M059J), as well as their respective parent cell lines (V79 and M059K), do express a Ku- or DNA-PKcs-deficient cells provides compelling evidence for a direct role for DNA-PK in regulating this response.

To assess the role of DNA-PK in the repression of octamer-motif-dependent transcriptional activation, we repeated the transfection experiments in the V79 and XR-V15B cells. In the wild-type V79 cells, the octamer-dependent activation of transcription was again significantly reduced after IR treatment (Fig. 7D). In contrast, reporter gene expression was unaffected by irradiation in the Ku80⁺ XR-V15B cells.

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Our results identify a role for DNA-PK in modulating specific gene transcription in response to DSBs by regulating the levels and activity of Oct-1. These results suggest that the stimulation of DNA-PK activity from DSBs can rapidly and directly affect cellular transcription by targeting transcription factors for phosphorylation.

Elevation in the expression of the histone H2B gene is a primary requirement for cellular proliferation because new DNA synthesis must be accompanied by new histone deposition (42, 63). Thus, down-regulation of histone synthesis would seem a natural consequence of conditions in which cell cycle progression is suspended, such as in response to the DNA damage caused by IR. The absence of down-regulation of H2B in Ku⁻ and DNA-PK⁻⁻ cells provides compelling evidence for a direct role for DNA-PK in regulating this response.

Down-regulation of U2 small nuclear RNA, an essential component of the splicing machinery, was found to coincide with the H2B mRNA decrease. U2 expression has been shown to be dependent on Oct-1 binding to the octamer sequence present in the distal sequence element of the U2 promoter (47, 64). Thus, inhibition of Oct-1 transcriptional activity by DNA-PK after IR would be expected to have a direct effect on U2 gene transcription, as observed in our experiments. However, the regulation of U2 transcription is a complex process because full activation of U2 also requires transcription factor Sp1, shown to interact with Oct-1, as well as the assembly of a multisubunit complex recruited to the proximal sequence element (43, 47, 64, 65). Therefore, complete understanding of how U2 is down-regulated after DNA damage will require further investigation.

Several reports have indicated that the absence of Ku or DNA-PKcs does not affect checkpoint activation and has no effect on the initiation of DNA replication induced by IR (66–69). Indeed, the ATM-dependent S checkpoint activated in response to DNA damage is stronger in Ku and DNA-PKcs-deficient cells (70–72). However, the Ku- and DNA-PK-deficient cell lines (XR-V15B and M059J), as well as their respective parent cell lines (V79 and M059K), do express a Ku- or DNA-PKcs-deficient cells provides compelling evidence for a direct role for DNA-PK in regulating this response.

To assess the role of DNA-PK in the repression of octamer-motif-dependent transcriptional activation, we repeated the transfection experiments in the V79 and XR-V15B cells. In the wild-type V79 cells, the octamer-dependent activation of transcription was again significantly reduced after IR treatment (Fig. 7D). In contrast, reporter gene expression was unaffected by irradiation in the Ku80⁺ XR-V15B cells.

We conclude that DNA-PK acts in response to IR to modulate expression of essential genes such as histone H2B and U2 through the promotion of a decrease in the transcriptional activation potential of Oct-1.

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Transcription of the histone H2B gene occurs in a cell-cycle-dependent manner and is largely dependent on Oct-1 (42, 77, 78).

Cell
cycle control of Oct-1 activity is regulated through phosphorylation of the octamer factor at Ser-385 in the homeodomain, which acts by inhibiting Oct-1 DNA binding at the onset of mitosis (79, 80). In contrast, DNA-PK modifies Oct-1 in the NH2 terminus, with preliminary results identifying multiple sites of phosphorylation within this region. This phosphorylation included sites several amino acids NH2-terminal to the POU-specific domain that were phosphorylated in our previous study using an Oct-1 construct focused on the POU-specific and POU homeodomains of Oct-1 (Ref. 38, and data not shown). These results suggest that modification of Oct-1 by DNA-PK is unlikely to affect Oct-1 DNA binding. Furthermore, an increase in Oct-1 DNA binding activity has been reported after DNA damage, consistent with elevated levels of the protein (50, 62). Thus, the modulation of Oct-1 by DNA-PK appears to be distinct from the normal cell cycle regulation of the factor.

In our experiments, the strong down-regulation of H2B mRNA observed somewhat exceeded the 2-fold reduction in transcription dependent on octamer motifs in a synthetic promoter analyzed by transient transfection. On the one hand, this suggests the possibility that effects on additional factors that are also involved in regulating H2B expression come together with the effect that we have characterized on Oct-1 to impart the strong effects observed in vivo. However, it should also be recognized that the nature of the transient transfection assay likely decreases the magnitude of the effect observed. Transfection was performed over a period of 5 h, followed by irradiation of the cells. Thus it is likely that at least some initial transcriptional response of the promoter occurred over the course of the incubation with transfection agent, before irradiation. The net effect of this activity would be to reduce the magnitude of the reduction observed in the level of the very stable CAT protein. Similarly, recovery of the cells as seen by the more modest reduction in transcription at the longer time point could also serve to blunt the overall magnitude of the effect observed.

It has previously been suggested that Oct-1 induction in response to DNA damage is a post-transcriptional mechanism because Oct-1 mRNA levels were not affected by DNA damage and the Oct-1 protein up-regulation was unaltered by the inhibition of RNA synthesis (62). We have confirmed here that Oct-1 up-regulation is likely attributable to a protein modification because ectopically expressed Oct-1 levels were increased similar to the levels of the endogenous protein. The Oct-1 cDNA in our constructs lacks the 5′ untranslated region, which could potentially modulate protein synthesis after IR. In addition, we can rule out that this up-regulation is attributable to a potential IR-induced activation of transcription from the CMV promoter because we observed a differential stabilization of the mutant Oct-1 proteins that were expressed from this same promoter. Oct-1 protein levels do not fluctuate throughout the cell cycle (79), which suggests that Oct-1 up-regulation after IR is likely not a consequence of cell cycle arrest, but rather a direct effect of a DNA damage-induced modification.

This is further supported by the increased in vivo phosphorylation of Oct-1 that occurs after DNA damage. This phosphorylation was rapid (within 30 min) and Ku dependent, suggesting that DNA-PK acts directly to modify Oct-1 stability and transcription activity. It is intriguing that down-regulation of the transcription activity of Oct-1 occurs concomitantly with overall stabilization of the protein. Initial analysis of the phosphorylation of Oct-1 by DNA-PK within the NH2 terminus indicates at least three clusters of phosphorylation (data not shown), suggesting that the effects on stability and transcription may be mediated through separate functional determinants. However, we also noted with interest recent reports that coupled ubiquitin-mediated protein turnover with transcriptional activation (81–85). Transcriptional activation mediated through several acidic activation domains, including that of the c-myc protein, has been shown to be linked to the rapid turnover of the proteins through the 26S proteasome, subsequent to ubiquitin modification within the transcriptional regulatory domains (84). Although Oct-1 contains a glutamine-rich rather than an acidic transcriptional activation domain in its NH2 terminus and its stability in normal cells is severalfold higher than those of rapidly turned-over proteins such as c-myc (79, 84), the possibility of an inverse relationship between Oct-1 stability and transcriptional potential merits further investigation.

A recurring theme in the study of DNA-PK phosphorylation targets is that colocalization of the kinase and substrate through protein–protein–protein–DNA interactions confers a selective advantage to phosphorylation (6, 38, 86–88). In this instance, the phosphorylation of Oct-1 and its stabilization in vivo in response to DNA damage induced by IR correlated directly with the ability of Oct-1 to interact with the Ku70 subunit of DNA-PK. Furthermore, the interaction of Oct-1 with Ku does not appear to lead to the recruitment of Ku to octamer motifs through which Oct-1 acts, but has been demonstrated to occur on DNA ends, from which DNA-PK is activated (38), supporting the emphasis on the potential for directed phosphorylation of Oct-1 in response to DNA damage. Moreover, the interaction with Oct-1 containing the homeodomain and the similar interactions demonstrated with other homeodomain proteins (38) imply that the activation of DNA-PK from DNA ends may act broadly to influence the function of homeodomain proteins in the cell in response to IR.

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