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Evidence for DNA-PK-Dependent and -Independent DNA Double-Strand Break Repair Pathways in Mammalian Cells as a Function of the Cell Cycle

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Mice homozygous for the *scid* (severe combined immune deficiency) mutation are defective in the repair of DNA double-strand breaks (DSBs) and are consequently very X-ray sensitive and defective in the lymphoid V(D)J recombination process. Recently, a strong candidate for the *scid* gene has been identified as the catalytic subunit of the DNA-dependent protein kinase (DNA-PK) complex. Here, we show that the activity of the DNA-PK complex is regulated in a cell cycle-dependent manner, with peaks of activity found at the G_1/early S phase and again at the G_2 phase in wild-type cells. Interestingly, only the deficit of the G_1/early S phase DNA-PK activity correlated with an increased hypersensitivity to X-irradiation and a DNA DSB repair deficit in synchronized *scid* pre-B cells. Finally, we demonstrate that the DNA-PK activity found at the G_2 phase may be required for exit from a DNA damage-induced G_2 checkpoint arrest. These observations suggest the presence of two pathways (DNA-PK-dependent and -independent) of illegitimate mammalian DNA DSB repair and two distinct roles (DNA DSB repair and G_2 checkpoint traversal) for DNA-PK in the cellular response to ionizing radiation.

Normal lymphoid development is marked by the somatic rearrangement of separate genetic elements to form functional immunoglobulin and T-cell receptor genes. This process, known as V(D)J recombination, is mediated by a site-specific DNA rearrangement mechanism that targets conserved signal sequences flanking the genetic elements (reviewed in reference 46). The initial steps of V(D)J recombination involve the recognition of the signal sequences and the introduction of DNA double-strand breaks (DSBs) adjacent to them. These steps are carried out by the products of two lymphoid-specific genes, *RAG-1* and *RAG-2* (20, 51).

Several studies have suggested that V(D)J recombination events may be restricted during the cell cycle. Analysis of RAG-2 protein demonstrated that it accumulated in cells preferentially in the G_0/G_1 phase of the cell cycle, declined more than 20-fold before entering the S phase, and remained low throughout the S, G_2, and M phases (47). Interestingly, the levels of RAG-1 protein, which fluctuated to a much lesser extent, still declined fivefold in the S phase (48). Lastly, the double-strand signal sequence breaks associated with V(D)J recombination can be detected only in the G_0/G_1 phase of the cell cycle (65). These data strongly imply that the initiation of V(D)J recombination is mostly, and perhaps entirely, restricted to the G_1 phase of cycling cells.

While *RAG-1* and *RAG-2* control the initial steps of V(D)J recombination, the later steps appear to be controlled by genes involved in generalized DNA DSB repair. In particular, a complex, DNA-dependent protein kinase (DNA-PK), which has DNA-dependent serine-threonine protein kinase activity and which consists of at least three components, the 465-kDa catalytic subunit ($DNA\text{-}PK_{cs}$) and the heterodimeric Ku protein, has been shown to be intimately involved in generalized DNA DSB repair and V(D)J recombination (reviewed in reference 36). $DNA\text{-}PK_{cs}$ is thought to be the product of the severe combined immune deficiency (*scid*) gene (9, 19, 28, 39), and it has long been known that mice homozygously mutant at this locus are X-ray sensitive and defective in DNA DSB repair and V(D)J recombination (reviewed in references 10 and 31). Ku is a heterodimeric protein of 70- and 86-kDa subunits which binds tightly to a variety of double-stranded DNA ends (reviewed in reference 36). Recently, it was shown that members of the fifth X-ray cross complementation group (XRCC5), which are defective in DNA DSB repair and V(D)J recombination, are mutated in the Ku86 gene (18, 21). Thus, DNA-PK has been unequivocally identified as an important mammalian DNA repair complex, and mutations in either $DNA\text{-}PK_{cs}$ or the 86-kDa subunit of Ku result in DNA DSB repair defects that manifest themselves as X-ray sensitivity and impaired V(D)J recombination.

Interestingly, the survival of certain mammalian mutant cells sensitive to ionizing radiation has also been shown to depend upon cell cycle position at the time of X-ray exposure, with maximal sensitivity to irradiation occurring at the G_1/S border (69, 75, 77). To see if this paradigm could be extended to DNA-PK in general and *scid* cells in particular, we set forth to test whether the activity of DNA-PK and the ionizing-radiation sensitivity of *scid* cells fluctuated during the cell cycle. Our results indicate that wild-type cells exhibit two distinct peaks of DNA-PK activity, one at the G_1/early S phase and another at the G_2 phase. In *scid* cells the lack of the G_1/early S phase peak correlated precisely with hypersensitivity to X-irradiation and a DNA DSB repair deficit. Unexpectedly, however, the loss of DNA-PK activity during G_2 in *scid* cells appeared to be unrelated to DNA DSB repair but instead correlated with cell cycle arrest. Thus, in addition to DNA-PK's well-characterized role in DNA DSB repair, which we demonstrate here occurs in the G_1/early S phase of the cell cycle, DNA-PK may also perform a novel function at a G_2 checkpoint.

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MATERIALS AND METHODS

Cells and cell culture. 300-19 and 8D are Abelson murine leukemia virus-transformed murine pre-B cell lines. 300-19 was derived from a wild-type mouse (2), while 8D was derived from a mouse homozygous for the *scid* mutation (33). Both cell lines were cultured in RPMI 1640 media supplemented with 10% fetal calf serum, 1% L-glutamine, 100 U of penicillin per ml, 0.1 mg of streptomycin per ml, and 50 μM 2-mercaptoethanol. HeLa cells (a generous gift of Surendra Sharma, Department of Pathology, Roger Williams Hospital, Providence, R.I.) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% L-glutamine, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml.

Cell synchronization. Exponentially growing 8D and 300-19 cells were seeded at a concentration of 3×10^5/ml in 25-cm^2 tissue culture flasks. Cultures were synchronized by incubating the cells for 8 h either with hydroxyurea (Sigma) at a final concentration of 375 μM or with methyl-(5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl)-carbamate (nocodazole) made from a stock solution in dimethyl sulfoxide (Sigma), at a final concentration of 250 ng/ml. Cell suspensions were then washed twice with 5 ml of fresh medium and resuspended in new flasks with 10 ml of medium. HeLa cells were synchronized as previously described (52), with the following modifications. Exponentially growing cells were subcultured at a concentration of 3×10^5 per 100-mm tissue culture dish; 12 h later, 1 mM hydroxyurea was added, and the cells were then incubated for an additional 24 h. Subsequently, the cells were washed twice with 5 ml of fresh media and then resuspended in 10 ml of media. For nocodazole synchronization, cells were synchronized first with hydroxyurea as described above, after which the hydroxyurea was removed; 5.5 h later nocodazole was added to a final concentration of 100 ng/ml, and the cells were further incubated for 5 h. The cells were then washed twice with 5 ml of fresh media and resuspended in 10 ml of media to resume cycling.

The positions of HeLa cells during the cell cycle were monitored by [^{3}H]thymidine incorporation. HeLa cells were incubated with 10 μCi of [^{3}H]thymidine per ml (80 Ci/mmol; ICN) for 1 h. The cells were then washed with phosphate-buffered saline, trypsinized, and pelleted by centrifugation. The cell pellet was resuspended in 0.5 ml of 0.1 N NaOH–10 mM EDTA–0.5% sodium dodecyl sulfate and incubated for 30 min at 70°C. After the sample had cooled to room temperature, an equal volume of 20% trichloroacetic acid was added and the sample was incubated an additional 30 min on ice. The sample was then applied to a filter (GF/C; Whatman), which was subsequently washed extensively with 10% trichloroacetic acid and 70% ethanol and then air dried. Radioactive incorporation was then determined by scintillation counting.

Propidium iodide staining and fluorescence-activated cell sorting (FACS) analysis. Approximately 10^6 cells were washed once with phosphate-buffered saline and resuspended in 1 ml of propidium iodide solution (50 μg of propidium iodide per ml, 0.1% Nonidet P-40, and 0.1% sodium citrate). The cell suspensions were stored at 4°C in the dark until they were analyzed with a Becton Dickinson FACScan instrument.

X-ray survival assays. At each time point after drug removal, an aliquot of synchronized cells was irradiated with a Philips 250-kV-peak X-ray machine set at 14 mA to emit 2.2 Gy/min by using a 0.35-mm-pore-size copper filter at doses ranging from 0 to 6 Gy. Cells were then plated in duplicate into 96-well plates under limiting dilution, and those cells surviving to form colonies were scored between 10 and 14 days postirradiation (22, 32, 43).

DNA DSB repair assays. DNA DSB repair assays using pulsed-field gel electrophoresis (PFGE) were performed as described elsewhere (26, 43).

Preparation of cell extracts. Whole-cell extracts were prepared from ~10^7 HeLa cells grown in monolayer culture (5, 45). Cells were harvested and washed twice with ice-cold phosphate-buffered saline by centrifugation. Cell pellets were resuspended in 100 ml of ice-cold LS buffer (25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 20 mM HEPES-KOH [pH 7.9], 10% [vol/vol] glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin [0.5 mg/ml; Sigma], trypsin inhibitor [1.0 mg/ml; Sigma], aprotinin [0.5 mg/ml; Sigma], bestatin [40 mg/ml; Sigma]) and subjected to a single freeze-thaw cycle. After microcentrifugation for 10 min at 4°C, the supernatant was transferred to a new microcentrifuge tube and designated S-10 extracts (5, 45). The pellets were resuspended in 50 ml of HS buffer (25 mM KCl, 500 mM NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 20 mM HEPES-KOH [pH 7.9], 10% [vol/vol] glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and leupeptin, trypsin inhibitor, aprotinin, and bestatin [at the concentrations for LS buffer]) and incubated on ice for 10 min. After microcentrifugation for 10 min at 4°C, supernatants were removed and designated P-10 extracts (5, 41). The amounts of protein in S-10 and P-10 extracts were determined with Bradford reagents (Bio-Rad).

DNA-PK assays. Peptide phosphorylation assays were performed as described previously (5, 45), with the following modification: S-10 and P-10 extracts (25 μg of each) were mixed and incubated at 30°C for 10 min with 0.05 μl of [γ-^{32}P]ATP (7,000 Ci/mmol; ICN Radiochemical), 1 mM ATP, 100 mM NaCl, and 4 nmol of peptide substrate (EPPLSQEAFADLLKK; Promega) in the presence or absence of 10 μg of sheared herring sperm DNA. Reactions were stopped and spotted onto Whatman P-81 paper. After washing, radioactivity was determined by liquid scintillation counting.

DNA-PK activity was defined as the counts per minute of ^{32}P incorporated in the presence of DNA minus the counts per minute of ^{32}P incorporated in the absence of DNA, divided by the counts per minute of ^{32}P incorporated in the absence of DNA—i.e., fold activation. This value was routinely in the range of 5 to 15 for all experimental samples at all time points. For ease of presentation, the data were normalized to a given time point (12-h data points in Fig. 1A and 6-h data points in Fig. 1B were made equal to 1.0-fold relative induction) and presented as relative fold induction.

Immunoblotting. For Western blotting, S-10 and P-10 extracts (25 μg of each) were mixed and resolved by sodium dodecyl sulfate–6% polyacrylamide gel electrophoresis and then were electrophoretically transferred to polyvinylidene difluoride membranes (Schleicher & Schuell). The membranes were then probed with anti-Ku70 (N3H10), anti-Ku86 (111) (both gifts from Westley Reeves, University of North Carolina), or anti-DNA-PK$_{cs}$ (42-psc; a gift of Tim Carter, St. John's University) monoclonal antibody. The antigen-antibody complexes were detected by using horseradish peroxidase-conjugated anti-mouse antibodies (Amersham) according to the manufacturer's instructions.

RESULTS

The activity of DNA-PK is regulated in a cell cycle-dependent manner. To investigate the mechanism of DNA DSB repair in mammalian cells, the activity of DNA-PK during the cell cycle was determined. Briefly, HeLa cells were first synchronized at the G_1/S border by using hydroxyurea (6, 52) and then released from this block and subsequently resynchronized at mitosis by using the microtubule inhibitor nocodazole (40, 67). After removal of the nocodazole, cells were harvested every 3 h, cellular extracts were prepared, and DNA-PK assays were performed with a synthetic peptide (3, 23, 27) as a substrate. To monitor cell cycle progression, cells were pulse-radiolabeled with [^{3}H]thymidine.

As expected, following removal of nocodazole, HeLa cells reentered the cell cycle and underwent a period of low DNA synthesis (0 to 6 h, corresponding to G_1), which was followed by a peak (9 to 15 h, corresponding to the S phase) and a subsequent reduction in DNA synthesis (18 to 21 h, corresponding to G_2) (Fig. 1A). During the course of the experiment, the activity of DNA-PK clearly fluctuated, although the difference between the highest and lowest values was only twofold (Fig. 1A). It should be noted, however, that the results reflect the averaging of three independent experiments, and although the absolute value of DNA-PK activity between experiments varied, the overall profile was highly reproducible. In addition, no fluctuations in DNA-PK activity were observed in the absence of added exogenous DNA (data not shown). Two distinct peaks of activity, one at 3 h and the other at 21 h after drug removal, were observed (Fig. 1A). These time points appeared to correspond to the G_1/early S and G_2 phases of the cell cycle, respectively.

To confirm that the G_1/early S and G_2 peaks of DNA-PK activity were not an artifact of the nocodazole treatment, HeLa cells were synchronized with hydroxyurea. As expected, HeLa cells treated with hydroxyurea (6) accumulated preferentially at the G_1/S border (Fig. 1B). Following removal of the drug, the HeLa cells immediately progressed into the S phase, with a peak of DNA synthesis observed at 6 h after drug removal (Fig. 1B). The cells then underwent a long period of low DNA synthesis, corresponding to G_2 (9 to 12 h) and G_1 (15 to 18 h), before the beginning of a second wave of DNA synthesis was observed (21 to 24 h) (Fig. 1B). Importantly, the DNA-PK activity profile observed with hydroxyurea-synchronized cells corroborated the pattern observed with nocodazole-synchronized cells. In particular, DNA-PK activity, which was initially high (0 h), immediately decreased as the HeLa cells began to replicate their DNA and reached a minimal value at 6 h after drug removal (Fig. 1B). Two subsequent peaks of DNA-PK activity, at 12 and 21 h after drug removal, were then observed, and these appeared to correspond to the G_2/M and G_1/S transitions (Fig. 1B). From these experiments, we concluded that

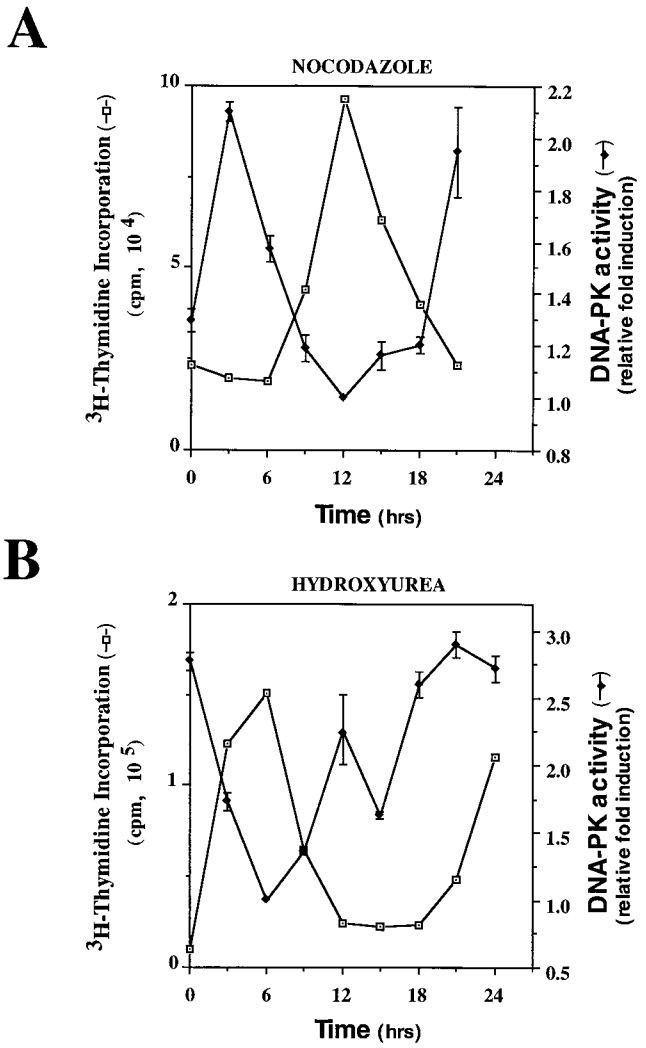

FIG. 1. DNA-PK activity fluctuates during the cell cycle. HeLa cells were synchronized either in mitosis by using the microtubule inhibitor nocodazole (A) or in the G_1/S phase by using the ribonucleotide reductase inhibitor hydroxyurea (B), as described in Materials and Methods. At time 0, the drug was washed out and the cells were resuspended in fresh media. At 3-h intervals, aliquots of cells were assessed for the levels of DNA synthesis (by [^{3}H]thymidine incorporation) and DNA-PK activity as described in Materials and Methods. The results shown for DNA-PK activity are the averages of three independent experiments.

DNA-PK activity fluctuates throughout the cell cycle, with peaks of activity observed at the G_1/early S and G_2 phases.

Fluctuations of DNA-PK activity are not related to the levels of the individual DNA-PK subunits. The DNA-PK complex consists of three proteins: the catalytic subunit (DNA-PK$_{cs}$) and the heterodimeric Ku proteins Ku86 and Ku70 (reviewed in references 4 and 36). To ascertain whether the cell cycle-specific alterations in DNA-PK activity correlated with the protein levels of any of the complex subunits, we performed Western analyses on the same extracts used to measure DNA-PK activity. In contrast to activity, none of the DNA-PK components showed any significant change in physical amount during the cell cycle (Fig. 2). These results were confirmed by using extracts from either nocodazole-synchronized (Fig. 2A) or hydroxyurea-synchronized (Fig. 2B) HeLa cells. It should be noted that the intensity of each band from each of the triplicate experiments was quantitated by densitometry, and no significant changes were observed (data not shown). Therefore, we concluded that the activity of DNA-PK was not directly related to the physical amounts of its constituent subunits.

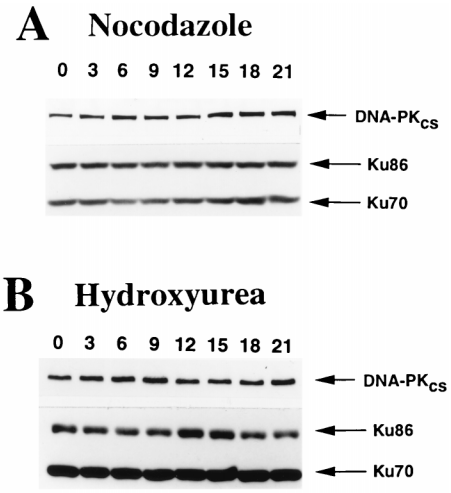

FIG. 2. Levels of the DNA-PK subunits do not fluctuate during the cell cycle. Whole-cell extracts were prepared from HeLa cells synchronized with nocodazole (A) or hydroxyurea (B) as described in the legend to Fig. 1 and were subjected to immunoblot analysis with monoclonal antibodies 42-psc (DNA-PK$_{cs}$), 111 (Ku86), and N3H10 (Ku70). The lane numbers correspond to the time points after drug removal indicated in Fig. 1. The intensity of each band was quantitated by densitometry (data not shown), and no significant changes were observed.

Cells defective in DNA-PK are hypersensitive to X-irradiation preferentially during the G_1/early S phase of the cell cycle. Having shown that DNA-PK activity fluctuates during the cell cycle, we anticipated finding cell cycle-specific defects in cell lines known to be genetically defective in components of DNA-PK. Recently, it has been shown that DNA-PK$_{cs}$ is the product of the *scid* gene (9, 19, 28, 39), and it has long been known that animals homozygously mutant at this locus are X-ray sensitive (24) and defective in DNA DSB repair (7, 32). Thus, the X-ray sensitivity of cells homozygous for the *scid* mutation was examined during specific phases of the cell cycle. Asynchronously growing populations of *scid* (8D) or wild-type (300-19) pre-B cells (2, 33) were either left untreated or synchronized at the G_2/M transition with nocodazole. Cells were then assayed for their cell cycle positions every 2 h following release from the chemical block. Untreated cells showed an asynchronous distribution throughout the cell cycle, with the majority of the cells residing in G_1 (Fig. 3A). In contrast, at time 0, which was defined as the time when the drug had been washed out, both *scid* and wild-type cells were found almost exclusively in G_2/M (0 h) (Fig. 3A). At later time points, both *scid* and wild-type cells progressed through the cell cycle in a highly synchronized manner (2 to 10 h) (Fig. 3A).

Consequently, starting at time 0, identical aliquots of synchronized *scid* and wild-type cells were X-irradiated at 2-h intervals. This corresponded to X-irradiating cells at approximately the G_2/M (0 h), G_1 (2 h), G_1/early S (4 h), S (6 h), late S/G_2 (8 h), and G_2/M phases (10 h) of the cell cycle (Fig. 3B). Cell survival was determined 2 weeks later by measuring colony-forming efficiency (32, 43). Following exposure to 150 rads (1.5 Gy) of X rays, survival of the nocodazole-synchronized *scid* cells varied between 15 and 20% at all time points, except when they had been X-irradiated at 2 and 4 h after drug removal (Fig. 3B, panel II). At these time points, which corresponded to the G_1 and early S phases of the cell cycle,

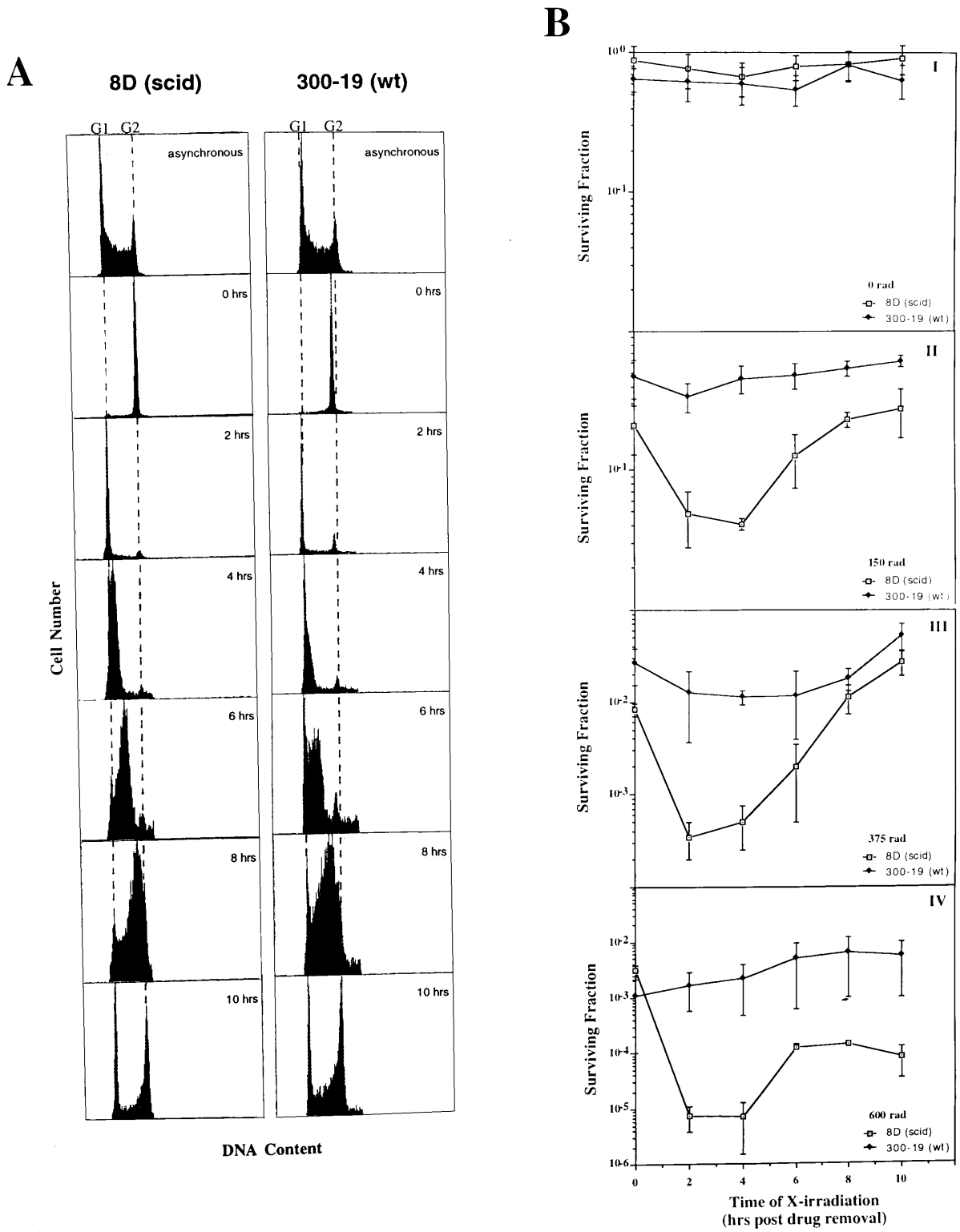

FIG. 3. Cells homozygous for the *scid* mutation are hypersensitive to X-irradiation during the G_1/S phase of the cell cycle. (A) Cell cycle distribution as assessed by flow cytometry of *scid* (8D) or wild-type (300-19) cells following nocodazole synchronization. Asynchronous populations of *scid* and wild-type cells were synchronized at the G_2 phase with nocodazole and then were analyzed for cell cycle position every 2 h after drug removal. (B) X-irradiation of synchronized *scid* cells reveals a G_1/S-phase hypersensitivity. Aliquots of the cells shown in panel A were X-irradiated at the indicated doses, plated by limiting dilution, and then scored for their ability to form colonies approximately 2 weeks postirradiation. The results shown are the averages of two to three experiments, each performed in duplicate. wt, wild type.

respectively, only 4 to 5% survival was seen (Fig. 3B, panel II). In contrast, 300-19 cells showed no such hypersensitivity during G_1 or early S phase; instead, survival ranged between 40 and 50% throughout the cell cycle (Fig. 3B, panel II). In addition, a control experiment, in which cell survival was assayed by using synchronized but unirradiated 8D and 300-19 cells, showed that both cell lines had nearly identical plating efficiencies and survival profiles that did not vary throughout the cell cycle, demonstrating that the nocodazole synchronization by itself did not induce any obvious cell cycle-specific lethality (Fig. 3B, panel I). Thus, it appeared that *scid* cells were hypersensitive to X-irradiation specifically in the G_1/early S phase of the cell cycle.

To confirm these results we repeated this experiment using increasing doses of X-irradiation. Importantly, the same pattern persisted, though the effect was more pronounced as the X-ray dose increased. For example, G_2 populations of *scid* cells exposed to 375 (3.75 Gy) and 600 (6.0 Gy) rads of X-irradiation showed survival profiles that were similar, although not identical, to those of G_2 populations of wild-type cells (Fig. 3B, panels III and IV). In striking contrast, G_1 populations of *scid* cells were 50- and 200-fold more X-ray hypersensitive than G_1 populations of wild-type cells following exposure to the same doses of X-irradiation (Fig. 3B, panels III and IV, respectively). In addition, in all cases the fraction of *scid* cells which survived X-irradiation increased dramatically when the cells were replicating or as the cells progressed into G_2 (Fig. 3B). From these experiments we concluded several things. First, wild-type murine pre-B cells do not exhibit hypersensitivity to X-irradiation during any phase of the cell cycle. Second, *scid* cells are particularly X-hypersensitive during the G_1/early S phase of the cell cycle. Third, at other phases of the cell cycle the survival of X-irradiated *scid* cells increased significantly but, in general, was still reduced from that of wild-type cells.

***scid* cells exhibit a reduced ability to carry out DNA DSB repair during the G_1 and early S phases.** It is well known that asynchronously growing *scid* cells are X-ray sensitive due to a reduced capacity for DNA DSB repair (7, 32). Therefore, we hypothesized that the X-ray hypersensitivity of *scid* cells in the G_1 and early S phases may have arisen from a reduced ability to carry out DNA DSB repair at those specific phases of the cell cycle. To test this experimentally, we examined DNA DSB repair activity throughout the cell cycle, using a PFGE assay that has been extensively described before (1, 26, 43, 68). Briefly, *scid* and wild-type pre-B cells were synchronized with nocodazole as shown in Fig. 3A. Every 2 h, an aliquot of each cell type was X-irradiated with 80 Gy, subsequently allowed to repair for 1 h, and then analyzed by PFGE. Under the conditions of this assay, smaller, broken DNA runs as a single band in the gel, while intact chromosomal DNA remains behind in the well (26, 43). Since the amount of broken DNA is directly proportional to the amount of unrepaired DSBs in the cell (1, 68), we were able to quantitate the amount of damage remaining after 1 h of repair during a specific phase of the cell cycle.

A distinctive cell cycle-dependent DNA DSB repair pattern was readily apparent. In *scid* cells, the percentage of DNA DSBs repaired was significantly reduced at the 2- and 4-h time points, which corresponded to the G_1 and early S phases, respectively (see Fig. 3A for comparison), while the repair rates throughout the rest of the cell cycle were almost indistinguishable from those of wild-type cells (Fig. 4). During the G_1 and early S phases, the DSB repair rate of *scid* cells was around 35%, compared to an average of 65% during the remainder of the cell cycle. In contrast, the DNA DSB repair rate of 300-19 cells did not vary extensively during the cell cycle (Fig. 4). Thus, *scid* cells appeared to be hypersensitive to X-irradiation during the G_1 and early S phases due to a reduced ability to carry out DNA DSB repair during these periods.

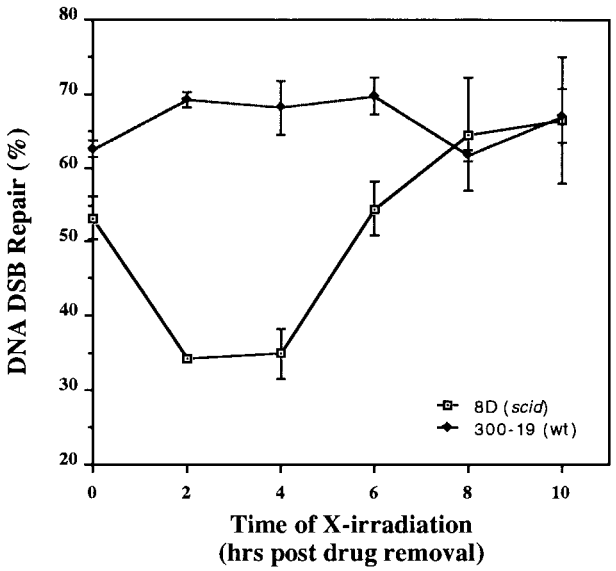

FIG. 4. The capacity of *scid* cells to repair DNA DSBs is greatly reduced during the G_1/S phase. Aliquots of cells synchronized as shown in Fig. 3A were X-irradiated with 80 Gy at the indicated times, and their ability to repair DNA DSBs was assessed by PFGE as described in Materials and Methods. The data are presented as the percentage of DNA DSBs repaired in 1 h. The results shown are the averages of three to four experiments, each performed in duplicate.

Cells defective in DNA-PK activity progress to the G_2 checkpoint and arrest following X-irradiation. The tight correlation between the reduced X-ray survival (Fig. 3B) and DNA DSB repair activity (Fig. 4) of *scid* cells during the G_1/early S phase of the cell cycle agreed very well with the G_1/early S phase-specific activation of DNA-PK (Fig. 1). However, the meaning of the G_2-specific activation of DNA-PK (Fig. 1) remained uncertain. Since *scid* cells were as proficient in DNA DSB repair as wild-type cells at G_2 in spite of their reduced survival following X-irradiation, we entertained the possibility that the *scid* gene product in particular, and DNA-PK in general, may have an additional or different role at G_2, which is nonetheless essential for cell survival following DNA damage. One such activity is G_2 cell cycle checkpoint control, since abnormalities in cell cycle checkpoints can also lead to X-ray hypersensitivity and reduced cell viability (74, 76; reviewed in reference 15).

Thus, asynchronous populations of *scid* (Fig. 5d) or wild-type (Fig. 5a) cells were X-irradiated with 5.0 Gy, and then their progression through the cell cycle was assessed by FACS analysis (Fig. 5). At 12 h postirradiation both *scid* (Fig. 5e) and wild-type (Fig. 5b) cells exhibited a normal G_2 arrest. In contrast, at 24 h postirradiation, *scid* cells were still arrested in G_2 (Fig. 5f), while the wild-type cells had resumed cycling (Fig. 5c). Additional experiments carried out with different doses of X-irradiation and for longer periods showed that the G_2 arrest of *scid* cells was invariably permanent (data not shown). Therefore, we concluded that *scid* cells exposed to DNA damage arrest normally at G_2 but are subsequently impaired or defective in their ability to resume cell cycling. Thus, DNA-PK may function in the exit from the G2 damage checkpoint control.

To extend the above observations, we repeated this experiment using the hamster cell line *sxi*-3, which is X-ray sensitive, defective in DNA DSB repair and lymphoid V(D)J recombination, and also defective in DNA-PK activity (43). Molecular analysis has shown that the phenotypes of *sxi*-3 cells are due to

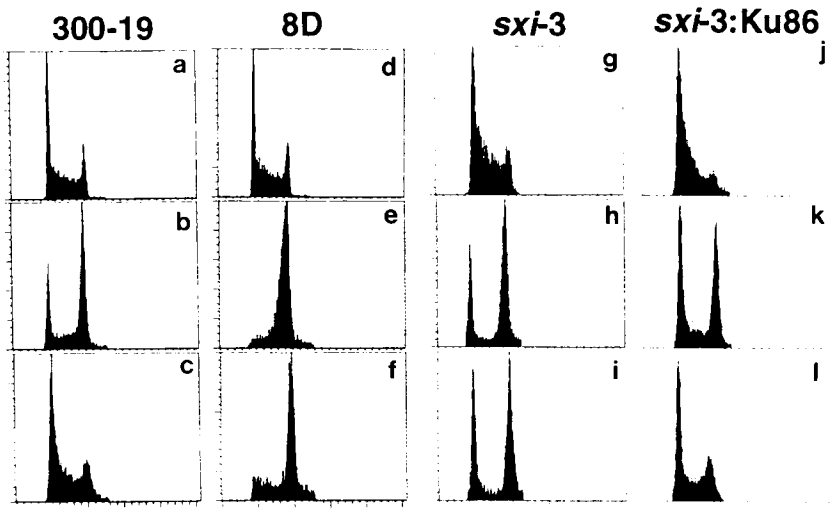

FIG. 5. Permanent G_2 arrest is a phenotype common to cells defective in DNA-PK activity. Asynchronous populations of the indicated cell lines were unirradiated (a, d, g, and j) or X-irradiated with 5 Gy, and the subsequent cell cycle distribution was determined by flow cytometry. Results at 12 (b, e, h, and k) and 24 h postirradiation (c, f, i, and l) are shown.

the lack of Ku86 gene expression (30). Twelve hours after X-irradiation of an asynchronous population, *sxi*-3 cells showed a cell cycle distribution profile similar to that of *scid* cells except for, interestingly, a much larger G_1-arrested population (Fig. 5h). Importantly, though, 24 h postirradiation, this profile was not altered (Fig. 5i). In contrast, a complemented *sxi*-3 cell line into which a functional hamster Ku86 gene had been reintroduced (*sxi*-3:Ku86) (30) resumed cell cycling by 24 h postirradiation (Fig. 5k and l). From these experiments we concluded that permanent G_2 arrest following DNA damage is a feature common to cell lines with defective or impaired DNA-PK activity.

DISCUSSION

Here we show for the first time that DNA-PK activity fluctuates in a cell cycle-dependent manner, being high at G_1/early S and again at G_2. In addition, by extension, we correlate the G_1/early S-phase DNA-PK activity in *scid* cells with increased X-ray hypersensitivity and reduced DSB repair ability. Importantly, the G_2-specific DNA-PK activity seemed to have no direct relationship with DNA DSB repair but instead appeared to be involved in the exit of cells from a checkpoint control following DNA damage.

Cell cycle-specific DNA DSB repair in *scid* cells—implications for different DNA DSB repair mechanisms. Wild-type murine pre-B cells did not exhibit any differences in DNA DSB repair activity during the cell cycle (Fig. 4). This observation is consistent with a report which showed that there were no differences in DNA DSB repair during the cell cycle when synchronized Ehrlich ascites tumor cells were X-irradiated (8) and with a recent report which showed that the repair of UV damage is cell cycle independent (61). In contrast, cells homozygous for the *scid* mutation showed a pronounced cell cycle variation in their capacity to repair DSBs. DNA DSB repair activity in these cells was greatly reduced during G_1/early S but was indistinguishable from that in wild-type cells during the late S and G_2 phases of the cell cycle (Fig. 4). This observation agrees well with an earlier study which found increased DNA DSB repair activity during G_2 in *xrs*5 cells (50), which are defective in Ku86 (38, 79). Together, these studies have important implications for the mechanism(s) of DNA DSB repair and suggest that there is a DNA-PK-dependent pathway which is active in G_1/early S and a DNA-PK-independent pathway(s) active in late S/G_2.

One possibility is that DNA-PK is involved primarily in nonhomologous (illegitimate) recombinational repair, where the ends of a DSB are joined together regardless of the sequences at the two ends, and that this type of DNA repair occurs primarily during G_1/early S. By extension then, late S/G2-phase DNA DSB repair would involve predominately homologous recombination, which requires a template of DNA for gene copying and repair (71). This model fits well with the physical constraints of the cell cycle in that during the G_1 and early S phases, the ploidy of the cell is merely 2n, and with one of two alleles already damaged, repair via illegitimate recombination might be more efficient. As the cells progress into the S and G_2 phases and the ploidy increases to 4n, homologous recombination may become the preferred mechanism. Indeed, there is abundant evidence that in yeast, sister chromatids (G_2-specific) undergo homologous recombinational repair much more readily than homologs (37).

Alternatively, we favor a model in which there exist two illegitimate recombinational repair pathways in mammals, one of them being DNA-PK dependent and restricted to the G_1/early S phase of the cell cycle and the other being DNA-PK independent and restricted to the late S/G_2 phase of the cell cycle. There is a precedent for such a hypothesis. In *Saccharomyces cerevisiae*, it has already been demonstrated that two pathways of nonhomologous end joining (i.e., illegitimate recombinational repair) exist. One of these pathways predominates at G_1 and proceeds primarily with associated deletions in the repaired products, whereas the other pathway is restricted to late S/G_2 and proceeds primarily with associated insertions (56). The G_2 pathway appears to be controlled by the genes *RAD50*, *XRS2*, and *MRE11* (56), and while the genes responsible for the G_1-specific pathway have not been identified, it is known that mutations in the *S. cerevisiae* genes homologous to those encoding Ku70 (12, 49, 66) and Ku86 (53) deleteriously affect illegitimate recombinational repair. In addition, given that mutations in the DNA-PK genes in mammals give rise to repair events where the products contain associated deletions

(reviewed in references 31 and 36), it is very tempting to speculate that the G_1-specific illegitimate recombinational repair is mediated by DNA-PK. Thus, mammalian DNA DSB repair may be mechanistically very similar to yeast DNA DSB repair, as has already been demonstrated for mismatch repair and excision repair (for reviews, see references 55 and 63). A corollary to this hypothesis is that homologous recombination and repair are DNA-PK independent and not restricted in the cell cycle. This may explain why *scid* cells exhibit residual DNA DSB repair activity during the G_1/early S phase (Fig. 4).

DNA-PK as a cell cycle-dependent kinase. DNA-PK activity is regulated in a cell cycle-dependent manner (Fig. 1). The G_1/early S phase-specific activation of DNA-PK correlated very well with the X-ray hypersensitivity (Fig. 3) and deficient DSB repair (Fig. 4) profiles of *scid* cells. These experiments thus support the notion that DNA-PK activity is required for DNA DSB repair during the G_1/early S phase. Moreover, mutations which deleteriously affect components of the DNA-PK complex also affect V(D)J recombination, and it has been suggested that V(D)J recombination occurs primarily during G_1 (48, 65). Thus, DNA-PK-dependent DNA DSB repair appears to be required for proper V(D)J recombination, and our data strongly suggest that this process is likely to occur primarily during the G_1/early S phase.

The function of DNA-PK activity in DNA DSB repair at the G_1/early S phase is still unclear. Most likely, DNA-PK phosphorylates some component(s) in the repair complex to induce DSB repair or signals the repair machinery to enrich where the DNA DSB is located. For example, it has been shown that DNA repair proteins RP-A (13, 60) and p53 (44) are substrates for DNA-PK phosphorylation. In addition, it has been proposed that the ATPase activity of Ku protein is up-regulated following phosphorylation by DNA-PK (14). Alternatively, DNA-PK may merely be involved in some indirect role, such as transcription inhibition, as it has been shown that DNA-PK can inhibit RNA polymerase I-mediated transcription (41). The development of a mammalian in vitro DNA DSB repair system should help illuminate the function of DNA-PK in DSB repair.

It is interesting, though perhaps not surprising, that the physical amounts of the DNA-PK subunits did not show any fluctuation during the cell cycle (Fig. 2). DNA-PK_{cs} and the Ku subunits are very abundant proteins (23, 54), and it is unlikely that their function is regulated at the protein level. In corroboration of this, we have also observed that Ku DNA end-binding activity, as defined by gel shift assays (62), was constant throughout the cell cycle (data not shown). Therefore, it is likely that posttranslational modification or subcellular compartmentalization of DNA-PK subunits during the cell cycle regulates DNA-PK activity (70). It has been demonstrated that Ku or DNA-PK_{cs} is autophosphorylated by DNA-PK in vitro (11, 16, 17, 44). Moreover, autophosphorylation of DNA-PK_{cs} appears to be inhibitory to its activity (16, 17, 70). Thus, the phosphorylation status of DNA-PK_{cs} and/or Ku may be regulated in a cell cycle-specific manner, and it will be interesting to test whether a cyclin-dependent kinase (reviewed in reference 57) is involved in the regulation of DNA-PK activity. Alternatively, DNA-PK activity may be regulated by sequestration, as Ku proteins are known to undergo subcellular compartmentalization during cell proliferation (78). Lastly, it is also possible that DNA-PK activity is regulated by cell cycle-specific proteolysis, as we have recently shown that site-specific proteolysis of Ku86 can inactivate the DNA-PK complex (27).

Cell cycle-specific X-ray sensitivity in *scid* cells. Cells homozygous for the murine *scid* mutation are hypersensitive to X-irradiation during the G_1/early S phase of the cell cycle (Fig. 3). This sensitivity was observed at multiple X-ray doses and in cultures that had been synchronized by several different growth arrest agents (e.g., hydroxyurea and mimosine) (data not shown). Genetically, *scid* cells belong to X-ray cross complementation group 7 (XRCC7) (79). Importantly, the hamster cell line V3 also belongs to this complementation group (72). Consistent with our observations, when V3 X-ray survival was assayed throughout the cell cycle, it was also found to be most sensitive during G_1 (77). In addition, the mutant *xrs*, which belongs to the XRCC5 (Ku86) complemetation group, also exhibited G_1/early S phase-specific X-ray hypersensitivity (35, 77). Lastly, another hamster cell line, XR-1, which belongs to the XRCC4 complementation group, displays DNA DSB repair and V(D)J recombination phenotypes nearly indistinguishable from those of XRCC5 and XRCC7 cell lines (69, 73), and though it is not known to be physically associated with the DNA-PK complex, it too is extremely sensitive to X-irradiation and shows a dramatically reduced ability to repair DSBs in the G_1/early S phase (25, 69). Thus, it seems very likely that the XRCC4 (XR-1), XRCC5 (Ku86), and XRCC7 (DNA-PK_{cs}) gene products are involved in the same pathway, which is most active in the G_1/early S phase of the cell cycle and which has overlapping functions in DNA DSB repair and V(D)J recombination.

A cell cycle checkpoint progression function for DNA-PK? Interestingly, we observed a second peak of DNA-PK activity during the G_2 phase of the cell cycle in wild-type cells (Fig. 1). Since *scid* cells showed a capacity for DSB repair at G_2 indistinguishable from that of wild-type cells (Fig. 4) in spite of reduced cell survival following X-ray irradiation (Fig. 3), we reasoned that the *scid* gene product may perform a second role at G_2 which is essential for cell survival following DNA damage but different from DSB repair. Since defects in a cell cycle checkpoint(s) can affect X-ray sensitivity and survival (reviewed in reference 29), we examined the cell cycle checkpoints in cells homozygous for the *scid* mutation following X-irradiation. X-irradiated *scid* cells progressed to G_2, where they were arrested (Fig. 5). Thus, the G_2 cell cycle checkpoint is functional in *scid* cells, a finding consistent with work from other laboratories (34). However, *scid* cells remained permanently arrested in G_2 before finally undergoing necrosis (Fig. 5 and data not shown). In addition, X-irradiated *sxi*-3 cells also were arrested permanently at the G_2 checkpoint (Fig. 5) (26). Since *sxi*-3 cells contain null DNA-PK activity due to a defect in Ku86 gene expression (30), permanent G_2 arrest may be a feature common to cells defective in DNA-PK (80). This conclusion is supported by recent data obtained with Ku86 knockout mice. Cells from $Ku86^{-/-}$ animals did not reenter the cell cycle following X-irradiation (59). Together, these observations suggest that DNA-PK may provide an activity, distinct from its activity required for DNA DSB repair, that is necessary for DNA-damaged cells to traverse a G_2 checkpoint.

DNA-PK is clearly required for the efficient rejoining of broken chromosomes following DNA damage (7, 32, 38, 43, 80). Consequently, it has been reasonably argued that DNA-PK_{cs} or Ku mutant cells are arrested in G_2 not because DNA-PK activity is needed to progress through the G_2 checkpoint but simply because the cells arrive at that checkpoint with a significant amount of unrepaired DNA damage (42, 58). However, it has been demonstrated in yeast that the existence of unrepaired DNA DSBs at G_2 is not always sufficient to cause cell cycle arrest (64). In addition, *scid* cells show a strong G_2 arrest even after very low doses of X-irradiation, where the level of DNA DSBs, and hence the requirement for DNA DSB repair, should be minimal (34). Lastly, the G_2 peak of DNA-PK activity (Fig. 1) appears unrelated to DNA repair

(Fig. 4). Together, these observations argue that DNA PK may have (at least) two roles in mammalian cells: a function required for DNA DSB repair and a second function required for G_2 checkpoint transition following DNA damage.

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