physically mapped on chromosome 8. We tested this novel marker through our radiation hybrid panel. The location of D8S359 is undistinguishable in our panel from D8S531 and D8S519. All three markers are contained on YAC 943g4, which we map by FISH to chromosome 8q11.1-11.2. We conclude that D8S359 and CEBPδ map to 8q11 in the same region as p350.

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35. For Western blot analysis, cell lysates were prepared by scraping cells from confluent, 100-mm^2 dishes, washing the cells three times in phosphate-buffered saline and freezing the dry cell pellet at −70°C. The pellet was resuspended in 150 μl of buffer containing 50 mM potassium phosphate, 1 mM EDTA and the following protease inhibitors: antipain (10 μg/ml), leupeptin (10 μg/ml), chymostatin (10 μg/ml), aprotinin (0.2 U/ml), pepstatin (10 μg/ml), capsic acid (2 mM), and phenylmethylsulfonylfluoride (350 μg/ml). It was then sonicated twice on ice for 20 s and frozen, followed by thawing and sonication for another 15 s. Unlysed cells and debris were removed by brief spinnng (30 to 60 s) at maximum speed in an Eppendorf centrifuge. Protein concentration was measured by a modification of the Bradford essay (*36*) (Bio-Rad). A total of 75 μg of protein was loaded in each lane, and electrophoresis was performed through a 5% denaturing SDS-polyacrylamide gel. Transfer of the proteins was by wet electrotransfer at constant 750 mAmp for 2 hours in ET buffer (25 mM tris; 192 mM glycine in 20% methanol) under cooling. Immunoblots were treated as previously described (*21*) except that the primary antibody incubation was at 4°C overnight. The secondary antibody was an alkaline phosphatase–conjugated goat anti-mouse IgG (Bio-Rad). Color development was in 0.45 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.27 mM nitroblue tetrazolium.
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39. For PCR, DNA was isolated by a salting out procedure (*37*). DNA (300 ng) was amplified in 50-μl reactions with Amplitaq (Perkin-Elmer Cetus) or with Taq polymerase (Promega) according to the manufacturer's instructions in a DNA Thermal Cycler (Perkin-Elmer Cetus). An initial denaturation of 2 min at 95°C was followed by 30 to 35 cycles of amplification for 1 min at 95°C, 1 min at 55°C, 2 min at 72°C and a final extension of 7 min at 72°C. PCR primer (40 picomoles per reaction) specific for chromosome 8 loci were obtained from Research Genetics; the centromere primers were synthesized (*13*); the primer sequences for LPL were 5′ GGAGAGCTGATCTCTATAAC and 5′AAGCTCTCCCTGAATTGTGA (amplifying a 330-bp product); the primers for POLB were 5′CTGGTTGTCAGTCCTGCCG and 5′TCGATTCTTGCTTTTTCCCG (amplifying a 350-bp product). Portions (20 μl) of the reaction product were separated by electrophoresis on a 1.5% agarose gel in TAE (0.4 M tris-Cl; 0.013 M sodium acetate; 0.2 mM EDTA, pH 8.0) and stained with ethidium bromide to visualize the amplification products.
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41. The authors thank L. Tosto, P. van Zijl, and T. McAffee for excellent technical assistance and A. Giaccia, M. Fox, J. Elwell, and J. Derry for valuable discussions. We wish to express our gratitude to M. Wagner, S. Wood, and S. Daiger for sharing their chromosome 8 knowledge; to T. Stamato and R. Fishel for helpful discussions; to L. Rowe and E. Birkenmeier for sharing unpublished information with us; and to J. Hesse and M. Gellert for supplying the V(D)J recombination substrates pJH200 and pJH290. Thanks to M. Bosma for his insightful comments and generosity. This work was supported by PHS grants CA 15201 (to J.M.B.), CA 37761 (to T.C.) and NIH GM48026 and PEW Scholar's Program (to M.A.O.).

21 December 1994; accepted 2 February 1995

Absence of p350 Subunit of DNA-Activated Protein Kinase from a Radiosensitive Human Cell Line

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The radiosensitive rodent mutant cell line *xrs-5* is defective in DNA double-strand break repair and lacks the Ku component of the DNA-activated protein kinase, DNA-PK. Here radiosensitive human cell lines were analyzed for DNA-PK activity and for the presence of related proteins. The radiosensitive human malignant glioma M059J cell line was found to be defective in DNA double-strand break repair, but fails to express the p350 subunit of DNA-PK. These results suggest that DNA-PK kinase activity is involved in DNA double-strand break repair.

DNA double-strand breaks (dsb) are induced by x-rays and by oxidative metabolism, and their repair is essential to cell survival. Several radiosensitive mutant cell lines have been identified in mammalian cells that are defective both in DNA dsb repair and in V(D)J recombination (*1*). V(D)J recombination is required for the assembly of antigen receptor gene segments and requires both lymphocyte-specific genes and general DNA repair activities. However, little is known about the genes and gene products involved in the DNA dsb repair process itself. Ku, the DNA targeting subunit of DNA-activated protein kinase, DNA-PK, may function in both DNA dsb repair and V(D)J recombination.

DNA-PK is a serine-threonine protein kinase that requires double-stranded DNA for activity (*2*–*6*). DNA-PK phosphorylates many protein substrates in vitro including the 90-kD heat-shock protein (hsp90), Sp1, SV40 T antigen, the tumor suppressor protein p53, serum response factor (SRF), fos, jun, and the CTD of RNA polymerase II (*6*–*8*). DNA-PK has been purified and characterized from HeLa cells (2, 3, 6), Raji Burkitt's lymphoma cells (*5*), and human placenta (*9*), and consists of at least two protein components; a large polypeptide of approximately 350,000 daltons (p350) and the Ku autoantigen. The p350 component binds adenosine triphosphate (ATP) analogs (*2, 3*), and is thought to contain the kinase domain of DNA-PK. Ku antigen is a dimer of 70- and 80-kD subunits, which are recognized by antibodies from sera of patients with auto-immune disorders including scleroderma-polymyositis (*10*). Ku interacts with the termini of double-stranded DNA (*11*) and by this means is thought to provide the DNA targeting required by DNA-PK.

The biochemical properties of both Ku and DNA-PK suggest it has a role in DNA damage detection or in repair or in both processes (*12*). The radiosensitive rodent cell line, *xrs-5*, lacks a DNA end binding activity that is characteristic of Ku and is defective in DNA dsb repair and in V(D)J recombination pathways (*13*–*15*). Transfection of *xrs* cells with the human Ku p80 gene was shown to restore partial radio resistance to these cells (*15, 16*). The human XRCC5 was identified as Ku p80 (*15*). These data suggest a role for Ku in dsb repair and V(D)J recombination. However, although DNA end binding activity like that of Ku occurs in rodent cells (*13*–*16*), simian cell lines (*17*), *Drosophila* (*18*), and yeast (*19*), little is known about DNA-PK activity in these organisms. We previously did not detect DNA-PK activity in cell lines from nonprimates using either a synthetic peptide or the protein hsp90 as substrate (*4*).

To date, only few human cell lines with radiosensitivities similar to those of the mutant rodent *xrs-5* cells have been characterized. Among these are fibroblast strains belonging to several ataxia telangiectasia complementation groups. Whereas ataxia telangiectasia cells are extremely sensitive to ionizing radiation, the underlying basis of this radiosensitivity is thought to involve misrepair of dsb rather than a reduced ability to rejoin dsb (*20*). Another radiosensitive human cell line is M059J. The M059J and M059K cell lines were isolated from different portions of the same human malignant glioma biopsy specimen. Whereas M059K cells are relatively radioresistant, M059J cells are ~10-fold more sensitive, with a radiosensitivity similar to that of *xrs-5* cells (*21*). We have examined the

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ability of M059J cells to repair DNA dsb using pulsed field gel electrophoresis (*22*) and find that, like *xrs-5* cells, M059J cells are deficient in dsb rejoining (Fig. 1).

Because the Ku results suggested that DNA-PK activity plays a role in DNA dsb repair, we examined radioresistant and radiosensitive human cell lines for DNA-PK activity and for the expression of Ku and p350 proteins (*23*). A549 (lung carcinoma), GM0637A (SV40 transformed normal fibroblast), and M059K (human malignant glioma) are relatively radioresistant, whereas GM5849 (SV40 transformed ataxia telangiectasia fibroblasts, complementation group D) and M059J (human malignant glioma) are radiosensitive. High levels of DNA-PK activity were seen in the A549, GM5849, GM0637A, and M059K cell extracts (Table 1). However extracts from the M059J cells contained no detectable DNA-PK activity (Table 1). DNA-PK activity was present in extracts from SV40 transformed ataxia telangiectasia cells (GM 5849), suggesting that in these cells the primary defect does not involve DNA-PK. We also assayed the CHO wild-type (radioresistant) and *xrs-5* (radiosensitive) cells for DNA-PK activity; however, consistent with our previous observations, little or no activity was observed (Table 1). To determine whether induction of DNA-PK is an early response to ionizing radiation, cells from rodent and human sources described above were irradiated with 5-Gy γ-rays, harvested after 15 min, and assayed for DNA-PK. No significant changes were observed in either DNA-PK activity or protein.

The same cell extracts were analyzed by protein immunoblotting. Both p350 and Ku were present in normal amounts in A549, GM5849, and GM0637A extracts (*24*). However, whereas p350 was readily detected in the M059K cell extracts, no p350 polypeptide was detected in the M059J extracts (Fig. 2). When M059J and M059K extracts were probed with antibodies to human Ku antigen, roughly equivalent amounts of the polypeptides corresponding to both p70 and p80 Ku were observed (Fig. 2).

The p350 and Ku components of DNA-PK can be fractionated biochemically and separately purified to homogeneity. Individually, the two components have little or no DNA-PK activity. However, full kinase activity is restored when the highly purified components are recombined (*6*, *9*). We therefore determined whether addition of highly purified DNA-PK components could restore kinase activity in the M059J extracts. Whereas the addition of highly purified Ku did not restore kinase activity, addition of p350 caused a large increase in DNA-PK activity in the M059J cell extracts (Fig. 3).

To study the genetic basis for the absence of DNA-PK in M059J cells, Southern (DNA) blots were prepared from M059J and K cells, and probed with a fragment (DPK1) of the complementary DNA (cDNA) that encodes the p350 polypeptide. These results (Fig. 4A) suggest that the absence of DNA-PK activity in M059J cells is not the result of the deletion of the entire p350 locus. On the

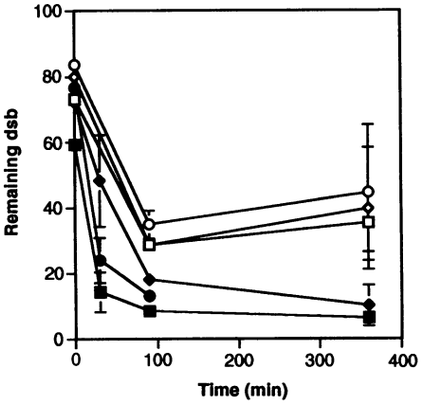

Fig. 1. The percentage of double strand breaks remaining in irradiated M059J (open symbols) and M059K (closed symbols) at repair intervals of 30 min, 90 min, and 4 hours after irradiation is shown. Squares, 20 Gy; diamonds, 30 Gy; circles, 50 Gy. Mean values from two of more experiments are shown; error bars, +/− standard error of the mean of three or more values.

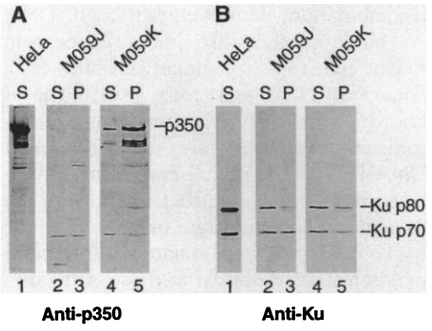

Fig. 2. Protein immunoblot analysis of M059J and K cell extracts: Crude extract (10 μg of total protein) was run on 10% acrylamide SDS-PAGE and transferred to nitrocellulose as described (*9*). The ECL method (Amersham) was employed for detection according to the manufacturer's instructions. Lane 1, HeLa S10 (cytoplasmic extract); lane 2, M059J S10; lane 3, M059J P10 (nuclear extract); lane 4, M059K S10; lane 5, M059K P10. The blots were probed with antibodies to p350 (**A**) (DPK1) or a human autoimmune serum 5888 (**B**) that contains antibodies to Ku as indicated. Only traces of p350 cross-reacting proteins were observed when 20 μg of crude extracts was loaded on the gels, or when a second antibody to p350 (C8-11) that was raised to a different region of the p350 cDNA was used (*35*).

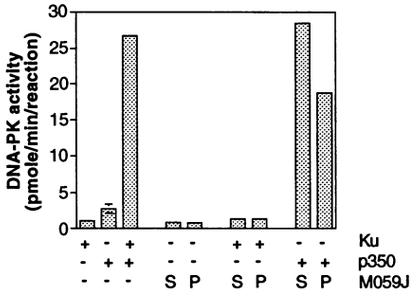

Fig. 3. Reconstitution of DNA-PK activity in M059J extracts by the addition of purified p350 protein. p350 and Ku were purified to homogeneity from human placenta and assayed as described (*9*). Where indicated reactions contained 0.06 μg of Ku; 0.06 μg of p350; 4.5 μg of protein (2 μl) from the cytoplasmic fraction of M059J extract (S); and 1.8 μg of protein (2 μl) from the nuclear fraction of M059J extract (P). Results are the average of duplicate assays. In most assays, the standard error of the mean was <0.1 activity units and was thus below the limits of resolution.

Table 1. DNA-PK activity in human and rodent cells lines. Extracts were prepared and assayed as described (*23*). Values are averages of duplicate assays.

Cell line	DNA-PK activity (nmol/min/mg)	
	Control	Irradiated
A549	4.67	4.65
GM0637A	5.82	5.71
GM5849*	4.13	3.94
M059K	6.27	5.01
M059J*	0.03	0.01
CHO	0.22	0.25
*xrs-5**	0.19	0.03

*Radiosensitive lines are indicated by an asterisk. †Cells were irradiated (5 Gy) and harvested 15 min later.

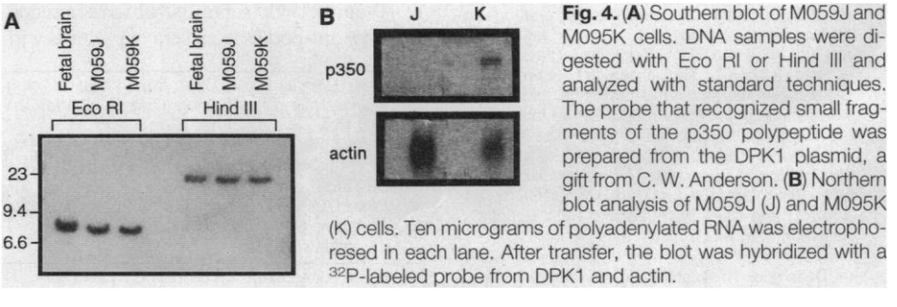

Fig. 4. (**A**) Southern blot of M059J and M095K cells. DNA samples were digested with Eco RI or Hind III and analyzed with standard techniques. The probe that recognized small fragments of the p350 polypeptide was prepared from the DPK1 plasmid, a gift from C. W. Anderson. (**B**) Northern blot analysis of M059J (J) and M095K (K) cells. Ten micrograms of polyadenylated RNA was electrophoresed in each lane. After transfer, the blot was hybridized with a ^{32}P-labeled probe from DPK1 and actin.

other hand, when the same probe was used for Northern (RNA) blot analysis of M059J and M059K samples, a transcript that hybridized to the p350 probe was clearly seen in M059K samples, but not in M059J (Fig. 4B).

Preliminary fluorescence in situ hybridization (FISH) studies suggest that M059J cells contain defects in human chromosome 8 which involve deletion or rearrangements or both (*25*). In contrast, no chromosome 8–associated abnormalities were observed in M059K cells. The p350 gene has been mapped to human chromosome 8 (*26*) at a region near the centromere (*27*). Defects in chromosome 8 in the region of the centromere have also been associated with the human equivalent of the mouse SCID gene (*28*). In mice, the SCID mutation confers hypersensitivity to ionizing radiation and deficiencies in both DNA dsb repair and V(D)J recombination (*29*).

We have assayed for DNA-PK in human cells lines with normal and increased sensitivity to the effects of ionizing radiation. The M059J cell line is a very radiosensitive cell line derived from a human malignant glioma. Extracts from these cells lack both detectable DNA-PK activity and p350 protein. In contrast, the Ku component of DNA-PK is unaffected in the M059J cell extracts. Thus, the addition of the p350 component alone, but not Ku, restored DNA-PK activity to normal levels in these extracts. We did not detect a p350 transcript in M059J cells, which suggests that the p350 defect was at the transcriptional level, or the result of a decrease in transcript stability. Further analysis of the p350 gene will be required in order to determine whether widespread disruption of the gene is responsible for the lack of p350 transcript in M059J. Such experiments will be possible when more of the p350 gene sequence is elucidated. To date, we have found that a >15 kb fragment that hybridizes to an internal portion of the gene is normal in M059J and M059K, indicating that the entire gene is not deleted (*30*).

Radiosensitive, dsb repair deficient rodent cell lines lack Ku (*13–16*). In contrast, our data show that the radiosensitive, dsb repair deficient human cell line M059J lacks the p350 component of DNA-PK. Together, these results suggest that it is DNA-PK activity which plays an important role in DNA damage repair.

Recent evidence has strongly linked the Ku component of DNA-PK with a role in DNA dsb repair (*13–16*). In addition, Ku has recently been identified as human DNA helicase II (*31*). Because Ku is known to bind to ends of DNA with high affinity, we can predict that upon the production of a DNA lesion, Ku binds to the free double-stranded DNA termini. The assembly of active DNA-PK on DNA is an ordered process in which Ku first binds to the DNA, followed by recruitment of p350 to the complex (*32*), which results in production of the active kinase complex. Once attached to DNA termini, Ku molecules translocate along the DNA in an ATP-independent manner (*33*). In vitro, DNA-PK phosphorylates a large number of DNA-binding proteins. It has also been suggested that some DNA-PK substrates, for example, p53, are phosphorylated more efficiently when bound to DNA (*8, 26*). Taking these data together we can propose a model in which the active kinase complex might translocate along the DNA to a suitable DNA bound substrate, for example, a protein complex associated with DNA repair, recombination, replication, or transcription. The challenge is to identify the critical DNA-PK substrates in vivo, and to determine the effects of phosphorylation on their activities.

Note added in proof: Recently, Finnie *et al.* (*34*) have shown that DNA-PK activity is present in cultured cells from rodent, *Xenopus*, and *Drosophila* but is approximately 50 times less abundant than in human cells.

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23. Cell extracts were prepared as described (*4*) with the following modifications. PMSF was added to both cytoplasmic (S10) and nuclear (P10) fractions to 0.5 mM, and the high salt wash (P10) buffer contained 10 mM magnesium chloride in addition to 0.5 M KCl. DNA-PK activity was assayed as described (*4, 8*), with a synthetic peptide corresponding to an amino-terminal region of human p53 (EPPLSQEAFADWLKK) at 0.25 mM. Sonicated calf thymus DNA was present as activator at 10 μg/ml. Reactions were for 6 to 8 min at 30°C. Protein concentrations were determined using the Bio-Rad protein assay using bovine serum albumin as standard. DNA-PK activity is expressed as nanomoles of phosphate transferred per minute in S10 and P10 fractions combined/mg total protein in S10 and P10 combined.
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36. We thank C. W. Anderson and S. P. Jackson for DPK1 and C8-11 plasmids, M. Fritzler for autoimmune antisera #5888, and J. Lee for technical assistance. This research was funded by grants from the National Science and Engineering Council (Canada), the Alberta Heritage Foundation for Medical Research, and the National Cancer Institute of Canada. S.P.L.-M. is a recipient of an NSERC Women's Faculty Award.

9 December 1994; accepted 3 February 1995