DISSERTATION

Phenotypic and Molecular Changes in Normal Human Cells Following Knockdown of DNA-PKcs by RNA Interference

Submitted by

Yuanlin Peng

Department of Environmental and Radiological Health Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2004

COLORADO STATE UNIVERSITY

March 12, 2004

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY YUANLIN PENG ENTITLED PHENOTYPIC AND MOLECULAR CHANGES IN NORMAL HUMAN CELLS FOLLOWING KNOCK DOWN OF DNA-PKCS BY RNA INTERFERENCE BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

James R. Bamburg

Martin F. Lavin

Ryuichi Okayasu

Robert L. Ullrich

Adviser Joel S. Bedford

Department Head John D. Zimbrick

ABSTRACT OF DISSERTATION

PHENOTYPIC AND MOLECULAR CHANGES IN NORMAL HUMAN CELLS FOLLOWING KNOCKDOWN OF DNA-PKcs BY RNA INTERFERENCE

A procedure based on the newly discovered RNA interference (RNAi) phenomenon was used to study the genetic control of cellular radiation responses in normal human cells. The aim was to study molecular and phenotypic effects in cultured normal human cells following knockdown of a single gene, *Prkdc* (DNA dependent protein kinase catalytic subunit), with small interfering double stranded RNAs (siRNAs). *Prkdc* is a key component of the non-homologous end joining process involved in rejoining DNA double strand breaks. This would enable the comparison of a single change on human cells with otherwise identical genetic backgrounds. The mRNA and protein products were successfully knocked down by transfection of cells with either of two siRNA sequences applied in nanomolar concentrations. The knockdown resulted in radiosensitization for cell killing, chromosomal aberration induction, and reduced the rejoining of interphase chromosome breaks after irradiation. It also resulted in a later reduction of ATM (mutated in Ataxia-Telangiectasia). *Prkdc* mRNA was reduced within a few hours after anti-*Prkdc* siRNA transfection, and DNA-PKcs protein began to decrease about 1.5 to 2 days later. Only after the DNA-PKcs had decreased did the *ATM* mRNA level drop, followed by a drop in the ATM protein starting at about 3.5 days. The mRNA and protein returned to pre-transfection levels at later sampling times since the transfections are transient. Control siRNA transfections using siRNAs with a slightly altered sequence did not produce any such molecular or phenotypic changes. No evidence

was seen of the induction of an interferon response. Reduced ATM protein was also observed in a *Prkdc* mutant hamster cell line, and the ATM deficiency was restored in a line stably transfected with a human *Prkdc*-containing vector. Another hamster line with reduced (but kinase-dead) DNA-PKcs did not display reduced ATM.

This is the first report of a phenotypic change, following knockdown of any DNA repair protein by RNA interference, and the first study showing, directly, that the levels of DNA-PKcs can influence the levels of ATM which in turn control a vast network of DNA damage checkpoint responses, apoptosis, and homology directed repair of DNA double strand breaks.

Yuanlin Peng
Department of Environmental and Radiological Health Sciences
Colorado State University
Fort Collins, Colorado 80523
Spring 2004

ACKNOWLEDGEMENTS

I would like to acknowledge many people for helping me throughout my doctoral study. First and foremost I must thank my advisor, Dr. Joel Bedford, for being such a pleasure to work with. Throughout my study he encouraged me to develop both my independent thinking and research skills. He has always been supportive concerning my career and a better advisor couldn't be found.

I am also very grateful for having an exceptional doctoral committee and wish to thank Dr. James Bamburg, Dr. Robert Ullrich, Dr. Ryuichi Okayasu, Dr. Martin Lavin, Dr. Micheal Fox and Dr. Charles Waldren. Special thanks to Dr. Hatsumi Nagasawa, my unofficial committee member, who has been both my friend and mentor. Everything I know about the Radiation Research Society I learned from Hatsumi.

I wish to extend many thanks to my colleagues and friends. I have rarely seen such an enjoyable group of smart and kind people who work together so well. I would like to thank my colleague Paul Wilson for helpful answers to my questions and great conversation, Dr. Maria Muhlmann-Diaz for teaching me chromosome microdissection, and Dr. Mohammed Al-Subael for his help when I arrived at CSU. My colleagues Mitra Jha, Brant Ulsh, Larry Dugan, Wendy Kuhne and Takamitsu Kato have also been marvelous companions. I would also like to thank Anna Montgomery for all the paper work involved in ordering supplies and reagents for me, Wei Zou for teaching me to use the Storm scanner, Dr. Farzan Siddiqui and Jennifer Malone for helping me get started with real-time RT-PCR.

In addition, I would like to thank the many Bedford lab assistants including Kevin Collin, Don Young, Christy Warner, and Zane Story. My special thanks also go to Sandy Wiggen, Julie Asmus, Norma Bulera for their administrative assistances.

Finally, I would like to thank my family. I am grateful to my parents for their support and care over the years, my sisters for their encouragement and enthusiasm, and my wife, Ming, and my daughter, Sydney, for keeping my life in proper perspective and balance.

This work was supported in part by grants DE-FG-03-01 ER63235 from the U.S. Department of Energy, and grants NAG9-1516 and NAG9-1569 from the U.S. National Aeronautics and Space Administration.

Table of Contents

Chapter 1

INTRODUCTION

Early phenomenological studies concerning the actions of radiations on living cells produced considerable knowledge of both fundamental and practical importance. While there are many beneficial medical and industrial uses of radiation, some radiation effects are detrimental and of general concern because of the potential hazards they pose to human health following accidental or deliberate exposures. Notable examples of radiobiological effects of special concern include the discoveries that ionizing radiations produce gene mutations (Muller 1927; Stadler 1928b), and are carcinogenic (Stadler 1928a; Goodspeed and Olson 1928) . In fact, not only was radiation the first known mutagenic agent, but it was also the first known carcinogenic agent.

Investigations into basic mechanisms underlying radiation effects principally dealt with broad characterizations that built the framework on which modern molecular approaches to understanding radiation action are based. Among the most important basic discoveries from radiation studies was that cells are able to process and repair damage to DNA and chromatin (eg. Sax K. 1938; Kelner 1949; Setlow *et al.* 1963; McGrath and Williams 1966; Cleaver 1968; Taylor *et al.* 1975).

These and many other studies helped to identify molecular targets whose damage is responsible for biological effects, or systems that may be involved in damage repair or other processing systems that govern radiosensitivity.

Biochemical Pathways and Networks Governing Cellular Radiation Responses

Arguably the most powerful and informative of all approaches for understanding biochemical pathways or processes, including biological actions of radiations, has involved studies of their genetic control. This "genetic approach" depends on the availability and means for studying and characterizing mutants with altered phenotypic properties for the characteristic of interest. For example, the first studies establishing gene-enzyme relationships, that initiated what became known as "biochemical genetics", originated with the isolation of radiation-induced *Neurospora* mutants affecting nutritional requirements (Beadle and Tatum 1941; Beadle and Tatum 1945). Isolation of mutants in microorganisms is facilitated by the availability of haploid cells, and much of our early knowledge of the genetic control of radiosensitivity derived from studies of radiosensitive mutants of bacteria or yeast. Extension of the genetic approach for similar studies in higher eukaryotic systems has been much more difficult; principally because the production and isolation of radiosensitive mutants arising from loss of gene function (homozygous recessives) in the laboratory is much more difficult in diploid cells.

Much has been learned by the study of cells from individuals with naturally occurring homozygous recessive mutations involving genes that confer radiosensitive phenotypes, such as Xeroderma pigmentosum (XP) or Ataxia-Telangiectasia. But the availability of cells from individuals with rare genetic diseases for all the various genes that are of potential interest makes progress extremely slow by this approach. In spite of the problem of "diploidy", certain mammalian cell culture systems such as the Chinese hamster CHO line, have lent themselves well to isolation of mutants. It is not entirely clear why the CHO genome appears to be functionally hemizygous for many loci, but

numerous mutants affecting nutritional requirements (auxotrophies), drug resistance, radiation sensitivity and others have been isolated from this cell line (Stamato and Waldren 1977; Jeggo and Kemp 1983; Stackhouse and Bedford 1993a; Zdzienicka *et al.* 1992). These classical approaches are sometimes known as "forward genetics", where the mutant gene is initially unknown, but is identified and characterized starting from a knowledge of the phenotype it confers.

Other approaches have been developed, such as gene knock-out strategies in mice where the gene or DNA sequence is known, but its function is not. The function is then studied by determining the phenotype of the mice when animals are developed with the both alleles of the gene knocked out (Lim *et al.* 2000b; Chen *et al.* 2003). This is known as "reverse genetics". For human cells, neither approach of waiting for naturally occurring rare mutant human phenotypes or inducing mutants in cultured normal human cells is entirely satisfactory. Certainly the knock out approach is not possible. A further problem for human cells is that even if mutants are found from individuals of different radiosensitivity phenotypes, these and the wild-type phenotypes will have considerably different genetic backgrounds, so the effect of altering one particular gene and using cells with identical backgrounds for comparison is not possible.

Possibilities such as the use of antisense RNA to reduce the level of a particular mRNA, or the introduction of so-called dominant-negative constructs that operate by producing a protein or subunit that interacts to prevent proper function of the gene product, have met with some reasonable success in certain cases, but there have been a number of drawbacks for broad general usage of these approaches.

As will be outlined in greater detail later, the present study had two principal aims. The first aim was to apply a new "reverse genetics" methodology known as RNA interference (RNAi) for general applications in studying repair defects in low passage normal human cells. The second aim was to further examine an interesting but inconclusive observation that there may be a causal connection between reduced levels of DNA-PKcs (DNA dependent protein kinase catalytic subunit) of the non-homologous DNA end joining (NHEJ) system, and ATM (mutated in Ataxia-Telangiectasia), that is a central element of a network of pathways involved in DNA damage processing, one of which is known as homology directed repair (HDR). Prior to this study, no solid evidence existed connecting these pathways or networks.

DNA Double Strand Breaks (DSBs) and DNA repair

The mammalian genome is at constant risk of DNA damage. One particularly severe form of DNA damage are double-strand breaks (DSBs). These may be induced by insults such as ionizing radiation (IR) or exposure to certain chemicals, or from the occasional collapse of replication forks when the replication machinery encounters single-stranded breaks (SSBs) in the DNA that can be caused by reactive oxygen species generated in the cell by normal respiration. DNA DSBs may also result from programmed cleavage by specific endonucleases during meiotic recombination, or they may arise normally as intermediates in V(D)J recombination, a process that is critical for the generation of the highly diverse antigen-binding sites for antibody and T-cell receptor proteins during development of the immune system (Gellert *et al.* 1999; Bassing *et al.*

2002b). It has been estimated that a mammalian somatic cell suffers some 10 spontaneous DSBs per day (Haber 2000).

If left unrepaired, DSBs can result in apoptosis, or mitotic cell death caused by loss of genomic materials (Olive 1998). If rejoined or repaired inaccurately, in addition to generating small deletion or insertion mutations at the site of the DSBs, they can result in chromosomal aberrations including translocations, deletions and inversions. Some of these are lethal and some are not, but a sub-set of non lethal aberrations can lead to mutations and/or carcinogenesis (Hoeijmakers 2001). A classic example of a harmful translocation is that seen in Burkitt's lymphoma, where an (8:14) translocation juxtaposes *c-myc* and strong transcriptional elements within the IgH locus, leads to the overexpression of *c-myc* and cell transformation. Cells have evolved several mechanisms to repair or at least rejoin DNA DSBs. Non-homologous end joining (NHEJ) and homology directed repair (HDR) are the two most widely studied repair pathways. A third, single-strand annealing (SSA), shares components with both NHEJ and HDR (Valerie and Povirk 2003).

Sensing Damage and Signalling Responses

The mechanism of DSB detection is still not clear although many candidate sensor proteins have been identified through cytological, biochemical and genetic studies (Petrini and Stracker 2003). One of the crucial early initiator is the protein kinase ATM, which is mutated in the cancer-prone, X-ray sensitive syndrome ataxia telangiectasia (see below). It has been demonstrated that ATM binds to DNA in vitro (Smith *et al.* 1999), and localizes to the nucleus in response to agents that cause DSBs (Andegeko *et al.*

2001). ATM activation, as indicated by autophosphorylation and dimer dissociation, can be triggered in minutes by changes in chromatin structure (Bakkenist and Kastan 2003; Kozlov *et al.* 2003). Once activated, ATM phosphorylates various down stream substrates, including p53, the checkpoint kinase CHK2, BRCA1 and NBS1, leading to various effects on DNA repair, cell cycle arrest and apoptosis.

Other DNA-damage surveillance proteins are ATR (ATM and RAD3-related) DNA-PK, P53, BRCA1, and BRCA2, and all these have broad effects on cellular biochemistry. Cell cycle arrest in G1 after radiation is mediated via p53 (Hirao *et al.* 2000).

An early and critical event which depend on the giant protein kinases ATM, ATR and DNA-PKcs, is phosphorylation of histone H2AX in the chromatin domain next to the nascent DSBs over a distance of thousands of base pairs from the break (Paull *et al.* 2000; Burma *et al.* 2001). γ-H2AX, the phosphorylated form of H2AX, has been proposed to recruit several proteins to DSB sites (Paull *et al.* 2000; Bassing *et al.* 2002a). After exposure to IR, foci of the DNA repair proteins Mre11/RAD50/NBS1 (the MRN complex), DNA-PKcs, RAD51, 53BP1, and BRCA1 colocalize with γ-H2AX foci (Schultz *et al.* 2000; Bassing *et al.* 2002a; Chan *et al.* 2002).

Many proteins, including not only ATM, but ATR, DNA-PKcs and p53, for example, also participate in triggering cells toward pathways leading to apoptosis (Herzog *et al.* 1998; Woo *et al.* 2002). The decision between repair and apoptosis in response to ionizing radiation depends on the cell cycle and the extent of damage and also depends on the disassembly of a complex formed between ATM, c-ABL, and BRCA1 (Foray *et al.* 2002; Valerie and Povirk 2003).

Non-homologous end-joining (NHEJ)

The major pathway for rejoining of DSBs in mammalian cells is non-homologous end-joining (NHEJ). In contrast to HDR, this pathway is "error-prone" and does not depend on the presence of homologous DNA sequences. This process can rejoin broken DNA ends directly end-to-end. It may be relevant that for organisms such as higher eukaryotes, in which a large portion of their genome is non coding, small errors in the base sequence at the site of rejoining may be better tolerated that would be the case for more compact genomes. Genetic studies, using radiosensitive mammalian cell lines defective or partially defective in DSBs rejoining have identified six different molecules which have been shown to be required for NHEJ: Ku70, Ku80, DNA-PKcs, XRCC4, DNA ligase IV and Artemis.

A working model for the steps involved in NHEJ (Lees-Miller and Meek 2003) is that the Ku70/80 heterodimer binds to the ends of double strand DNA at the site of a DSB or even normal telomeric chromosome ends. DNA-PKcs then interacts with the DNA end bound to Ku, forming the DNA-PK serine/threonine protein kinase complex. On its own, the 450 kDa catalytic subunit is inactive and relies on the other DNA-PK components to direct it to bind to the DNA and trigger its kinase activity (Smith and Jackson 1999). The two molecules of DNA-PKcs, one bound to each end of the DSB/Ku may serve to hold the ends together (Lees-Miller and Meek 2003). The DNA ligase IV/XRCC4 complex is responsible for ligation of the DNA ends. The chemical nature of the broken DNA ends are very unlikely to be identical and could very well require processing before the ligation step. Artemis is a protein that has a 5' to 3' exonuclease activity, and when bound to DNA-PCcs it has been shown to possess an endonuclease

activity. It is known to be required in V(D)J recombination, and individuals that are mutants for the Artemis gene are immunodeficient and are also radiosensitive.

Since the central focus of the present study involves DNA-PKcs, summarizing some additional features of this protein and its gene may be useful. The gene coding for the DNA-PKcs protein is called *Prkdc*. It was first identified as a strong candidate for the mouse Scid gene (Kirchgessner *et al.* 1995). The human *Prkdc* gene was mapped to 8q11 by fluorescent *in situ* hybridization (FISH) (Sipley *et al.* 1995; Ladenburger *et al.* 1997). It transcribes a 12,228-bp open reading frame that encodes a polypeptide consisting of 4,127 amino acids with, as mentioned above, a molecular weight of 470 kDa. Analysis of the gene structure revealed 86 exons. Cloning of its 14kb cDNA indicated that the carboxyl-terminal ~500 amino acid residues of this subunit falls into the phosphatidylinositol 3 (PI 3)-kinase family. However, it phosphorylates proteins and has no detectable activity toward lipids (Hartley *et al.* 1995). PI 3-kinase family proteins can be divided into two subclasses. One class has lipid kinase activity and a conserved PI 3-kinase domain at the extreme C-terminus. The other class, termed PI 3-kinase related kinases, tend to be large proteins with protein rather than lipid kinase activity and have a highly conserved region downstream of the PI-3 kinase domain (Hunter 1995). Included in this latter group are proteins that function in controlling transcription, the cell cycle and/or genome stability and DNA repair, such as DNA-PKcs, ATM and ATR. The amino-terminal ~3500 amino acid residues of DNA-PKcs does not appear to have significant homology to other characterized proteins (Hartley *et al.* 1995; Poltoratsky *et al.* 1995).

DNA-PKcs appears to be restricted to higher eukaryotes. Homology has been identified in mouse (Araki *et al.* 1997), horse (Shin *et al.* 1997), and *Xenopus laevis* (Labhart 1997), but it is not present in the genome of *Saccharomyces cerevisiae* and has not been identified in the genomic sequences available for *Caenorhabditis elegans* (Smith and Jackson 1999).

DNA-PK phosphorylates a large number of nuclear proteins, including TP53 (LeesMiller *et al.* 1992) and replication protein A (RPA) (Brush *et al.* 1994), both of which are phosphorylated in response to DNA damage, XRCC4 (Leber *et al.* 1998) which is required for NHEJ and V(D)J recombination, and Artemis (Ma *et al.* 2002). The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is critical for DNA repair via the nonhomologous end joining pathway.

Defects in DNA-PKcs not only affect radiation sensitivity with respect to cell killing, but also render cells more sensitive to the induction of chromosomal aberrations (Stackhouse and Bedford 1994; Evans *et al.* 1996). Recent work has implicated DNA-PK components in a variety of other processes, including the modulation of chromatin structure and telomere maintenance. Defects in this protein also result in telomere-telomere fusions, fusions between telomeres and DNA DSBs, and these can lead to chromosomal instability (Bailey *et al.* 1999; Bailey *et al.* 2001), More importantly, a defect in BALB/c mice that renders them more sensitive than other mice to radiation induced breast cancer and to radiation induced chromosomal instability, has been shown to result from a polymorphism involving a defective DNA-PKcs.

Homology Directed Repair (HDR)

In higher eukaryotes, the predominant of pathway for DNA DSB rejoining is NHEJ (Jeggo 1998b), while homology directed repair (HDR) sometimes referred to as homologous recombinational repair, dominates in lower eukaryotes such as yeast (Lee *et al.* 1997; Sonoda *et al.* 1998). However, recent evidence indicates that some vertebrate cells, notably chicken DT cells, are also quite efficient at HDR (Liang *et al.* 1998; Pfeiffer *et al.* 2000). Aside from repairing radiation damage, the process of homologous recombination is still fundamentally important in higher eukaryotic cells, and the utilization of this system can vary during development and in meiosis. In somatic cells its utilization depends on the stage of the cell cycle. It plays a more prominent role when sister chromatids are in close proximity during late S and G2 stages of the cell cycle (Morrison *et al.* 2000). NHEJ occurs in all cell phases but a key protein in HDR, rad 51, is very low or absent in G1 or G0 cells (Flygare *et al.* 1996; Chen *et al.* 1997; and personal observation).

HDR promotes accurate repair of DSBs by copying intact information from an undamaged homologous DNA template. This requires the presence of a homologous chromosome, sister chromatid or DNA repeats. Because the two homologous chromosomes of a diploid mammalian cell are not often in close proximity, the repair of DSBs through interaction of homologous chromosomes is orders of magnitude less efficient than between chromatids (Helleday 2003).

HDR involves a large number of proteins. In human cells, the main steps are thought to be mediated by the single-strand binding protein RPA (Baumann and West 1997), the human homologs of *Saccharomyces cerevisiae* Rad51, Rad52, and Rad54

(Sung *et al.* 2000), the rad51 paralogs XRCC2, XRCC3, Rad51B, Rad51C, and Rad51D, and the MRN complex (reviewed in Thompson and Schild 2001).

While the disruption of Rad52 confers no sensitization (Rijkers *et al.* 1998), inactivation of Rad54 in mouse cells causes a small increase in radiosensitivity that is mainly associated with the late S/G2 phase (Essers *et al.* 1997). The disruption of Rad51 in mouse ES cells leads to loss of cell viability (Lim and Hasty 1996; Sonoda *et al.* 1998). Knockout of Rad51B, Rad51D (Pittman and Schimenti 2000), Rad50 (Luo *et al.* 1999), MRE11 (Xiao and Weaver 1997) and Nbs1 (Zhu *et al.* 2001) are embryonic lethal in mice. The mutations in the Rad51 paralogs XRCC2 and XRCC3 confer significant radiosensitivity (Liu *et al.* 1998; Takata *et al.* 2001; Yoshihara *et al.* 2004), at least in chicken DT40 cells.

The initial step in HDR is thought to be a 5' to 3' exonuclease trimming of the DNA end to produce a 3' single-stranded DNA overhang by the MRE11/Rad50/Nbs1 (MRN) complex. This could be controlled by ATM because Nbs1 is a substrate for ATM phosphorylation. The RAD50 subunit of MRN is believed to facilitate DNA unwinding. RAD51 is the eukaryotic homologue of RecA protein in *Escherichia coli*, and it forms nucleoprotein filaments on single-stranded DNA tails. These become coated by RPA and are thought to catalyse the search for homologous sequences, strand pairing and strand exchange. The RAD51 paralogs are believed to facilitate the action of RAD51 to replace the RPA proteins on the ssDNA overhang. RAD52 helps RAD51 form DNA exchange intermediates. BRCA2 may play a role in loading Rad51 onto ssDNA (Powell *et al.* 2002). BRCA1 is also involved at an early point of DSB repair.

Following sister-chromatid pairing and strand invasion of DNA overhangs, DNA synthesis is initiated at the 3' end by a DNA polymerase. Finally, the breaks are sealed by ligase (reviewed in Valerie and Povirk 2003; Helleday 2003).

Some additional comments focused on ATM are of interest since much of the present project involves this gene, its protein, and its role in the complex network of cellular damage processing pathways.

ATM

Ataxia telangiectasia (A-T) is a rare autosomal recessive disorder. It is characterized by progressive cerebellar degeneration, premature aging, growth retardation, gonadal atrophy, immunodeficiency, high sensitivity to ionizing radiation, genomic instability and cancer predisposition (Boder and Sedgwick 1958; Harnden 1994; Lavin and Shiloh 1997). The characteristic features of A-T are cerebellar ataxia and dilation of small blood vessels and capillaries (telangiectasis) of the sclera, face, and ears. Some 0.5-1% of the general population may be heterozygotes for mutation of the AT gene (Swift *et al.* 1987). Approximately 30% of AT homozygotes develop cancer (Boultwood 2001). The increased risk of cancer is estimated to be 60-180-fold higher than the general population, with ~70% of malignancies being of the lymphoid system. Lymphoid malignancies in patients with AT are of both B cell and T cell origin, including Hodgkin's and non-Hodgkin's lymphomas, and several forms of leukaemia (Meyn 1997; Khanna 2000).

Cell lines derived from A-T patients are also radiosensitive and exhibit defects in various cell cycle checkpoint responses to IR including p53-dependent G_1 cell cycle

arrest and rapid p53-independent S and G_2 phase arrests (Canman and Lim 1998; Rotman and Shiloh 1999). The cellular phenotype is characterized by chromosomal breakage (Elson *et al.* 1996), telomere instability, radiosensitivity, radioresistant DNA synthesis, and reduced p53 response.

It was thought for a time that the cellular defect leading to x-ray hypersensitivity in A-T cells was possibly related to a difference in chromatin structure and was reflected in a radioresistant DNA synthesis permitting replication on a damaged chromosome before it could repair (Painter and Young 1980). Two independent experiments about this time resulted in this idea being abandoned. First, Nagasawa and Little showed that A-T cells held in a non-cycling confluent plateau phase were unable to repair "potentially lethal" damage (Little and Nagasawa 1985). Second, Cornforth and Bedford (Cornforth and Bedford 1985; Cornforth and Bedford 1987) showed that the all the difference in chromosome damage development for normal vs AT cells necessary to account for the difference in cell killing (from the initial x-ray breakage of interphase chromatin to the mis-rejoining or lack of chromosome rejoining to form lethal chromosome aberrations) occurred while the cells were in a non-cycling G0 state. No cell cycling or checkpoints triggering apoptosis were involved. While this literature was apparently overlooked by newer workers in the field (Meyn *et al.* 1994; Meyn 1995), the current view now being arrived at by these workers, independent of this 1980's literature, is that a checkpoint in AT does not underlie the defect leading to hyperradiosensitivity (Xu *et al.* 2002).

The magnitude of the hypersensitivity A-T fibroblast and other A-T cells relative to wild- types is of the same order as that for the more severe mutants of the NHEJ

system. Normal cells typically require radiation doses 3-5-fold greater for a given level of cell killing than are required for that level of killing for A-T cells (Thacker 1989).

The gene that is defective in A-T cells is designated ataxia telangiectasia mutated (*ATM* in human and *Atm* in mice). The AT locus was mapped to the chromosomal region 11q22–23 in the late 1980s (Gatti *et al.* 1988) and the gene was identified by positional cloning several years later (Savitsky *et al.* 1995a; Savitsky *et al.* 1995b; Savitsky *et al.* 1996). It encodes a 3056-amino acid protein with a molecular weight of 370kD (Chen and Lee 1996). The location of the mouse *Atm* gene was mapped to chromosome 9 (Xia *et al.* 1996) and refined to band 9C (Pecker *et al.* 1996) by fluorescent *in situ* hybridization (FISH). The mouse *Atm* gene encodes a deduced 3066-amino acid protein with 84% identity to the human sequence (Pecker *et al.* 1996).

The *ATM* gene was determined to contain 66 exons spanning approximately 150kb of genomic DNA by long distance PCR between exons. The processed transcript is 13kb, which has a 9.3kb open reading frame (Uziel *et al.* 1996).

The majority of patients with A-T are compound heterozygotes, ie, they are homozygous recessive for functional gene products, but the mutations are different in the two alleles. A large majority of the mutations in *ATM* involve truncation and destabilization of the protein, but certain missense and splicing errors have been shown to produce a less severe phenotype (Banin *et al.* 1998; Lavin 1999).

The ATM protein resides predominantly in the cell nucleus, with a small amount detected in microsomal fractions (Brown *et al.* 1997). ATM protein levels and localization remain constant throughout all stages of the cell cycle. Exposure of normal human cells to gamma-irradiation and the radiomimetic drug neocarzinostatin have no

effect on ATM protein levels (Brown *et al.* 1997), in contrast to a noted rise in p53 level over the same time interval (Kastan *et al.* 1991)

The ATM protein is a member of the phosphatidylinositol-3 kinase family of proteins that respond to DNA damage by phosphorylating key substrates involved in DNA repair and/or cell cycle control (Lavin *et al.* 1995).

ATM protein activity is dormant in unirradiated cells. It exists as a dimer or higher-order multimer, with the kinase domain bound to a region surrounding serine-1981. Cellular irradiation induced intermolecular autophosphorylation of serine-1981 that cause dimer dissociation and initiated cellular ATM kinase activity (Kozlov *et al.* 2003; Bakkenist and Kastan 2003). The ATM kinase activity is elevated ~2.5 fold immediately after exposure of cells to IR and decline to a basal level one hour after irradiation (Canman and Lim 1998; Pandita *et al.* 2000).

ATM is a key mediator in the pathways that signal cell cycle checkpoint arrests after DNA damage. It exerts many effects through its interaction with different proteins, phosphorylating one or more substrates in response to DNA damage to activate radiation signal transduction pathways and/or recruit proteins to sites of DNA repair. ATM has been shown to phosphorylate proteins such as p53 (Canman *et al.* 1998; Banin *et al.* 1998), c-Abl (Shafman *et al.* 1997; Baskaran *et al.* 1997), the p34 subunit of replication protein A (RPA) (Gately *et al.* 1998), H2AX, LKB1 (Sapkota *et al.* 2002), PHAS-1, Chk1, Chk2 (Matsuoka *et al.* 2000; Ahn *et al.* 2000), NBS1 (Lim *et al.* 2000a; Zhao *et al.* 2000), FANCD2 (Taniguchi *et al.* 2002), Mdm2 (Khosravi *et al.* 1999), Cdc25, and BRCA1 (Cortez *et al.* 1999; Gatei *et al.* 2000). While some of these are directly involved in HDR (RPA and NBS1), most of them participate in other signaling process and

checkpoint functions (H2AX, Tp53, Mdm2, BRCA1, Chk1, Chk2) or other regulatory responses directing cells toward pathways of apoptosis or cell proliferation (c-Abl and E4-BP1). Most ATM-mediated signaling involves phosphorylation, but some proteins, such as MKP5 (Theodosiou *et al.* 1999) and p53 (Waterman *et al.* 1998) undergo dephosphorylation at specific sites.

Connections Between NHEJ, HDR, and ATM?

There have been detailed and extensive studies concerning pathways and protein interactions involved in the DNA DSB sensing, signaling, apoptosis, and HDR network (eg., reviewed in Thompson and Schild 2001; Valerie and Povirk 2003; Helleday 2003). ATM is a key protein in this network and accordingly, its status controls virtually this entire system. Likewise, similarly extensive studies have been carried out regarding the NHEJ system, and some regarding alternative end joining (ALT) pathways. There has been relatively little evidence of direct connections and interrelationships between the systems. In 1993, Allalunis-Turner and her colleagues isolated a radiosensitive clone designated M059J from a culture arising from a glioblastoma biopsy (Allalunis-Turner *et al.* 1993). Other clones from the biopsy (M059K) were normal in radiosensitivity. Later, the genetic defect underlying the hypersensitivity to radiation in the M059J cells was identified as a mutation in the *Prkdc* gene, leading to a virtual absence of DNA-PKcs (Lees-Miller *et al.* 1995). In 1998 Chan and colleagues noticed that these M059J cells were also deficient in the ATM protein, although RT-PCR showed that *ATM* mRNA was present. Four years later, Tsuchida and colleagues isolated and sequenced both alleles of the *ATM* in M059J cells and found a different mutation in each allele that would be

predicted to truncate the message and result in protein degredation (Tsuchida *et al.* 2002). It is still not clear why M059J cells should have different mutations in both alleles of both *ATM* and *Prkdc*. In retrospect, it may not be surprising why *ATM* mRNA should be found by RT PCR, because enough cycles of PCR can amplify very small amounts to achieve saturation of the PCR reaction. Real-time RT PCR was not available at that time.

Some further observations on reduced ATM in scid mice were made by Dr. Lavin and his colleagues in Brisbane (personal communication). Since our laboratory was using siRNA technologies to knock down DNA-PKcs, and had already reported its successful application, conversations with Dr. Lavin led to his suggestion that it might be worth looking at the ATM levels in our samples. This would possibly overcome the serious objection from the previous suggestive observations that the reduced ATM levels may reflect a natural variation based on different genetic backgrounds and perhaps other factors. The ATM levels in DNA-PKcs knocked down cells would at the very least be from measurements in identical and normal human cells.

The application of the siRNA technology for reverse genetics studies in mammalian cells only emerged three years ago and the subject is so new that I felt it worth some general review and discussion at this point.

RNA interference

The recent discovery of RNA interference (RNAi) has offered a powerful new way to inhibit the expression of virtually any gene in living cells (Sharp 2001). It is a process involving degradation of mRNAs by a dsRNA having a sequence that matches a sequence in the mRNA, and has been observed in a variety of eukaryotic organisms

(Hannon 2002). It has been speculated that it may have evolved as a mechanism for cellular protection against molecular parasites such as viruses and mobile elements, while removing abundant but aberrant nonfunctional messenger RNA (Tijsterman *et al.* 2002). RNAi may also have a normal functional role in cells to inhibit the expression of some genes during growth and development.

Tuschl and colleagues discovered that the approach could be utilized in mammalian cells by introduction of short interfering RNA (siRNA) duplexes into mammalian cells (Elbashir *et al.* 2001a). Longer dsRNAs that had induced the RNAi response for organisms such as *C. elegans* had not been successfully used for mammalian cells (Hammond *et al.* 2001). The use of the RNAi approach is increasing rapidly (Bedford and Liber 2003). As RNA interference relies on the sequence-specific interaction between siRNA and mRNA, siRNAs can be tailored to silence almost any gene. Rapid progress has been made to attenuate the expression of specific proteins both *in vitro* and *in vi*vo, where the cDNA sequence is known.

A brief history of RNA interference

The phenomenon of RNAi was first discovered in plants as post-transcriptional gene silencing, or (PTGS). Our understanding of the role of RNA has improved dramatically in recent years.

In retrospect there were several studies a decade ago where the introduction of dsRNAs or DNAs into cells resulted in an unexpected suppression of gene expression and which might well be attributed to the RNAi phenomenon (Napoli *et al.* 1990; Romano and Macino 1992; Cogoni *et al.* 1994; Cogoni *et al.* 1996).

In 1995, Guo and Kemphues made the surprising observation that when they used either antisense or sense RNA to block the production of par-1 protein in the nematode worm *Caenorhabditis elegan* both gave the result of embryonic lethality. However, the inhibition of gene expression was thought to be caused by the saturation of the factors needed for *par-1* translation (Guo and Kemphues 1995).

The apparent paradox was resolved by Andrew Fire and Craig Mello in 1998 (Fire *et al.* 1998). They had also observed that either sense or antisense RNA inhibited the gene function after being injected into the worm. But they demonstrated that silencing was the result of dsRNA contamination in the prepared single-stranded RNA population. Pure single-stranded RNA of sense polarity was unable to trigger gene silencing, while the mixture of sense and antisense strands (double-stranded RNA) silenced expression of a target gene roughly ten-fold more efficiently than either strand alone. Furthermore, injection of dsRNA into the gut of the worm caused gene silencing not only throughout the worm, but also in its first generation offspring. The term 'RNA interference' or 'RNAi' was coined to describe this dsRNA-induced effect (Fire *et al.* 1998).

RNA interference in *C. elegans* was also carried out by soaking the worms in a solution containing dsRNAs (Tabara et al. 1998) or by feeding the worms *Escherichia coli* that expressed the dsRNAs (Timmons and Fire 1998). RNase protection assays on RNA isolated from *C. elegans* fed on bacteria that generate dsRNA homologous to the *C. elegans unc-22* gene revealed small RNA molecules of 20-25 nt in length and these worms developed an *unc-22* null-like phenotype (Timmons *et al.* 2001; Sijen *et al.* 2001). These strategies have enabled large-scale screens to select for RNAi-defective *C. elegans* mutants and have led to large numbers of gene knockout studies within this organism.

RNA interference has also been observed in *Drosophila melanogaster* using a variety of techniques to deliver the dsRNAs to the cells or embryos (Kennerdell and Carthew 1998; Kennerdell and Carthew 2000).

Many research groups have diligently worked over the last few years to attempt to understand how these dsRNA silence genes. Baulcombe and Hamilton discovered an important clue that during the process of cosuppression or virus-induced gene silencing in *Arabidopsis*, a short dsRNA species of around 25 nucleotides appeared, corresponding to both the sense and antisense sequence of the targeted mRNAs. These short RNA species were not found in non-silenced plants (Hamilton and Baulcombe 1999).

Further insight for a role for short RNAs came from discovery of these small RNAs in *Drosophila* embryo lysates (Zamore *et al.* 2000) and an *in vitro* system derived from *Drosophila* S2 cells (Hammond *et al.* 2000). It was shown that long dsRNA substrates (>500 nucleotide) added *to Drosophila* embryo extracts were cleaved to 21-23 nucleotide species. Further, the homologous endogenous mRNA was cleaved only in the region corresponding to the introduced dsRNA (Zamore et al. 2000). The cloning and sequencing of the endogeneous RNAs from Drosophila revealed that they had a very specific structure: 21-23 nt dsRNAs with 2-nt 3'-end overhangs (Elbashir *et al.* 2001b). And the introduction of chemically synthesized 21-nt and 22-nt siRNAs to these extracts facilitated the degradation of the homologous RNA (Elbashir *et al.* 2001b).

The mechanism of the RNA interference

The precise mechanisms behind RNAi are not yet fully understood, but what was established early on is as follows:

Upon introduction, long dsRNAs enter a cellular pathway that is commonly referred to the RNA interference pathway. In the initiation step, the dsRNAs get processed into 20-25 nucleotide double-stranded RNA duplexes with symmetric 2-3-nt 3' overhangs in an ATP-dependent reaction. This structure is characteristic of a RNase III-like enzymatic cleavage pattern, which led to the identification of the highly conserved Dicer family of proteins (Bernstein *et al.* 2001; Ketting *et al.* 2001).

In the next step, the siRNA duplexes are assembled into ribonuclease-containing complexs known as RNA-induced silencing complexes (RISCs). Although the uptake of siRNA by RISC is independent of ATP, the unwinding of the siRNA duplex requires ATP. Once unwound, the antisense strand guides the RISCs to the complementary target mRNA molecules by base pairing interaction. Cleavage of mRNA takes place at a single site in the center of the duplex region between the mRNA and the guide siRNA strand (Elbashir *et al.* 2001b). The antisense siRNA could then be recycled (Bernstein *et al.* 2001).

Scilencing by siRNA

Since RNA interference was first discovered in the worm in the late 1990s, it was immediately adopted as a method to investigate gene functions in various organisms including plants, planaria, hydras, *trypanosmes*, *Drosophila*, and mosquitoes with varying degree of success. In *C. elegans*, *Drosophila* and plants it seems to be an effective and

specific. A second class of organisms, including zebrafish and *Xenopus*, has been shown to have some capacity for RNAi (Hammond *et al.* 2001).

In lower eukaryotes, dsDNA is cleaved by Dicer to siRNA, which then mediates post-transcriptional gene silencing (PTGS). In mammals, the dsRNA mediated gene silencing is only applicable to a limited number of cell types such as mouse oocytes, pre-implantation embryos and a few cell lines derived from embryos. The applicability of this approach is limited in mammals because introduction of dsRNA longer than 30nt into mammalian cells induces sequence-nonspecific interferon response. Interferon triggers the degradation of mRNA by inducing 2'-5' oligoadenylate synthase, which in turn activates RNase L. In addition, interferon activates the protein kinase PKR (Der *et al.* 1997), which phosphorylates the translation initiation factor eIF2α, leading to a general inhibition of protein synthesis (Moss and Taylor 2003; Sledz *et al.* 2003).

This mammalian antiviral response can be bypassed by the introduction or expression of siRNA. It has also been known that dsRNAs less than 30nt in length do not activate the PKR kinase pathway, although this may not always be the case (Sledz *et al.* 2003). This observation, as well as knowledge that long dsRNAs are cleaved to form siRNAs in worms and flies and that siRNAs can induce RNAi in *Drosophila* embryo lysates, prompted researchers to test whether introduction of chemically synthetic siRNAs could mediate effective gene-specific silencing without inducing interferon response in mammalian cells (Elbashir et al. 2001b). The chemically synthetic siRNAs introduced by transient transfection were found to be functional and comparable to that obtained with long dsRNA in *Drosophila* S2 cells, in several mammalian cultured cells in a sequence-specific manner without activating the nonspecific effects. But the

effectiveness of synthetic siRNAs varies. The most potent siRNAs result in >90% reduction in target RNA and protein levels (Elbashir et al. 2001a; Caplen et al. 2001). The most effective siRNAs turn out to be 21nt dsRNAs with 2 nt 3' overhangs, which resemble the naturally active products of Dicer. Sequence specificity of siRNA is very stringent, as single base pair mismatches between the siRNA and its target mRNA dramatically reduce or eliminate silencing (Elbashir et al. 2001a; Elbashir et al. 2001c).

Selection and generation of effective siRNA sequences

Choosing siRNA sequence is an empirical process. General guidelines have been proposed for synthesis of siRNAs by several groups (Elbashir *et al.* 2002; Dykxhoorn *et al.* 2003; Reynolds *et al.* 2004). Generally, it requires avoiding such regions of mRNA as 5' and 3' untranslated regions (UTRs), regions within 75 bases of start codon, and sequences having a GC content of <30% or >70%. Unfortunately, not all siRNAs with these characteristics are effective. On average, only 1 in 5 of the siRNA selected for targeting a region of a gene show efficient gene silencing (Kumar *et al.* 2003). It is known that siRNAs that target different regions of the same gene vary remarkably in their effectiveness (Holen *et al.* 2002; Kawasaki *et al.* 2003; Yoshinari *et al.* 2004). The reasons for this are unclear but may be a result of instability of an siRNA *in vivo*, inability to interact with components of the RNAi machinery, and inaccessibility of the target mRNA due to local secondary structure. In one study, RNase-H-sensitive sites were used to determine siRNA target sites. It was found that the RNase-H-sensitive sites on the mRNA are in a conformation that is susceptible to siRNA-directed silencing (Vickers et al. 2003). Moreover, empirical approaches such as the use of a recombinant

human Dicer (Kawasaki *et al.* 2003; Myers *et al.* 2003) to cleave *in vitro* transcribed long dsRNA into a group of siRNAs have been shown to be very effective in gene silencing after such pools are transfected into mammalian cells. This approach allows for the presentation of siRNAs with multiple specificities to the target without activating an interferon response. Still there are possible problems with so called "off target" effects.

The transfection of siRNA into cells leads to only a transient knockdown of the gene of interest. The silencing generally effect lasts for ~3-5 cell doublings, that is 3-5 days for most cell lines, and eventually diminishes. The siRNAs are diluted during each cell cycle. In actively dividing cells, the duration of silencing is directly related to the number of cell doublings (Dykxhoorn et al. 2003), indicating the loss of effectiveness of siRNA is due to the dilution below an effective level rather than degradation of siRNA. Normal gene expression resumes in ~7-10 cell doublings (McManus and Sharp 2002). If the cells are serum starved or their growth is otherwise arrested, this window might be extended. Another factor that could limit the transient siRNA-mediated silencing is the protein turnover rate. It might be difficult to effectively knockdown genes that encode proteins with long half-lives by transient transfection of siRNA. So, differences in siRNA effectiveness, transfection efficiency, cell type and protein stability might all contribute to the efficiency of siRNA-mediated RNAi. The transfection approach that results has a great advantage if permanent knockdown of expression of a gene is lethal to cells but the transient knockdown is not. It may thus be possible to study the function of the protein of interest during the period of its knockdown. There is a disadvantage that usually 100% transfection of cells cannot be achieved. In some cases this could be more of a problem than in other cases.

Expression vectors have been developed to express substrates that can be converted to siRNAs *in vivo*. One approach is to devise a system where the sense strand and antisense strand of the siRNA are transcribed under different promoters and the two strands anneal inside the cells. In the other approach, transfected plasmids that produce dsRNA hairpins, called short hairpin RNAs (shRNAs), get processed *in vivo* into siRNAs-like molecules capable of carrying out gene-specific silencing (McManus *et al.* 2002; Paddison *et al.* 2002a). Many vector based expression systems still demonstrate only transient knockdown of gene expression and low transfection efficiency.

To circumvent these problems, shRNAs expressed in various viral vectors, including retroviral (Liu *et al.* 2004) and lentiviral (Rubinson *et al.* 2003) vectors, have been developed, and these have shown great promise for many applications.

The number of papers published on RNAi has exploded over the last few years and the journal *Science* proclaimed it the biggest scientific breakthrough of 2002.

Setting up Sufficient siRNA Controls

To ensure the validity of results utilizing RNA interference for knockdown of gene expression, a number of proper experimental controls are necessary. The editors of *Nature Cell Biology* have recommended several controls (Editorial 2003). Two kinds of negative controls may be used. First a scrambled siRNA can be used to reveal any changes to the gene expression profile that may result from the siRNA delivery method. These should have the same nucleotide composition as the input siRNA sequence but lack significant sequence homology to any mRNA in the mRNA pool for a particular organism. Comparing cells transfected with the scrambled siRNA control to

nontransfected cells can reveal changes caused by siRNA delivery. Second, since a single mismatch in the center of an siRNA sequence can abolish silencing effects (Miller *et al.* 2003), a one- or two-base-pair change in the middle of the antisense strand can be used as a negative control. This control allows us to check the specificity of the siRNA of interest. There has been one report indicating that an siRNA with a region of 14-15 contiguous bases of sequence homology between the siRNA and an mRNA can induce silencing without perfect matching of the other bases (Jackson *et al.* 2003).

For positive controls, some validated and published sequences can be used to monitor siRNA transfection efficiency. Another useful control to help determine whether an "off-target" effect is occurring is to attempt to knock-down the gene product with two independent siRNA duplexes.

A very low concentration (1-100 nM) of siRNA is sufficient for specific gene silencing (Elbashir *et al.* 2001a). The concentration of siRNA required will depend upon the method and efficiency of siRNA delivery, the cell line used, and the effectiveness of the siRNA sequence. Concentrations of siRNA of 100nM or higher in mammalian cultured cells, however, can lead to nonspecific changes in gene expression due to the induction of an interferon response that affects the expression of many proteins (Semizarov *et al.* 2003; Sledz *et al.* 2003). Certainly it seems prudent to test a range of concentrations of siRNA and use the minimum concentration that still provides maximal target gene silencing.

It is also important to show reduction of the expression of both mRNA and protein. Protein reduction in the absence of mRNA reduction may indicate that an siRNA is mediating its effects at the translational level as has been suggested for the action of

micro-RNAs (Nelson *et al.* 2003). The global translational repression through the interferon response can also be monitored by measuring the level of 2'5'-oligoadenylate synthetase mRNA (Bridge *et al.* 2003). This enzyme catalyzes the synthesis of 2'-5' polyadenylic acid, which activates the nonspecific ribonuclease RNase L. RNase L is a nonspecific enzyme that targets all mRNAs. Alternatively, another path of an interferon response involves the phosphorylation and activation of dsRNA-dependent protein kinase PKR (Khabar *et al.* 2003). Activated PKR phosphorylates the small subunit of the eukaryotic translation initiation factor 2-alpha (eIF2α) and results in the shut down of all protein synthesis.

Statement of the Problems Addressed

As mentioned briefly earlier in this introduction there has been a need for better understanding of the genetic control of radiation responses in human cells, but from a practical viewpoint neither the forward nor the reverse genetic approaches have been available until very recently. While genetic defects in DNA repair underlie some rare genetic diseases, it is still not possible to assess phenotypic changes in cell radiation responses with only one genetic change, i.e., in two cell populations with otherwise identical genetic backgrounds. Further, it would be virtually impossible to obtain and study individuals with various combinations of double or even triple mutations of different genes. When it became possible to knock down gene expression by RNA interference in mammalian cells using small double stranded siRNAs (Elbashir *et al.* 2001a) our laboratory immediately began to explore its use to begin such applications for studying the genetic control of cellular radiosensitivity.

With these possibilities in mind, the first problem addressed was to determine whether a radiation hypersensitive phenotype similar to that known to be characteristic of a loss of the *Prkdc* gene product DNA-PKcs in other cells could be produced in cultured low passage normal human fibroblasts by knockdown of DNA-PKcs following transfection of an siRNA targeted to the *Prkdc* mRNA. The phenotypic changes I followed were increased radiosensitivity to cell killing, chromosome aberration induction, and a reduced capacity to rejoin interphase chromosome breaks as measured in prematurely condensed chromosomes 4 hours after irradiation.

The second problem addressed was whether there is a causal connection between loss or deficiency in DNA-PKcs and the expression of ATM. There have been one or two observations indicating that there may be such a connection, but the circumstances made the observations only suggestive. Dr. Martin Lavin, who was aware of our siRNA work involving DNA-PKcs knockdown, suggested a more definitive answer may be possible using this approach. As this did indeed turn out to be very interesting, I placed much of my efforts during the past 18 months on this portion of the project.

MATERIALS AND METHODS

Cells and Culture Conditions

In all cases described below, the various cells were cultured in an incubator maintained at 37℃ in a humidified atmosphere of 5% CO_2 in air, except for brief periods at room temperature required for subculture or other procedures as described for particular experimental protocols.

The "test cells" for most of the experiments were cultured low passage human fibroblasts. These fibroblasts, designated GM08399, were obtained from the National Institute of General Medical Sciences Coriell Cell Repositories. These cells were originally isolated from a skin biopsy from a 19-year old apparently normal female. Cells were cultured in 6-well plates with 2 ml culture medium per well. After the cells grew to form confluent monolayers, the cells were resuspended by trypsin treatment and ¼ of the cells in a well were used to reinoculate each well of a new 6-well plate.

Low passage (P4-P5) normal human fibroblasts AG1521A were obtained from American Type Culture Collection (ATCC). For subsequent serial subculture in our laboratory, a passage is defined as 1:10 dilution of a confluent T25 flask. The cells were grown in α-MEM (Gibco BRL, Grand Island, IL, USA) supplemented with 15% fetal bovine serum (Sigma, St. Louis, MO, USA), and containing 50 U/ml penicillin-G and 50 μg/ml streptomycin sulfate (Sigma, St. Louis, MO, USA).

For studies involving analysis of expression of the *ATM* gene (Mutated in Ataxia-Telangiectasia), we used negative and positive control cells supplied by Dr. Martin F. Lavin, Queensland Institute of Medical Research, Brisbane, Australia. The negative

control "L3" cells are an EBV-transformed lymphoblastoid cell line established from a North African Jewish Ataxia-Telangiectasia patient. L3 cells fail to produce the ATM protein due to a homozygous truncation mutation in both alleles of the gene. For positive controls, we used "C3 ABR" cells, which are a normal lymphoblastoid cell line that expressed normal ATM protein. The lymphoblastoid cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, IL, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and contained 50 U/ml penicillin-G and 50 μg/ml streptomycin sulfate (Sigma, St. Louis, MO, USA).

Human glioblastoma tumor cell line M059J that does not express the DNA-PKcs protein was originally isolated by Dr. Allalunis-Turner (Cross Cancer Institute, Alberta, Canada) and were kindly provided by Dr. Ed Goodwin, Los Alamos National Laboratory. They were maintained as a monolayer cultures in DMEM/F12 (1:1) (Gibco, Grand Island, IL, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and containing 50 U/ml penicillin-G and 50 μg/ml streptomycin sulfate (Sigma, St. Louis, MO, USA).

CHO 10B2 is a subclone derived from a Chinese hamster ovary cell line originally obtained from Dr. W.C. Dewey, now at The University of California, San Francisco. V3/A+ and V3/vector cells were generously provided by Dr. Susan P. Lees-Miller, University of Calgary, Alberta, Canada. V3 cells are a radiosensitive DNA-PKcs mutant line derived from the AA8-4 line of CHO cells that are wild-type with respect to DNA-PKcs and have normal radiosensitivity. DNA-PKcs protein is absent in V3 cells (Peterson *et al.* 1995), although the precise nature of the mutation is unknown. V3/A+ cells are V3 transfectants expressing human DNA-PKcs. They were derived by stably

transfecting cells with a plasmid vector containing and expressing wild-type human DNA-PKcs. V3/vector cells are V3 cells with the same vector but not containing the DNA-PKcs gene (Ding *et al.* 2003).

Irs-20 is DNA-PKcs defective CHO cell line isolated from a mutagenized culture of CHO 10B2 cells (Stackhouse and Bedford 1993a; Stackhouse and Bedford 1993b; Stackhouse and Bedford 1994; Lin *et al.* 1997). The mutation causes substitution of a lysine for a glutamic acid in the fourth residue from the DNA-PKcs C-terminus. The level of expression of DNA-PKcs is also very low in irs-20 cells. And DNA-PKcs protein from irs-20 cells is able to interact with DNA but lacks kinase activity (Priestley *et al.* 1998). Irs-20/YAC is an irs-20 cell line corrected with a yeast artificial chromosome (YAC) containing a human DNA-PKcs gene (Priestley *et al.* 1998). All of these cell lines were maintained in α-MEM (Gibco, Grand Island, IL, USA) supplemented with 15% fetal bovine serum (Sigma, St. Louis, MO, USA), and contained 50 U/ml penicillin-G and 50 μg/ml streptomycin sulfate (Sigma, St. Louis, MO, USA).

HeLa cells, originally derived from the cervical carcinoma, were cultured in α-MEM (Gibco, Grand Island, IL, USA) supplemented with 15% fetal bovine serum (Sigma, St. Louis, MO, USA), 50 U/ml penicillin-G and 50 μg/ml streptomycin sulfate (Sigma, St. Louis, MO, USA).

siRNA Transfection

siRNA

The siRNAs used were chosen as described by Elbashir (Elbashir *et al.* 2002) and synthesized by Dharmacon Research Inc, Lafayette, CO. Two siRNA sequences were used for targeted silencing of *DNA-PKcs*. One targeted a sequence in *DNA-PKcs* mRNA 293 bases downstream from the start codon.

GAUCGCACCUUACUCUGUUdTdT (siRNA sequence A)
dTdTCUAGCGUGGAAUGAGACAA

the other siRNA, targeted to the kinase domain, was

CUUUAUGGUGGCCAUGGAGdTdT (siRNA sequence B)
dTdTGAAAUACCACCGGUACCUC

The control double-stranded siRNA sequence was a *DNA-PKcs*-derived sequence with the following small changes relative to the first siRNA described above.

GAACGCACCUUCAUCUGUUdTdT
dTdTCUUGCGUGGAAGUAGACAA

The siRNA sequence to target Ku80 is

CCAUGUUUGUACAGCGACAdTdT
dTdTGGUACAAACAUGUCGCUGU

The siRNA sequence to target Lamin A/C is

CUGGACUUCCAGAAGAACAdTdT
dTdTGACCUGAAGGUCUUCUUGU

A Blast-search (www.ncbi.nlm.nih.gov/BLAST) of these sequences against GenBank® human mRNA database were carried out to ensure the sequences chosen would not target other gene transcripts. The search returns sequence matches with greater

than 15 base identical matches. For the DNA-PKcs targeting siRNAs, no such matches were found for the human genome.

Transfection

Oligofectamine™ Reagent (Invitrogen, Carlsbad, CA, USA) was used for the transfection of siRNAs into GM08399 normal human fibroblast cells.

For optimization of the transfections and siRNA knockdown conditions, a standard seeding protocol was maintained from experiment to experiment and cells used were within 7 to 10 subculture passages. To obtain the highest transfection efficiency and best knockdown, transfection conditions were optimized by varying siRNA, Oligofectamine™ concentration, and cell density. Cells were transfected when they had reached about 50% confluence. Antibiotics were omitted from the culture medium during transfection as it was suggested by Tuschl and colleagues that antibiotics can reduce transfection efficiency and cause cell death (Elbashir *et al.* 2002). The quantities of reagents given below were used for the transfection of cells in one well of a 6-well plate.

One day before transfection, cells were trypsinized and counted. 1 x 10^5 cells per well were plated into 6-well plates with 1 ml α-MEM and 15% fetal bovine serum (Sigma, St. Louis, MO, USA) and 0.1g/L penicillin-G and 0.1g/L streptomycin sulfate. Twelve hours after inoculation, medium was changed to α-MEM without serum or antibiotics. After another 12 hours, the medium was changed again, this time using 2 ml of medium without serum or antibiotics. The transfection was then carried out 2-3 hours after the second medium change.

For each transfection sample, Oligonucleotide-Oligofectamine™ complex was prepared as follows: depending on the particular batch of siRNA or the test concentration of siRNA needed from 1 to 64 μl of the 20 μM siRNA solution (or for some tests, a further dilution of the stock siRNA solution was used) was diluted in Opti-MEM® I (Invitrogen, specially formulated medium for transfection, made without serum) to a final volume of 280 μl and mixed gently. Then 15 μl Oligofectamine™ was added in 60 μl Opti-MEM® I, mixed gently and incubate for 7 minutes at room temperature. The diluted siRNA and diluted Oligofectamine™ (total volume is 340 μl) were combined into 1.7 ml Eppindorf tubes and mixed gently by inverting the tubes 5 times. The mixture was incubated 20 minutes at room temperature to allow the oligonucleotide-Oligofectamine™ complex to form. Successful complex formation results in the appearance of a cloudy solution. The total volume (355 μl) of oligonucleotide-Oligofectamine™ complex was added drop by drop with shaking to each well containing the cells with 2 ml of serum and antibiotic free medium. The cells were then incubated at 37℃ in a CO_2 incubator for 4 hours. After the initial 4 hour incubation, 2.5 ml α-MEM containing 30% FBS containing antibiotics (penicillin and streptomycin) was added to the well without removing the transfection mixture. After 12 hours further incubation at 37℃ in a CO_2 incubator, cells were trypsinized and split 1:2 and replated into new six-well plates. After another two days, all these steps were repeated to perform a second transfection. In some cases, as described later, only one transfection was carried out. Approximately 16 hours after the second transfection, instead of splitting the cells, the medium was again changed to normal α-MEM with antibiotics and 15% serum (Sigma, St. Louis, MO, USA). Cells

were further incubated 2-3 days before performing assays for gene expression, or phenotypic expression of cell or chromosomal radiosensitivity.

Western Blot Analysis of Proteins

After various siRNA treatment conditions and time-course protocols, levels of the proteins of interest in cells relative to those in control cells were measured by western blot analysis as follows.

Sample Preparation: Cell Lysis and Extraction

Culture medium was removed from the *DNA-PKcs* siRNA or control siRNA treated wells by aspiration. After rinsing with Dulbeco's phosphate buffered saline (PBS) twice, the cells were harvested by treatment with a 0.25% trypsin/ 0.1% EDTA solution and resuspended in chilled α-MEM medium with 15% fetal bovine serum (Sigma, St. Louis, MO, USA). Cells were collected by centrifugation at 300 x g for 4 min and after discarding the supernatant, the cell pellet was resuspended in ice-cold PBS, and the cells were centrifuged again and the PBS was aspirated. The cells were then either processed further or for later processing the cell pellet was immediately frozen at - 80°C and stored for up to a month.

For protein extraction, 100ul Lysis/Extraction buffer[1] was added to each cell pellet. The tubes were vortexed and placed on ice for 10 minutes. The samples were then centrifuged at 12,000 rpm (10,000 x g) for 10 minutes at 4°C. The supernatant, referred

[1] The composition of all buffers and other solutions is given in Appendix I under the same sub-heading where they are referred to in this chapter; eg, the lysis/extraction buffer composition is listed in Appendix I.

to as the total cell lysate, was then collected and the protein concentration of each sample was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA).

Protein Separation by SDS-PAGE Gel Electrophoresis

An equal amount of total protein (20-100 μg) from the *DNA-PKcs* siRNA or control siRNA treated samples was mixed in 250 μl Eppendorf tubes on ice with 4μ 5X gel-loading buffer[1] and water to a final volume of 20μl. Samples were denatured in a PCR thermocycler at 95°C for 5 minutes. The samples were then centrifuged briefly and loaded onto a 6% pre-cast Tris-Glycine SDS-polyacrylamide gel (Invitrogen Novex Tris Glycine EC6068). At the same time, protein molecular markers were also loaded in the first lane. Electrophoresis was carried out on a Novex® electrophoresis module (Invitrogen, Carlsbad, CA, USA) at room temperature for 75 minutes using a 125 volt power supply (BioRad, Hercules, CA, USA).

Transfer of Proteins from SDS-polyacrylamide Gels to Nitrocellulose Membranes

Gloves should be worn when handling gels or blot membranes.

When the SDS-polyacrylamide gel was approaching the end of its run, 500 ml transfer buffer[1] was poured into a glass tray, and 4 transfer sponge pads were soaked in the transfer buffer. The pads were placed 0.5 cm below the surface of the buffer and the air was excluded by applying pressure to the pads. One piece of Hybond™ ECL nitrocellulose membrane (Amersham Pharmarcia Biotech, Piscataway, NJ, USA) and two pieces of Whatman™ 3MM filter paper (Whatman International, Ltd, Maidstone, UK)

were then cut the exact size of the sponge pad and these were pre-wetted with deionized water. The membrane and filter papers were then soaked in transfer buffer.

After the gel had finished running, as judged from the position of the dye marker, the clear plastic front plate covering the gel was carefully removed and then the stacking gel and loading well regions of the gel were cut away and removed. The slotted part at the bottom of the gel was also cut away and removed. In addition, a small portion of the upper left-hand corner of the running gel was removed to mark its orientation during the transfer process. The gel was then rinsed with distilled water to remove the running buffer. One piece of buffer-wetted filter paper was then placed on the exposed running gel, making sure that air bubbles between the gel and filter paper were excluded. The gel with filter paper was then inverted and carefully placed in a tray containing the transfer buffer[1] and on top of the two layers of sponge pads. At this time a nitrocellulose membrane, also soaking in the tray was placed on top of the gel followed by placement of one layer of pre-wetted filter paper. At this stage a roller was used to apply pressure to the stacked filter paper, membrane, and gel to make sure that no air bubbles have been trapped between the gel and the membrane. Then two more layers of the pre-wetted sponge pads were added to the stack. Care was taken to ensure the entire stack of gel, membrane, filter papers and sponge pads were aligned. The full stack was then loaded and clamped into the Invitrogen XCell II electroblotting- module (Invitrogen, Carlsbad, CA, USA). The transfer module was then locked back into the gel box and the module was and filled with 50 ml transfer buffer. The gel box surrounding the transfer module was filled with 500 ml cooling water, and transfer was carried out with a voltage setting of 25V at room temperature for 2 hours or at 4°C overnight.

Immunological Detection of the Proteins

The first step for detecting the protein of interest on the membrane blots was to block non-specific binding of sites of the antibodies used for detection by immersing the membrane in 7% non-fat dried milk solution in TBS[1] for one hour at room temperature on an tilting shaker. In some instances the membrane was left in the blocking solution overnight in a refrigerator at 2-4°C.

DNA-PKcs

After the non-specific blocking step, the membrane was briefly rinsed using two changes of washing buffer (TBST 1X). The primary anti-DNA-PKcs antibody Ab4 (Neomarkers, Fremont, CA, USA) was diluted 1:800 in 5% non-fat dried milk solution in PBST[1] and the membrane was incubated in 20 ml of the diluted primary antibody for 2 hours at room temperature with gentle agitation on a tilting shaker. The primary antibody solution was then removed and the membrane was rinsed first with two changes of washing buffer and then washed again in 20 ml of washing buffer for 15 minutes at room temperature. Finally, the membrane was washed 3 more times with fresh changes of wash buffer for 5 minutes each at room temperature. The primary antibody was detected with horseradish peroxidase (HRP) labeled goat anti-mouse secondary antibody (Amersham Pharmarcia Biotech, Piscataway, NJ) diluted 1:2000 in 5% non-fat dried milk solution in PBST[1]. The membrane in diluted secondary antibody was then processed for incubation and rinsing in exactly the same manner as described for the primary antibody.

ATM

For the detection of ATM protein, the application of the primary antibody and labeled secondary antibody were identical to the procedure described above for DNA-PKcs detection.

1:2500 diluted rabbit anti-ATM (Catalog number NB 100-104) obtained from Novus (Novus, Fremont, CA, USA) was used in the detection of ATM protein in human cells.

For detection of ATM protein in CHO cells, the rabbit anti-ATM primary antibody APH397 (Serotec, Oxford, UK) 1:1000 diluted or 4BA 1: 2000 diluted was used. The primary antibody 4BA originally described by Dr Martin F. Lavin (Queensland Institute of Medical Research, Brisbane, Australia) was kindly supplied by Dr Susan P. Lees-Miller (University of Calgary, Alberta, Canada).

Horseradish peroxidase conjugated goat anti-rabbit secondary antibodies were used (1:2000 diluted) to detect the ATM primary antibody (Calbiochem/Oncogene, San Diego, CA, USA).

Ku 80 and β-Actin

Generally Ku-80 and β-Actin proteins were used as the loading controls or standards for relative measurements of the proteins of interest such as DNA-PKcs or ATM. The Ku-80 antibody was mouse anti-Ku80 Ab-2 obtained from Neomarkers (Neomarkers/Lab Vision, Fremont, CA, USA) and the β-actin antibody was mouse anti-β-actin (Catalog number NB 600-501) obtained from Novus (Novus, Fremont, CA, USA).

The horseradish peroxidase-labeled secondary antibodies were goat antimouse (Amersham Pharmarcia Biotech, Piscataway, NJ) diluted 1:2000 in 5% non-fat dried milk solution in PBST.

Detection: Chemiluminescence and Film Densitometry

The washing buffer on the nitrocellulose membrane was drained on a paper towel and then placed on a piece of saran wrap. The ECL reagent (x-ray film detection) or ECL Plus reagent (Storm Scanner detection system) was then prepared (Amersham Pharmarcia Biotech, Piscataway, NJ). About 2 ml was poured on the membrane which was rocked back and forth for 1 minute to ensure even coverage over the membrane. For the case where x-ray film was used to detect light emission by ECL reagent, the membrane was then drained but not dried on a paper towel, and was then sealed in saran wrap. Then the sealed membrane was taped to the inside of a film exposure cassette with the protein-bearing side up. After overlaying a Kodak BioMax x-ray film (Eastman Kodak, Rochester, NY, USA) in the darkroom, the cassette was closed and the x-ray films were exposed for different times depending on fluorescence intensity of the particular sample. The exposed x-ray films were then developed in Kodak M35-A X-OMAT processor (Eastman Kodak, Rochester, NY, USA). For cases in which Storm Scanner System (Amersham Biosciences, Piscataway, NJ, USA) was used for detection, the ECL plus reagent was used, the excess ECL plus reagent was more thoroughly removed such that the membrane was only damp but not completely dry. The chemiluminescence signal was then scanned and the image was quantified by ImageQuant 5.1 software (Amersham Biosciences, Piscataway, NJ, USA).

The Storm Scanner detection method has some advantages over "classical" film densitometry; principally the much larger range of linearity in the relationship between probe signal intensity and the corresponding response output of the detector. This is because the film blackening is not proportional to light exposure for very low light levels or short exposure times, and the level of blackening (film optical density) saturates after higher intensities or longer exposures. For quantitative measurements with film densitometry, it is therefore important to ensure the densitometry measurements are within the linear range of exposure signal vs. densitometry response.

It appears to be common practice to "trim away" all bands and signals on the film or digital image (eg, Storm scan) of the western blot except that near the band(s) judged to be a result of the protein of interest. The judgment is usually from a set of molecular weight markers, with the assumption that the protein of interest will have an identical electrophoretic mobility as that of the marker set, or better, by comparison with standards known to contain the protein of interest. However, this practice obscures other bands that may result, for example, from degradation products of the protein of interest, or from other cross-reacting proteins with epitopes similar enough for recognition by the antibody used. For illustrative purposes, to indicate the degree of specificity of the antibodies used and at the same time showing both the proteins of interest as well as the loading controls, in some cases I have adjusted concentrations of the different antibodies so that simultaneous incubation with all antibodies together followed by secondary antibody detection would not result in overexposure of the films. When even with this concentration adjustment it was not possible to obtain film densities in the linear range for all the bands with a single film exposure time, several exposure times of the same

western blot filter were carried out so that band densities in the linear range could be found with a corresponding reference loading control band. In other cases, I have used Storm digital image scans where the linear range of signal input vs. detection output is several orders of magnitude broader than is the case for film densitometry.

The question then is: why not use digital image scans and analysis exclusively, and discard the use of photographic films altogether? A very good argument can by made for exclusive use of the direct image analysis approach, but I did not use this exclusively for the following reasons. First, when I started the project I was obtaining very "clean" western blots with the x-ray films and the ECL chemiluminescence approach, and by knowing the limitations and difficulties that have to be considered with this approach the resulting quantification could still be obtained. Second, neither our laboratory nor our department had a Storm scanner or other image analysis system. The Biochemisry and Molecular Biology Department does have such a system and I have used this for some of the work reported. Early attempts to use the Storm scanning direct image analysis with the ECL Plus reagent resulted in blots that had a high and very uneven background. This problem has lessened with more experience, but even at the time of writing, the images using the Storm system are not as "clean" as my x-ray film exposures. While good quantitative data has been obtained by this method, and it may be inherently better in principle, this does not invalidate film densitometry, so long as the precautions noted above are taken. A more complete reference and discussion on film densitometry was recently reviewed by Fournier *et al* (Fournier *et al.* 2003).

Details and examples of the film densitometry and Storm 860 Scanner digital imaging methodologies are given in Appendix II.

Immunocytochemistry

Immunocytochemistry for DNA-PKcs

Cells were grown on Lab-Tec™ 2-chamber-slides (Nunc, Rochester, NY) in α-MEM medium. Immunocytochemistry was carried out three days after the second transfection. After each step, reagents were removed by aspiration, with care being taken to avoid drying of slides between steps.

Cell cytoplasm was first labeled with Vybrant™ CFDA SE Cell Tracer kit (Molecular Probes, Eugene, OR, USA) that contains CFDA SE (carboxyfluorescein diacetate, succinimidyl ester). It passively diffuses into the cells and is not fluorecent until its acetate groups are cleaved by intracellular esterases to yield a highly fluorescent, amine-reactive carboxyfluorescein succinnimidyl ester. The fluorescent conjugates in the labeled cells are retained and can be fixed with aldehyde fixatives.

Approximately 30 minutes before the start of the immunocytochemistry procedure, the culture medium was removed from the chamber slide and pre-warmed PBS containing the dye was added to each chamber. After incubating 15 minutes at 37℃, the dye solution was replaced with fresh medium and the slides were incubated another 30 minutes at 37℃.

The medium was then removed, and the chambers were rinsed with PBS at room temperature. Cells were then fixed for 10 minutes in 4% paraformaldehyde in PBS at 4℃. After washing with PBS, cells were permeabilized to allow penetration of the antibodies by adding 0.2% Triton X-100 in PBS for 5 min at room temperature. After three rinses in PBS to remove the Triton X-100, 10% goat serum (Sigma, St. Louis, MO, USA) in PBS was added for 45 minutes to suppress non-specific binding of IgG

(blocking). The chambers were then detached from slide, and 50 μl of 1: 200 diluted mouse anti-DNA-PKcs Ab-4 (Neomarkers, Fremont, CA, USA) diluted in 3% BSA in PBS were added to each sample and covered with a coverslip to prevent evaporation during a one-hour incubation in a wet chamber at 37℃. After incubation with the primary antibody, the slides were washed with three changes of PBS in a glass Coplin jar for 5 minutes each. To detect the primary antibody, the slides were incubated for 45 minutes at 37℃ with Rhodamine labeled goat anti-mouse (Sigma, St. Louis, MO, USA) secondary antibody that was diluted 1:2000 in 1.5% blocking serum. The secondary antibody solution was then removed by washing with three changes of PBS in a glass Coplin jar for 5 minutes each. Slides were then counterstained with 0.4 μg/ml 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) in antifade (10mg p-Phenylenediamine and 9 ml glycerol in 1ml 1 x PBS solution) and mounted with a coverslip. Slides were viewed under an Olympus Provis fluorescent microscope (Olympus, Melville, NY, USA) with FITC, Rhodamine and DAPI excitation and emission filter. Photographs were taken by a Photometrics SenSys CCD camera (Photometrics, Tucson, AZ, USA) and processed with Applied Imaging's MacProbe imaging software (Applied Imaging, Santa Clara, CA, USA).

Immunocytochemistry for DNA-PKcs and ATM

For the simultaneous immunocytochemistry for detection of both DNA-PKcs and ATM, the DNA-PKcs procedure was carried out as described above, but in addition at the stage of primary antibody addition, a 1:200 diluted mouse anti-DNA-PKcs Ab-4 (Neomarkers, Fremont, CA, USA) and 1:2000 diluted rabbit anti-ATM polyclonal

antibody (Novus, Littleton, CO, USA). These dilutions were in 3% BSA in PBS. For the secondary antibodies, 1:2000 diluted Rhodamine labeled goat anti-mouse (Sigma, St. Louis, MO, USA) and 1:800 FITC labeled goat anti-rabbit (Novus, Littleton, CO, USA) antibodies diluted in 1.5% blocking serum were used.

Induction of Interferon Response

It has been reported that in some instances, especially using certain shRNAs [2] or high concentrations of certain siRNAs that an "interferon response" can be induced. This results in the general shut-down of protein synthesis. To ensure this was not the case for the siRNA treatments described here, I deliberately induced an interferon response as described by others (Conn 2003) and measured this by induction of the appearance of phosphorylated protein kinasePKR which is a key element induced during this response. I then measured the level of phosphorylated PKR in the extracts of siRNA treated cells to determine whether this might be a problem in my studies. To induce the response for measurements of phosphorylated PKR, normal human fibroblasts GM08399 cells were transfected (Oligofectamine) with 1 μM poly(I)·poly(C) (Sigma, St. Louis, MO, USA). Two days after transfection, cells were harvested and Western blotting analysis of phosphorylated PKR was performed after 20 μg of total protein was loaded and separated in a 10% SDS-PAGE gel.

Rabbit anti-phosphorylated PKR antibody (Sigma, St. Louis, MO, USA) diluted 1:1000 was used to detect the induced phosphorylated PKR protein followed by a labeled mouse anti-rabbit secondary antibody as described above for other protein detections

[2] Short hairpin RNAs (shRNAs) are linear RNAs produced by incorporated vectors containing reverse complementary sequences separated by a short spacer so that a short hairpin dsRNA is produced that acts in the RNAi pathway

Chromosome Damage Assay

Interphase Premature Chromosome Condensation (PCC)

GM08399 human fibroblasts cells were transfected twice with two days between transfections either with the two DNA-PKcs siRNAs or control siRNA. There was no subculture between these two transfections. Three days after the second transfection, cells were confluent (density-inhibited monolayers) and were irradiated with a dose of 5 Gy.

After irradiation the cells were incubated for 4 hours to allow for completion of the majority of post-irradiation DNA damage processing, and the cells were then harvested and processed for induction of premature chromosome condensation (PCC). The PCC procedures closely followed those described by Bedford (Bedford and Mitchell 1977; Cornforth and Bedford 1983a; Bedford and Goodhead 1989; MuhlmannDiaz and Bedford 1994).

Before starting the induction of PCC, a 37℃ water bath (beaker) was prepared in a CO_2 incubator. Concentrated UV inactivated Sendai virus and mitotic HeLa cells (HeLa-M; see below) were taken from a -80℃ freezer and after thawing, were kept on ice. Serum free medium for washing cells was also prepared and kept on ice.

Cells were harvested from six-well plates by treatment with 0.25% trypsin / 0.1% EDTA (Gibco, Grand Island, IL, USA) and resuspended in cold α-MEM medium with 15% fetal bovine serum (Sigma, St. Louis, MO, USA) and put on ice.

The cell number was counted, and for each fusion, 0.5 x 10^6 interphase GM08399 cells were mixed with an equal number of mitotic HeLa cells (see below) in cold medium in a 15-ml plastic centrifuge test tube. Cells were mixed well, centrifuged at 200 x g for 4 minutes, the supernatant was discarded, and the cell pellet was resuspended in 10 ml of

ice-cold serum-free medium. The cells were centrifuged again, and the supernatant was decanted and the tube drained by keeping it upside down on a paper towel. UV inactivated Sendai virus was then added directly to the pellet to give a final concentration of 100 hemagglutinating units (HAU)/ml after immediate addition of 0.7 ml of ice-cold serum-free medium. The cells were mixed with virus by tapping the tube many times (not vortexed). The mixed cells/virus were kept on ice for 15 minutes with the tube cap closed to allow cell agglutination by the virus. Then the tubes were incubated for 10 minutes in a 37℃ water bath with cap closed to start the cell fusion and PCC process. After 10 minutes, 5 ml medium with fetal bovine serum (Sigma, St. Louis, MO, USA) and 0.2 μg/ml Colcemid® was added to aid in eluting the virus to prevent excessive lysis of the cells. The tubes were incubated in a 37℃ water (beaker) bath with cap open in a CO_2 incubator for an additional 60 minutes.

The cell fusion and induction of PCC is mostly completed after 15 minutes of 37℃ incubation, but by delaying the fixation until 1 h of incubation, somewhat more condensation and compaction of the PCCs is obtained. After this, the cells were centrifuged down and the supernatant were removed. The cell pellet was gently resuspended in 7 ml 37℃ prewarmed 0.075 M KCl hypotonic solution, and the tubes were allowed to stand in a 37℃ water bath for 15 minutes. After the hypotonic treatment, 7 ml of freshly prepared methanol: acetic acid (3:1) was added to each tube without removing the KCl solution. The tubes were then centrifuged and the supernatant was removed. The cells were washed twice in fresh fixative and resupended in a small volume. Cells were dropped onto chilled wet slides and air dried.

The slides were stained with 5% Gurr® R66 Giemsa stain (BDH Chemicals Ltd, Poole, UK) in Sorensen's buffer (pH 6.8) for 5 minutes and rinsed briefly with distilled water. After the slides were dried, they were scored under a Nikon Microphot® microscope (Nikon Inc, Garden City, NY, USA) and HR-2000 high resolution monitor (Gate-Mti Inc, Michigan City, IN, USA).

The total number of PCCs and fragments was scored. The frequency of "excess PCC fragments per cell" was then estimated by subtracting the total number per cell in unirradiated samples from that for the irradiated samples. For unirradiated cells, the total number of PCCs per cell was tightly distributed around 45-46.

Preparation of Mitotic HeLa Cells

HeLa cells were maintained in α-MEM supplemented with 15% fetal bovine serum (Sigma, St. Louis, MO, USA). HeLa cells have a cell cycle of G1 phase about 10-12 hours, S about 8 hours, G2 phase about 2 hours and M phase about 1 hour.

Approximately 2.5 x 10^6 HeLa cells were inoculated into T150 flasks. After 12 – 25 hours, thymidine was added to a final concentration of 2.5mM. Thymidine at this concentration arrests all DNA synthesis (Bostock *et al.* 1971). Cells originally in G2, M or G1 phase accumulate at the G1/S border, while cells in S phase remain in S. After 16+ hours, flasks were rinsed with TdR-free medium twice, then α-MEM with 15% FBS was added. After 9-12 hours, a thymidine block was applied as before. This second block accumulates all cells at the G1/S border. After 16+ hours, flasks were rinsed with TdR-free medium twice, α-MEM with 15% FBS was added and Colcemid® was added to a final concentration of 0.2 μg/ml. This step allowed all cells to progress through S and G2

and to be blocked in mitosis at metaphase. After 12-15 hours from the last rinse, mitotic cells were shaken off (should be nearly all of the population). A small aliquot was removed for the determination of mitotic index. Usually more than 90% of the HeLa cells collected in this manner were in mitosis. Cells were centrifuged at 1000 rpm for 10 min at 4°C and resuspend in 10 ml Hanks' balanced salts solution made up without the addition of glucose (HBSS-G), and containing 0.2 μg/ml Colcemid®. The cells were centrifuged again and resuspended in HBSS-G plus Colcemid®. Cells were dispensed in aliquots of 1.0 x 10^6 each and frozen in -80°C in Borrelli's freezing medium (Borrelli *et al.* 1987).

Large batches of Sendai virus have been prepared previously in this laboratory and inactivated by exposure to U.V. light (Cornforth and Bedford 1983a) closely following procedures described by Johnson and co-workers (Johnson and Rao 1970).

Aberrations in Mitotic Chromosomes

In the same experiment described for PCC break rejoining measurements, other parallel samples were subcultured and allowed to progress to mitosis for scoring aberrations in metaphase cells. For cytogenetic analysis of the aberrations initially produced in cells, it is important to examine only the metaphases of first post-irradiation mitotic cells. About 50% of the cells carrying "unstable" chromosome aberrations are expected to be eliminated during each mitosis, so the frequency of cells bearing aberrations and the level and kinds of aberrations seen will vary depending on the number of cell generations that have elapsed since irradiation treatment. By adding Colcemid® and fixing cells that were irradiated in G1 or G0 at various time intervals after subculture,

we have seen that for the first 20 to 24 hours after subculture the mitotic index remained near zero and then began to increase in the 30 to 36 hour interval. By thus following the time of appearance of the first wave of mitotic cells after subculture we can ensure that virtually all mitotic cells scored for aberrations were first post-irradiation mitotic cells.

Preparation of Mitotic Chromosome Spreads

Metaphase chromosome spreads were prepared as described by Cornforth and Bedford (Cornforth and Bedford 1987). Briefly, Colcemid® was added to samples in a final concentration of 0.1 μg/ml during the intervals 18-22, 22-26, 26-30 and 30-34 hours after subculture. Each sample was incubated for 4 hours and cells were harvested from six-well plates by treatment with 0.25% trypsin / 0.1% EDTA and resuspended in α-MEM medium with 15% fetal bovine serum (Sigma, St. Louis, MO, USA). After centrifugation for 5 minutes at 1000 rpm, cell pellets were resuspened in 7 ml 0.075 M KCl hypotonic solution for 15 minutes at 37℃ waterbath. A total of 7ml fresh fixative (methanol: glacial acetic acid 3:1, room temperature) was added to the suspension. After centrifugation, the cells were washed twice with 7 ml fixative. After the last wash, cells were resuspended in approximately 1 ml fixative, and cells were then dropped onto chilled wet slides (VWR, West Chester, PA, USA). The remaining cell suspension was stored in 15 ml test tubes at -20℃.

The slides were stained with 5% Gurr® Giemsa stain (BDH Chemicals Ltd, Poole, UK) in Sorensen's buffer (pH 6.8) for 5 minutes and rinsed briefly with distilled water. After the slides were dried, Slides were sealed with Cytoseal 60 (VWR, West Chester, PA, USA) under a coverslip. After the slides were dried, they were scored under a 100X

oil immersion lens using a Nikon (Nikon Inc, Garden City, NY, USA) (Nikon Inc, Garden City, NY, USA) equipped with a video camera and HR-2000 high-resolution monitor (Gate-Mti Inc, Michigan City, IN, USA) to aid in scoring.

Chromosome Aberrations

All slides analyzed in this study were coded and randomized as a precaution to reduce or eliminate any unintended bias that may be present when scoring samples for aberrations. Each slide was scored by the same observer for both mitotic index (MI, the percentage of mitotic cells) and extent of chromosome damage (frequency of chromosomal aberration in 100 metaphase cells). The MI's were obtained from counting 1000 cells. Chromosome damage was assessed in mitotic cells that appeared to be intact. Cells with less than 46 chromosomes were considered to have arisen from broken cells and were not counted. Chromosome aberrations were scored based on the classification of Savage (Savage 1976). Only dicentrics, centric rings, interstitial deletions, terminal deletions, chromatid breaks and chromatid gaps were scored. Very few chromatid exchange aberrations were seen. A dicentric along with an accompanying acentric fragment was considered as one aberration (a dicentric). An excess acentric fragment was considered an interstitial deletion if it was smaller in length than the width of a chromatid and as a terminal deletion if it was larger. A centric ring along with its accompanying acentric was also considered as one aberration (a centric ring). A chromatid break was considered to be separated by a large gap and usually displaced whereas the distal portion of chromatid with a chromatid gap had a smaller separation from the proximal portion.

Statistical Analysis of Aberration Frequency Estimates

At least 100 metaphases were evaluated, and 95% Poisson confidence limits were determined for the mean number of aberrations per cell according to the method of Johnson and Kotz (Johnson and Kotz 1969).

Cell Survival

Irradiation Procedure and Dosimetry

Irradiation was carried out using a 6000 Ci ^{137}Cs JL Shepherd Mark-1 irradiator (J. L. Shepherd & Associates, San Fernando CA, USA). The dose rate was 250 rads / minutes. The irradiator setup allowed for the simultaneous irradiation of 6 T-25 flasks. All irradiation were at room temperature.

Dosimetry was obtained using both ionization chambers and by thermoluminescence dosimetry (TLD), and has been checked periodically in this laboratory.

Colony Formation Assay

Cell survival responses were measured after irradiation of log phase cultures using colony formation as the criterion for cell survival. The siRNA transfection protocol for log phase cultures was similar that for the treatment of the contact-inhibited plateau-phase cultures, except cells were subcultured after the first siRNA transfection so they would still be growing and would be about 80% confluent two days after the second transfection. Two days after the second transfection, cells were trypsinized with 0.25% trypsin / 0.1% EDTA (Gibco, Grand Island, IL, USA) and resuspended in fresh α-MEM

medium with 15% fetal bovine serum (Sigma, St. Louis, MO, USA). The cells were passed through a 21-gauge needle to minimize cell clumping. Cell numbers were counted with a hemocytometer and appropriate number of cells were inoculated into T-25 flask to give 25–200 colonies per flask. Three flasks were prepared per dose point. 8 hours after plating, flasks were irradiated with doses of 0, 0.125, 0.25, 0.375, 0.5, 0.75, 1, 2, 4, 5 and 7Gy.

Flasks were then incubated at 37°C for 11 days for lower dose exposures of 0, 0.125, 0.25, 0.375, 0.5, 0.75, 1Gy doses, for 12 days for 2 Gy doses and 14 days for 4, 5 and 7 Gy doses. After colony formation the flasks were fixed with methanol and stained with Gurr® crystal violet (BDH Chemicals Ltd, Poole, UK). Flasks were coded and colonies counted under a low power dissecting microscope without knowing the treatment received by the flask being scored. Colonies containing more than 50 cells were considered to have arisn from single surviving cells.

Data Analysis for Survival Assays

The plating efficiency (PE) was measured in several untreated control flasks as the number of colonies counted divided by the number of cells plated. Surviving fractions (SF) were calculated by determining the ratio of the number of colonies in each irradiated flask to the product of the number of cells plated times the mean PE in zero dose controls.

Fitted lines were obtained by using standard regression analysis programs within Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA, USA) and Sigma Plot 2001 (SPSS, Inc. Chicago, IL, USA). Standard deviations were calculated for each value based on the variances for the respective numbers.

Real-time RT-PCR

General considerations

Since contamination risks from nucleic acids other than from the target sample exist for sensitive molecular biological methods like real-time RT-PCR, some precautions have to be followed regarding the laboratory equipment and good laboratory practice.

Separate working rooms or areas were used for the preparation and extraction of RNA, as well as the master mix for reverse transcription and real-time PCR. Separate sets of micropipettes were used, especially for the extraction of RNA and the preparation of master mix. RNase Zap wipes (VWR, West Chester, PA, USA) were used very time to clean the pipettes and working area. RNase free pipette tips with barrier were used. Disposable gloves were changed between every step.

Sample Preparation and RNA Extraction

Normal human fibroblast GM08399 cells were transfected with DNA-PKcs siRNA or control siRNA twice in a two days interval schedule. Samples were taken 4 hours after the first transfection and then every day until day eight.

RNA was extracted from cells using the QIAGEN RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) spin protocol for small volumes. Briefly, cells were harvested by 0.25% Trypsin / 0.1% EDTA. Cells were centrifuged down at 300 x g for 4 minutes. The supernatant was discarded and cell pellet was lysed and homogenized in a QIAshredder spin column. Total RNA was extracted according to the manufacturer's instructions. The

extracted RNA was eluted in 80 µl RNase-free water and frozen at -80 °C until further use.

The integrity and size distribution of purified total RNA were checked by electrophoresis in 1.5% agarose gel and ethidium bromide staining to visualize the 28*S* and 18*S* subunits of the ribosomal RNA bands under UV transillumination. These bands should appear sharp. If not, but appear as a smear of smaller sized RNAs, it is likely that the RNA samples suffered major degradation during preparation. This did not occur for the samples described for this study. The RNA content of the samples was quantified by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. The purity was estimated by the ratio of the reading at 260 nm and 280 nm (A_{260}/A_{280}).

The RNA was high quality and free from genomic DNA contamination. This was confirmed by RT-PCR for the GAPDH gene, using two primers located in two different exons. If there was a generalized cellular DNA contamination in the presumed pure RNA sample, then the RT-PCR analysis for the gene product in question could be erroneous because a product would be formed not only from the mRNA but from the contaminating DNA as well. Suitable RT-PCR primers in adjacent introns could not be found for DNA-PKcs or ATM, but good primer candidates were found for the GAPDH gene. If there was DNA contamination in the supposedly pure RNA samples there should be a 2000bp product from the presence of GAPDH DNA in the samples being tested for DNA-PKcs or ATM mRNAs. The polyacrylamide gel electrophoresis of the PCR products showed a single band of about 200bp which is the expected size for the spliced mRNA product. The PCR from genomic DNA sequence should yield a product of about 2000bp, but no such product was produced (see Appendix III).

cDNA synthesis

An equal volume of total RNA (10 µl) was reversed transcribed to first strand cDNA using SuperScript II (Invirogen, Carlsbad, CA, USA). Reverse transcription was carried out in a final volume of 20 µl two-step RT-PCR reaction. Reverse transcription master mix (See Table 2-1) was assembled on ice. Briefly, 10 µl of isolated RNA were mixed in a 250 µl PCR tube with 10 µl reverse transcription master mix on ice within a laminar flow. In a final volume of 20 µl, the reaction conditions were 1x first strand buffer, 500 µM each dNTP, 10 units RNaseOut® RNase inhibitor (Invirogen, Carlsbad, CA, USA), 50 units SuperScript® II, 600 ng random hexamers (Invirogen, Carlsbad, CA, USA) and 10 µl template-RNA. Strip caps were vigorously pressed on PCR tubes. Tubes were immediately placed in a preheated Hybaid thermocycler (Hybaid Limited, Teddington, Middlesex, UK).

Table 2-1. Reverse Transcription Mixture

Reagent	Concentration	Amount needed per reaction	Final concentration
First Strand Buffer	5X	4 µl	1X
dNTP	10 mM	1 µl	500 µM
DTT	0.1 M	1 µl	5 mM
RNase Out	40 U/µl	0.25 µl	0.5 U/µl
SuperScript II	200 U/µl	0.25 µl	2.5 U/µl
Random Hexamers	300 ng/µl	2 µl	30 ng/µl
RNA		10 µl	
H_2O		1.5 µl	
Total		20 µl	

The reverse transcriptase reaction was carried out at 5 minutes at 25 °C, 50 minutes at 42 °C followed by 5 min at 95 °C to inactivate the reverse transcriptase. Samples were frozen at -80 °C until further use.

Primers and Probes Design

The real-time RT-PCR probes and primers sets for the detection of human *DNA-PKcs*, *ATM* and *α-Tubulin* genes were selected from open reading frame regions of the genomic sequences through the use of Primer Express software 1.5A (Applied Biosystems, Inc, Foster City, CA, USA). The TaqMan probes were designed so that the predicted melting temperature was at least 10°C higher than the predicted melting temperature of the primers. All primer and probe sequences were BLAST searched in GenBank to ensure unique sequences for the target genes.

The primer set used to detect *DNA-PKcs* (GenBank accession no. NM_006904, gi no. 31340617) cDNA generates an 118bp (nucleotides 11822-11939) DNA product after amplification. The forward primer was 5'-ACAGACATCTGAACAACTTTATGGT -3' and the reverse primer was 5'-AAAGGCATCAACTCAGGGACT -3'. The TaqMan probe, 5'-CCATGGAGACTGGCGGCGTGAT -3', which corresponds to the region from nucleotides 11849-11870, was synthesized and labeled with fluorescent dye FAM® (6-carboxyfluoroscein) on the 5' end and quencher BHQ_1® (blackhole 1) on the 3' end.

The primer set used to detect *ATM* (GenBank accession number NM_000051, gi no. 20336202) cDNA generates a 91bp (nucleotides 1481-1571) DNA product after amplification. The forward primer was 5'- AGCTGTCTCCATTACTGATGATACT -3' and the reverse primer was 5'- TCCGTAAGGCATCGTAACACATA -3'. The TaqMan

probe, 5'- CAGCTTCTACCCCAACAGCGACATGG -3', which corresponds to the region from nucleotides 1510-1535, was synthesized and labeled with fluorescent dye FAM® (6-carboxyfluoroscein) on the 5' end and quencher BHQ_1® (Blackhole 1) on the 3' end.

The primer set used to detect *α-tubulin* (GenBank accession number BC000696, gi no.12653814) cDNA generates an 118bp (nucleotides 463-580) DNA product after amplification. The forward primer was 5'- GCAAGCTGGCTGACCA -3' and the reverse primer was 5'- CCATAATCAACTGAGAGACGT -3'. The TaqMan probe, 5'- CACCGGTCTTCAGGGCTTCTTGGTT -3', which corresponds to the region from nucleotides 482-506, was synthesized and labeled with fluorescent dye FAM® (6-carboxyfluoroscein) on the 5' end and quencher TAMRA® *(N,N,N',N'*-tetramethyl-6-carboxyrhodamine) on the 3' end. These primer sets are shown in Table 2-2.

The probes and primer sets of *DNA-PKcs* and *ATM* were synthesized by IDT (Integrated DNA Technology, Coralville, IA, USA). The probes and primer sets *α-tubulin* were synthesized by MWG Biotech Inc (High Point, NC, USA).

TaqMan PCR Reagents

TaqMan Master Mix containing nucleotides, AmpliTaq Gold® Polymerase, $MgCl_2$, AmpErase® Ung was purchased from ABI (Applied Biosystem, Inc, Foster City, CA, USA). The plates, optical sealing film and tubes were purchased from BioRad (Hercules, CA, USA). The probes and primers were synthesis at MWG Biotech (High Point, NC, USA) and IDT (Coralville, IA, USA).

Table 2-2. Primer sequences used in real-time PCR

Gene Name	GenBank No	Forward Primer	Sequences	Tm (°C)	Amplicom
DNA-PKcs	NM_006904	Forward Primer	5' ACAGACATCTGAACAACTTTATGGT 3'	57.8	118bp
		Reverse Primer	5' AAAGGCATCAACTCAGGGACT 3'	57.5	
		Probe	5' CCATGGAGACTGGCGGCGTGAT 3'	65.7	
ATM	NM_000051	Forward Primer	5' AGCTGTCTCCATTACTGATGATACT 3'	58.3	91bp
		Reverse Primer	5' TCCGTAAGGCATCGTAACACATA 3'	58.4	
		Probe	5' CAGCTTCTACCCCAACAGCGACATGG 3'	66.8	
α-Tubulin	BC_000696	Forward Primer	5' GCAAGCTGGCTGACCA 3'	59	118bp
		Reverse Primer	5' CCATAATCAACTGAGAGACGT 3'	58.6	
		Probe	5' CACCGGTCTTCAGGGCTTCTTGGTT 3'	68	

Optimization of real-time QRT-PCR procedure

All PCR products were initially confirmed by size using polyacrylamide gel electrophoresis. Using serial dilutions of cDNA to generate a standard curve, I determined the optimal primer and probe concentrations that exhibited the best efficiency for *DNA-PKcs, ATM* and *α-Tubulin* detection over a broad dynamic range.

Real-time PCR Protocol

Total RNA derived from normal human fibroblast cells was reverse transcribed as described above. PCR reactions for the *DNA-PKcs, ATM* and *α-Tubulin* genes were carried out by using these cDNA and PCR primers.

PCR reaction conditions were optimized for each gene evaluated, with the variables including cDNA, probe, and primer concentrations, as well as and annealing temperature.

A five-tube RT-PCR was optimized for the detection and quantitation of *DNA-PKcs*. The PCR was carried out in a 0.2 ml 96-tube-plate covered with an optical sealing film. The reaction mixture was made up to a volume of 25 μl containing 12.5 μl 2x TaqMan universal PCR mix, probe, primer sets and 0.5 μl cDNA. *DNA-PKcs* was amplified with a final concentration of 600 nM of each primer and 600 nM of probe in a 25 μl volume. *ATM* was amplified with a final concentration of 600 nM of each primer and 600 nM of probe in a 25 μl volume. *α-Tubulin* was amplified with a final concentration of 600 nM of each primer and 600 nM of probe in a 25 μl volume. 10 μl mineral oil was added to each tube to prevent evaporation.

After samples were sealed with an optical sealing film, the 96-tube-plate centrifuged 5 seconds at 700x *g* at room temperature.

The PCR reaction was carried out at 25°C for 2 minutes and 95°C to activate the AmpliTaq Gold® Polymerase, then followed by 50 cycles of 95°C for 15 seconds, 60°C for 1 minute using an iCycler iQ® real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer specification.

During amplification, the plate was automatically scanned at 518 nm to measure the amount of FAM released at each PCR cycle. An amplification plot was generated after the PCR was done. The threshold cycle (C_T) represents the cycle at which the fluorescence signal generated during amplification by cleavage of the probe passes above a threshold baseline.

Serial dilutions of cDNA were used as a standard curve for the relative quantification of mRNA in each sample. The starting copy number in samples was calculated by comparison of their C_T values to those of a standard curve.

Analysis of Real Time RT-PCR Data

The relative *DNA-PKcs* and *ATM* mRNA levels were calculated by normalizing the mRNA level of the *α-Tubulin* and PCR efficiencies for each individual sample. Gene expression data were processed following algorithms described by Muller (Muller *et al.* 2002).

In order to obtain accurate and reproducible results, reactions should have efficiencies as close to 100% as possible. Several design factors can influence efficiency such as the length of the amplicon, the G/C content of the amplicon and secondary

structure. The dynamics of the reaction itself can also influence efficiency. Variations in the dynamics can result from such sources as the enzymes used in the reaction and non-optimal reagent concentrations.

During exponential amplification, the yield Y_N after N cycles of initial Y_0 copies is $Y_N = Y_0 (1 + E)^N$, where N is the number of PCR cycles, E is the PCR efficiency. At efficiency of 100% the template doubles after each cycle during exponential amplification.

The slope of the serial dilutions of the cDNA standard curve can be used to determine the PCR amplification efficiencies (E) for the exponential amplification as follows:

$$E = 10^{-1/\text{slope}} - 1 \qquad \text{(Eq. 1)}$$

The equation for the calculation of the normalized gene expression (NE) as given by Muller (Muller *et al.* 2002) is:

$$NE_{\text{DNA-PKcs}} = \frac{(E_{\alpha\text{-}Tubulin} + 1)^{CT_{\alpha\text{-}Tubulin}}}{(E_{DNA\text{-}PKcs} + 1)^{CT_{DNA\text{-}PKcs}}} \qquad \text{(Eq. 2)}$$

where $E_{\alpha\text{-}Tubulin}$ is the PCR efficiency of the *α-Tubulin* gene, $E_{DNA\text{-}PKcs}$ is the PCR efficiency of the *DNA-PKcs* gene, $CT_{\alpha\text{-}Tubulin}$, is the threshold cycle of the *α-Tubulin* gene (10 times the mean standard deviation of the fluorescence collected in all wells over the baseline cycles), and $CT_{DNA\text{-}PKcs}$, is the threshold cycle of the *DNA-PKcs* gene;

The standard error of the mean, is an estimate of the standard deviation of a sampling distribution of means. It is based on determining the means from several different independent samples from a population and then estimating the mean of these

means and standard deviation of this sampling of means. Standard errors reflect how much sampling fluctuation a statistic such as a mean value will show if one or more additional means are taken from other samples from the same parent population.

The standard error (SE) is calculated as:

$$\text{SE} \qquad \sigma = \sqrt{\sum (x_i - \mu)^2 / n \bullet (n-1)} \qquad \text{(Eq. 3)}$$

Where X_i, is value of independent measurements; μ, is the mean value of the independent measurements; n, is the number of independent measurements.

The equation for the calculation of the mean normalized gene expression (MNE) using the mean C_T value for *DNA-PKcs* and *α-Tubulin* gene given by Muller (Muller *et al.* 2002) is:

$$\text{MNE}_{DNA\text{-}PKcs} = \frac{(E_{\alpha\text{-}Tubulin} + 1)^{CT_{\alpha\text{-}Tubulin,\ mean}}}{(E_{DNA\text{-}PKcs} + 1)^{CT_{DNA\text{-}PKcs,\ mean}}} \qquad \text{(Eq. 4)}$$

So the equation for the calculation of the standard error of mean normalized gene expression is:

$$\Delta\sigma = \text{MNE}_{DNA\text{-}PKcs} \bullet \sqrt{(\ln(E_{DNA\text{-}PKcs}+1) \bullet C_{T DNA\text{-}PKcs,\ mean})^2 + (\ln(E_{\alpha\text{-}Tubulin}+1) \bullet C_{T \alpha\text{-}Tubulin})^2} \qquad \text{(Eq. 5)}$$

The following is an example of the relative quantification of the *DNA-PKcs* mRNA at day 5, which was performed3 days after the second *DNA-PKcs* siRNA (Table 2-3) relative to the DNA-PKcs mRNA in cells after the control siRNA altered sequence transfection (Table 2-4).

The slopes from the standard curves of the PCR for the DNA-PKcs and *α-Tubulin* gene were –3.4068 and –4.542, respectively. The calculated PCR efficiencies in this case were

$$E_{DNA\text{-}PKcs} = 10^{-1/-3.4068} - 1 = 10^{0.2935} - 1 = 96.6\%$$

$$E_{\alpha\text{-}Tubulin} = 10^{-1/-4.542} - 1 = 10^{0.2202} - 1 = 66\%$$

Table 2-3. Gene expression for the *DNA-PKcs* siRNA transfected

Description	C_T of *DNA-PKcs* Gene	C_T of *α-Tubulin* Gene	Normalized Expression	Mean Normalized Expression
Well # 1	32.6	27.3	2.74×10^{-4} [b]	
Well # 2	32.6	27.9	3.72×10^{-4} [b]	
Well # 3	33.0	27.7	2.56×10^{-4} [b]	
Well # 4	32.9	27.9	3.03×10^{-4} [b]	
Mean	32.775	27.7	3.01×10^{-4}	2.98×10^{-4} [c]
Standard Error (σ)	+/-0.103[a]	+/-0.141[a]	$+/-2.55 \times 10^{-5}$ [a]	$+/-2.97 \times 10^{-5}$ [d]
σ in %	+/-0.31	+/-0.51	+/-8.47	+/-9.97

[a]According to Equation 3; [b]According to Equation 2; [c]According to Equation 4; [d]According to Equation 5.

Table 2-4. Gene expression for the control siRNA transfected

Description	C_T of DNA-PKcs Gene	C_T of *α-Tubulin* Gene	Normalized Expression	Mean Normalized Expression
Well # 1	31.0	29.1	2.01×10^{-3}	
Well # 2	31.9	28.8	0.94×10^{-3}	
Well # 3	31.6	29.0	1.28×10^{-3}	
Well # 4	31.3	29.0	1.56×10^{-3}	
Mean	31.45	28.975	1.45×10^{-3}	1.39×10^{-3}
Standard Error (σ)	0.194	0.063	2.26×10^{-4}	1.88×10^{-4}
σ in %	0.62	0.22	15.6	13.5

So, the relative quantitation for the *DNA-PKcs* mRNA at this time points is

$$R = \frac{\text{Gene expression for the } \textit{DNA-PKcs} \text{ siRNA transfected from Table 3}}{\text{Gene expression for the control siRNA transfected from Table 4}}$$

$$= 2.98 \times 10^{-4} / 1.39 \times 10^{-3} = 0.2143$$

The standard error for this time points is calculated according to Bevington's book (Bevington 1969).

$$\sigma = \sqrt{(\Delta\sigma_{siRNA})^2 + (\Delta\sigma_{control\ siRNA})^2}$$

$$= \sqrt{(9.97\%)^2 + (13.5\%)^2}$$

$$= 16.78\ \%$$

For the PCR standard curves, fitted lines were obtained by using standard regression analysis programs within Microsoft Excel 2000 (Microsoft Corporation,

Redmond, WA, USA). Time course expressions of DNA-PKcs mRNA and ATM mRNA were plot using Sigma Plot 2001 (SPSS, Inc. Chicago, IL, USA).

CHAPTER 3

DNA-PKcs Silencing by siRNA Sensitizes Normal Human Cells for Radiation-Induced Chromosome Damage and Cell Killing

ABSTRACT

Targeted gene silencing in mammalian cells by RNA interference (RNAi) using small interfering RNAs (siRNAs) was utilized in cultured normal human cells to reduce expression of the *Prkdc* (*DNA-PKcs*) gene coding for the catalytic sub-unit of the DNA-dependent protein kinase, DNA-PKcs. This protein is involved in the nonhomologous end joining (NHEJ) of DNA double strand breaks. The siRNA knockdown of this protein in low passage normal human fibroblasts resulted in a reduced capacity for restitution of radiation-induced interphase chromosome breaks as measured by premature chromosome condensation (PCC), an increased yield of acentric chromosome fragments at the first post-irradiation mitosis, and an increased radiosensitivity for cell killing.

INTRODUCTION

Isolation and characterization of mammalian cell mutants hypersensitive to ionizing radiation has provided key evidence that a principal underlying defect involves faulty processing of DNA double strand breaks (Jeggo 1998a; Thompson and Schild 2001; Bedford and Dewey 2002). There are major difficulties associated with applying similar approaches for studying the genetic control of radiosensitivity in normal human cells. In 2001, Elbashir and Tuschl and colleagues reported a highly specific targeted gene silencing in mammalian cells by RNA interference (RNAi) using small interfering RNAs (siRNAs) (Elbashir *et al.* 2001a). To examine the potential general use of this approach for studying the genetic control of radiosensitivity in human cells, where comparisons could be made easily with cells of identical genetic backgrounds, the present study was initiated to target the mRNA transcript of the *Prkdc* (*DNA-PKcs*) gene coding for the catalytic sub-unit of DNA dependent protein kinase (DNA-PKcs). This protein is central to the NHEJ process as well as being involved in the maintenance of telomere stability (Bailey *et al.* 1999; Bailey *et al.* 2001). Specifically, the aims were to measure the effectiveness of this siRNA approach for "knocking down" DNA-PKcs levels using western blot analysis and immunocytochemistry, and to measure functional (phenotypic) effects on radiosensitization. The latter included measurements to determine whether the knockdown resulted in 1) reduced interphase (G_1) chromosome break restitution as measured by premature chromosome condensation (PCC) 4 hours after irradiation, 2) increased frequencies of chromosome aberrations in the first post-irradiation mitosis, and 3) increased cell killing in low passage normal human fibroblasts.

MATERIALS AND METHODS

siRNA Transfections

The siRNA sequence used for targeted silencing of *DNA-PKcs* was chosen as described by Elbashir (Elbashir *et al.* 2002) and recommended by the siRNA supplier (Dharmacon, Lafayette, CO, USA). Two duplex siRNA sequences were used separately or combined together. One was targeted 293 bases downstream from the start codon in the *DNA-PKcs* mRNA. This double stranded siRNA was

GAUCGCACCUUACUCUGUUdTdT (siRNA sequence A)
dTdTCUAGCGUGGAAUGAGACAA.

The other siRNA, targeted to the kinase domain, was

CUUUAUGGUGGCCAUGGAGdTdT (siRNA sequence B)
dTdTGAAAUACCACCGGUACCUC.

The complete *Prkdc* mRNA sequence is given in Appendix IV, with the sequences targeted by sequences A and B above highlighted, as well as the start codon position and the kinase domain.

A control sequence with the following modifications from sequence A was:

GAACGCACCUUCAUCUGUUdTdT (control siRNA)
dTdTCUUGCGUGGAAGUAGACAA

Searches of the human genome database (BLAST) were carried out to determine whether the sequences might obviously target other gene transcripts. The BLAST search takes consecutive sequences of lengths varying from the full 19 base length sequence chosen down to any 18, 17, or 16 base sequence contained in the chosen sequence and searches for any matches in mRNAs expressed from any other genes in any known

organism. No such matches were found in the human genome (Appendix IV). The concentration of siRNAs was 0.15 μM or in one case 0.03 μM during transfections, which were facilitated by Oligofectamine (Invitrogen). The concentrations were "nominal", in the sense that they are based on the quantity of siRNA stated by the supplier (Dharmacon). In later experiments reported in chapter 4, a new batch of anti-*Prkdc* siRNA was obtained from the supplier and another experiment to determine the siRNA concentration dependence of the knock down was carried out. For reasons that are not clear, this gave a different, and in fact lower, optimum concentration of siRNA for maximum knockdown. The transfection protocol followed that outlined by Elbashir and the siRNA supplier (Elbashir *et al.* 2002). For controls we used either Lamin A/C or Ku80-targeted siRNAs, or in more recent experiments, a *Prkdc*-derived sequence in which 2 or 3 base pairs were transposed. None of these control siRNAs affected DNA-PKcs protein levels, or the radiosensitivity phenotypes studied. Since protein turnover rates will determine the optimal transfection and irradiation or assay timing protocol, I carried out preliminary experiments which established that two successive transfections of log phase of cultures (~50% confluent) at two day intervals produced marked reductions in DNA-PKcs. Of course, other protocols I have not tried may also produce similar or superior DNA-PKcs knockdowns. Transfections were carried out in 6-well tissue culture plates (9.62 cm^2 surface per well) after inoculating 5 x 10^4 cells per well. A fully confluent well contains approximately 6 x 10^5 cells. The doubling time of these cells during log phase growth is approximately 24 to 30 hours. In most experiments with the fibroblasts, two days after inoculation and one day after siRNA transfection, cells were subcultured to one-half the cell density and transfected again one day later. Then,

three days later when irradiations or other assays were performed, cells were still growing and cultures were about 80 to 90% confluent. In one of the human normal fibroblast experiments, the cells were not subcultured after the first transfection, and following the second transfection cells were allowed to reach confluence and the following day the irradiations were carried out. Then, one day later, the cells were subcultured and aberrations were scored after colcemide collection of cells in the first post-irradiation mitosis, as reported in figure 3-7.

During the 6-day siRNA transfection protocol for the fibroblasts, in preparation for irradiation or other assays, the growth rate (doubling time) of the DNA-PKcs targeted siRNA transfected cell populations decreased by approximately 10% relative to control (altered sequence) siRNA transfection, but the growth was interrupted twice for approximately 20 hours for each transfection which was carried out in serum-free medium, and in most cases cells were subcultured to one-half density between transfections.

Western Blot and Immunocytochemistry

For western blot analysis, cells were prepared as described by Song and colleagues (Song *et al.* 1996). Gels were loaded with 20μg protein and after electrophoresis in 6% Tris-Glycine polyacrylamide gels, proteins were transferred to nitrocellulose membranes. After blocking in Tris buffered saline (TBS) containing 7% nonfat dry milk, membranes were incubated in TBS with 5% nonfat dry milk containing mouse antibodies against DNA-PKcs (AB-4), Ku 80 (Ab2) obtained from Neomarkers (Neomarker, Fremont, CA, USA), Lamin A/C (sc-7292) (Santa Cruz Biotechnology,

Santa Cruz, CA, USA), β-actin (NB 600-501) (Novus, Littleton, CO, USA). Goat anti-mouse secondary antibodies labeled with horseradish peroxidase were then used to bind the primary antibodies, and detection was by chemiluminescence (ECL kit, Amersham Pharmacia). X-ray films to detect the chemiluminescence were then exposed for appropriate times depending on luminescence intensity. For cases in which more accurate quantification was necessary, exposures were adjusted to give film densities that were in the linear range for densitometry measurements. The use of film dosimetry for quantification of western blots was recently discussed by Fournier (Fournier *et al.* 2003). In some cases described later a Storm 860 scanner image analysis instrument (Amersham Pharmarcia Biotech, Piscataway, NJ, USA) was used for quatifications. Further details are presented in Appendix II.

For immunocytochemistry, after the siRNA transfections and at the time selected for optimum knockdown, human fibroblast cultures of attached cells (grown on chamber slides for later microscopic observation) were fixed *in situ* in 4% paraformaldehyde in PBS, and then permeablized in PBS containing 0.2% TritonX-100. To detect DNA-PKcs, the same primary antibodies were used but detection in this case was with rhodamine labeled goat anti-mouse IgG and after staining cytoplasm Vybrant™ CFDA SE (Molecular Probes, Eugene, OR, USA) the cells were viewed by fluorescence microscopy.

Chromosome Damage Assays

Interphase PCC

At the time of irradiation, the cultures were confluent or near-confluent so that most of the cells were in a G1 or G0 state. Four hours after irradiation of confluent cultures, to allow for completion of much of the post-irradiation damage processing, cells were resuspended and fused to mitotic HeLa cells to induce premature chromosome condensation, or prematurely condensed chromosomes (PCC) and the total numbers of PCCs and fragments were scored as described on numerous previous occasions from work in this laboratory (Cornforth and Bedford 1983a; Cornforth and Bedford 1983b; Cornforth and Bedford 1985; Cornforth and Bedford 1987). The frequency of "excess PCC fragments per cell" was then estimated by subtracting the total number per cell in unirradiated samples from that for irradiated samples. For unirradiated cells the total number of PCCs was tightly distributed around 45-46. It has been shown on previous occasions that immediately after a dose of 5Gy there are about 25 excess PCC fragments produced in normal human fibroblasts in G0, and that the number of excess fragments decreases with a half-time of about 1 to 2 hours after irradiation due to rejoining of breaks. In repair deficient cells the number of residual excess fragments after long rejoining times is higher that for normal cells (Cornforth and Bedford 1983a; Cornforth and Bedford 1983b; Cornforth and Bedford 1985; Cornforth and Bedford 1987).

Mitotic Cells

In the same experiments described for PCC break measurements, other parallel samples were sub-cultured and allowed to progress to mitosis for scoring aberrations in

metaphase cells. Samples were fixed after incubation in the presence of 0.1 μg/ml Colcemid during intervals from 18-22, 22-26, 26-30, and 30-34 hours after subculture. We have shown that virtually no second division cells are present during these fixation intervals after irradiation, incubation, and subculture of these contact-inhibited cells. Procedures for hypotonic treatment of cells, methanol:acetic acid fixation, slide preparation and chromosome aberration scoring were carried out as described previously (Bender *et al.* 1973; Bender *et al.* 1974; Bedford *et al.* 1978; Cornforth and Bedford 1987). Aberration scoring criteria followed those outlined by Savage (Savage 1976).

Cell Survival

Cell survival responses were measured after irradiation of log phase cultures using colony formation as the criterion for cell survival. The siRNA transfection protocol for log phase cultures was similar to that for treatment of the contact-inhibited plateau phase cultures except, as mentioned earlier, cells were sub-cultured after the first siRNA transfection so that they would still be growing and cultures would be about 80% confluent two days after the second transfection. Since I knew that in many cases some 20% of cells are *not* successfully transfected, and if sensitization occurred in those 80% of cells that were successfully transfected, this would result in a cell population of mixed and heterogeneous radiosensitivity. In such cases, it is necessary to examine particularly the survival response of the population at the lowest doses possible because the rate of decrease in survival with dose is predominantly a reflection of the radiosensitivity of the most sensitive component or sub-population. In the higher dose range of dose-survival

response measurements, the rate of decrease in survival with dose is largely due to the most resistant sub-population.

RESULTS AND DISCUSSION

Some early literature had indicated that some siRNAs targeting different sequences of a given mRNA worked considerable better and some considerably worse than others, and that one strategy to improve the process of selecting the best sequence was to simply use Dicer generated fragments from much longer target sequences (Kawasaki *et al.* 2003). More recently some suppliers offer "Smart Pools" containing a mixture of 4 different siRNAs. In these studies, we decided at the start to use two anti-*Prkdc* sequences and combine them, but we also tested the efficiency of knockdown for the sequences used separately. As described below, while one sequence was better than the other, both worked fairly well and virtually all subsequent experiments used the mixture of both siRNAs.

Figure 3-1 shows a western blot analysis of GM08399 cells for an experiment in which siRNAs for two different targets in *Prkdc* were used; one (sequence A) being 293 bases downstream from the start codon, and the other (sequence B) being in the kinase domain. Samples were taken for analysis 5 days after the first transfection (3 days after the second transfection) and the concentrations of the siRNAs were 0.15μM. Lane 1 shows the sample using sequence A, lane 2 shows the sample using sequence B, lane 3 shows the sample where transfection was with a simultaneous transfection with both sequence A and sequence B siRNAs where the concentration was 0.15μM for each (total siRNA concentration 0.30μM). Lane 4 was for a mock transfected sample (transfected with Oligofectamine, etc, but with no siRNA present), and lane 5 was for a sample that was not transfected. The DNA-PKcs antibody detected only one discrete band at a

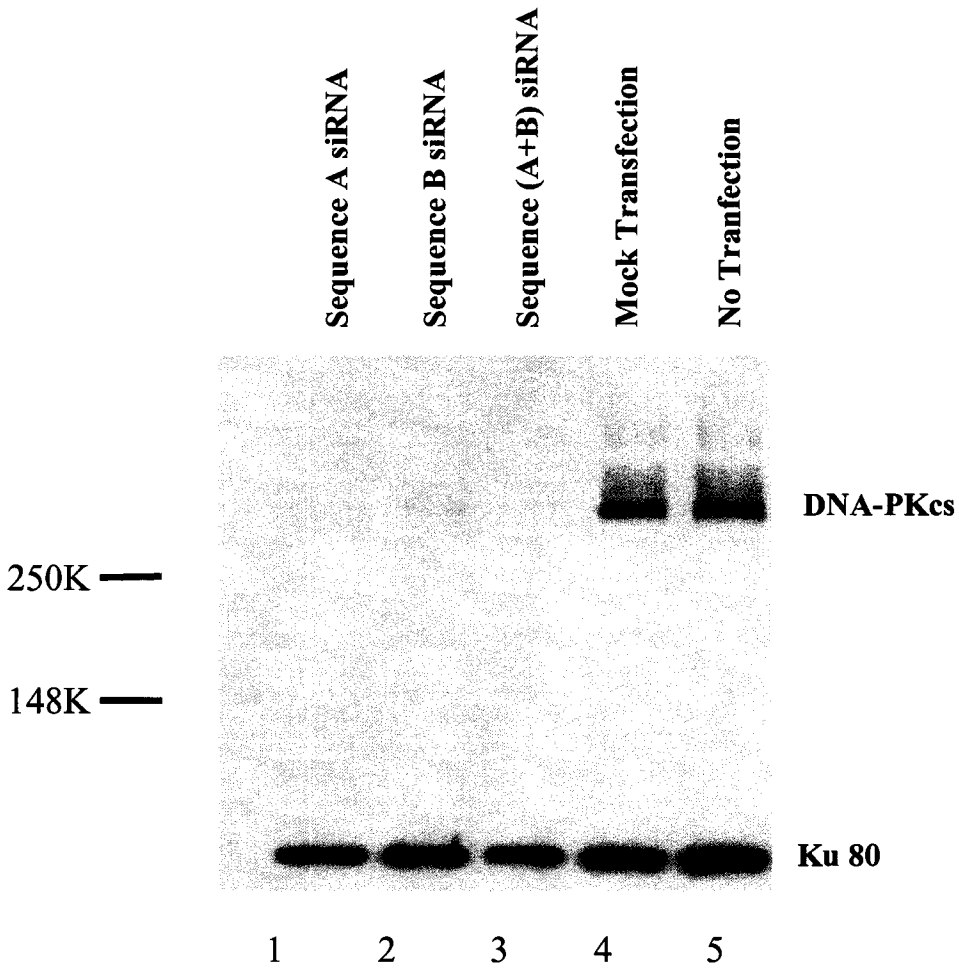


Figure 3-1. Expression of DNA-PKcs in low-passage normal human fibroblasts (GM08399) after 2 transfections of siRNA sequences A and B targeted to DNA-PKcs mRNA. Samples were taken for analysis 5 days after the first transfection (3 days after the second transfection) and the concentrations of the siRNAs were 0.15μM. Lysates prepared from indicated transfected cells were resolved on a 6% SDS-PAGE gel, blotted, and probed with indicated antibodies.

position in the gel corresponding to the expected high molecular weight of 460 kDa. The blots were simultaneously incubated with Ku80 antibody that served as a loading control. This experiment showed that both sequences were very effective in knocking down DNA-PKcs, although sequence A, targeting a sequence 293 bases downstream from the start codon appeared to be more effective than sequence B in the kinase domain. Both sequences together appeared to be as effective as sequence A alone, as might be expected since it would be difficult to achieve a better knock down that seen for A alone.

Figure 3-2 shows a western blot analysis for one experiment in which two different concentrations were used of the siRNAs targeted to *Prkdc* mRNA, as well as an siRNA targeted to *Ku 80* mRNA and a mock transfection without siRNA. The samples were taken 5 days after the first transfection, with a second transfection 2 days after the first. In this experiment, the blots were incubated simultaneously with mouse anti-DNA-PKcs and mouse anti-Ku80 antibody followed by detection with horseradish peroxidase (HRP) labeled goat anti-mouse IgG and a chemiluminescence assay. Again the same film exposure was used to show the entire western, rather that showing cut-outs that obscure cross-reacting bands. No such cross-reacting bands were observed. A greater knockdown of DNA-PKcs was seen for the 0.15μM concentration of siRNA targeted to DNA-PK_{cs} than for the 5-fold lower (0.03μM) concentration. As mentioned earlier, the concentrations were based on the stated quantity of siRNAs specified by the supplier, and as I later discovered, the supplier's stated quantity may not be completely accurate. In all cases, however, the concentrations were in the nanomolar (sub-micromolar) range. In several other experiments the level of knockdown of DNA-PKcs varied somewhat depending on the protocol. No effect of DNA-PKcs-targeted siRNA transfection on

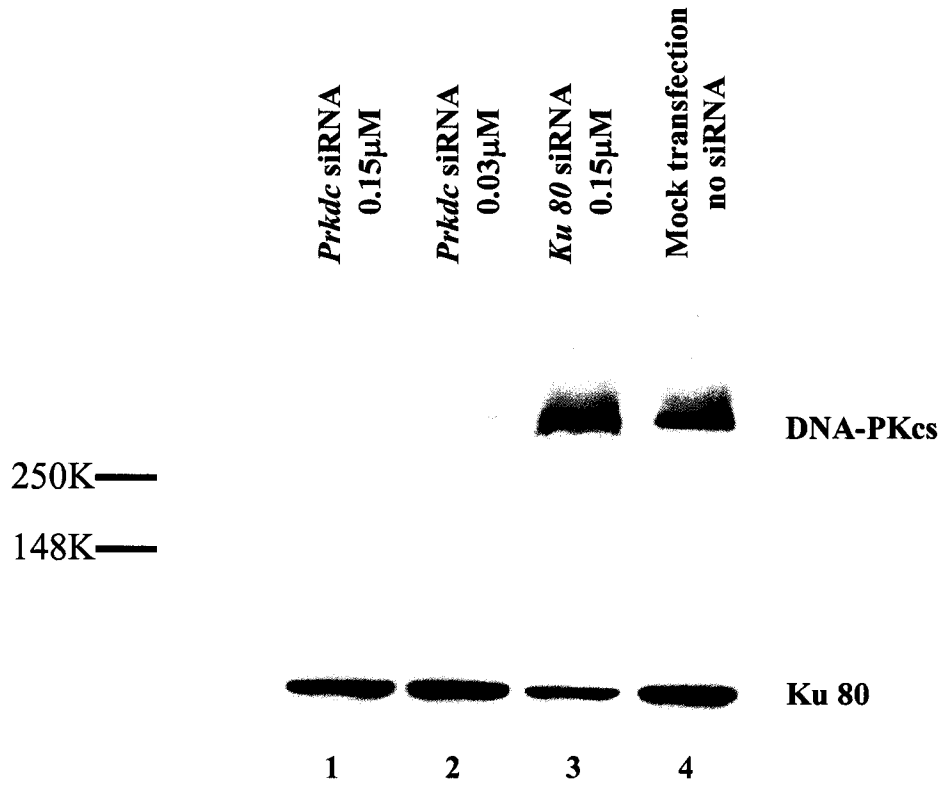


Figure 3-2. A Western blot from GM08399 cells after two Oligofectamine-mediated transfections (see "Materials and Methods"); Samples were taken for analysis 5 days after the first transfection (3 days after the second transfection). the measurement of both DNA-PKcs and Ku80 protein from cells using siRNA targeted to DNA-PKcs at a concentration of 0.15 μM (*Lane 1*) and at a concentration of 0.03 μM (*Lane 2*). *Lanes 3* and *4*, respectively, cells that were transfected with Ku80-targeted siRNA or mock transfected (two Oligofectamine treatments but without RNA).

Ku80 was seen, nor did Ku 80-targeted siRNA affect DNA-PKcs levels. Similar results were obtained in other experiments showing a lack of cross-reactivity with Lamin A/C-targeted siRNAs (data not shown), although in those cases, the Lamin A/C-targeted siRNA drastically reduced Lamin A/C protein. In the experimental result shown here, Ku80 protein was only partially reduced by Ku80-targeted siRNA. In this case, either the Ku80-targeted siRNA sequence was relatively ineffective, or the turnover time of Ku80 is sufficiently long that the timing, or other aspects of the transfection protocol are sub-optimal for that protein. To actually conclude that the anti Ku 80 siRNA was itself ineffective it would be necessary to carry out a real time RT-PCR analysis of the *Ku 80* mRNA, since it is the mRNA and not the protein that is targeted for break-down by the siRNA.

In another experiment to determine whether the same protocol would be effective in knocking down DNA-PKcs in other human cells, I repeated the procedure using both low passage AG1522 normal human cells and HeLa cells. Figure 3-3 shows a western blot analysis for DNA-PKcs for these cells 5 days after the start of the siRNA transfections. Lanes 1 - 4 were for AG1522 cells and lanes 5 - 8 were for HeLa cells. The results of siRNAs transfections targeting *Prkdc* mRNA are shown in lane 1 (AG1522) and lane 5 (HeLa). Lanes 3, 4, 7, and 8 were controls either mock transfected or not transfected. Lanes 2 and 6 were another control using an siRNA directed against Lamin A/C mRNA just to indicate the lack of actually including an siRNA not directed against *Prkdc*. I also did an experiment showing that the lamin A/C did knock down its expected target, but the anti *Prkdc* siRNA did not knock down lamin A/C. Clearly, the siRNA targeting *Prkdc* worked reasonably well for both these other cell strains.

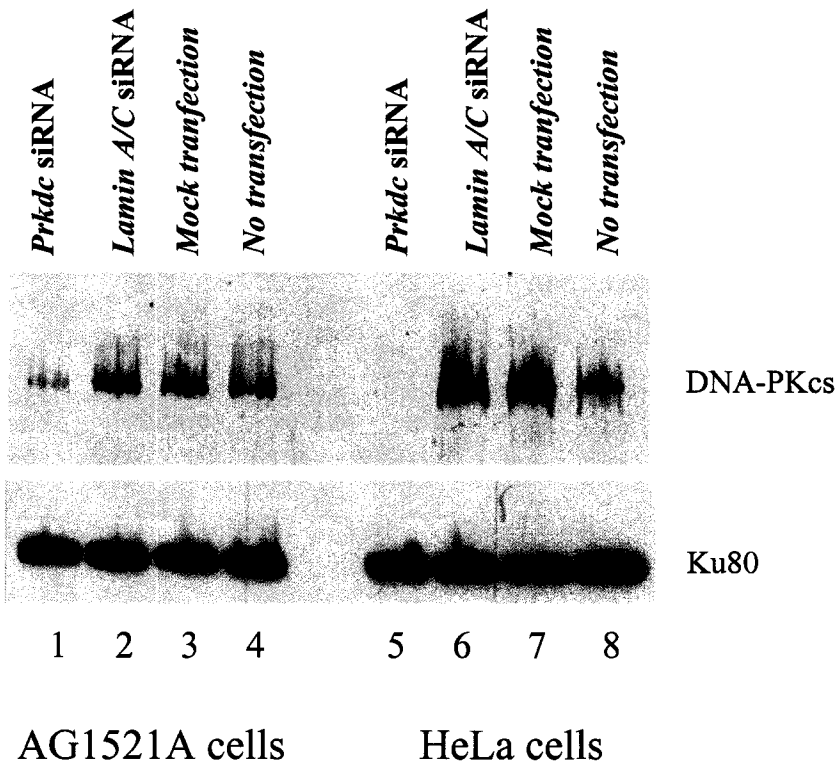


Figure 3-3. Expression of DNA-PKcs in low-passage normal human fibroblasts (AG1521A) and HeLa cells after transfection of two siRNA sequences targeted to DNA-PKcs mRNA (lane 1 and 5), siRNA sequences targeted to Lamin A/C (lane 2 and 6), mock transfection (lane 3 and 7) and no transfection (lane 4 and 8). Samples were taken for analysis 5 days after the first transfection (3 days after the second transfection). Lysates prepared from indicated transfected cells were resolved on a 6% SDS-PAGE gel, blotted, and probed with indicated antibodies.

Because the anti-DNA-PKcs antibody appeared from the western blots to be very specific with no evidence of significant cross reactivity with other proteins it was possible to carry out immunocytochemical studies to determine the efficiency of transfection, i.e., the proportion of cells that were or were not transfected. This is very important to know for these studies because a western blot by itself cannot distinguish between a situation where, for example, 50% knockdown occurred in 100% of the cells or 100% knockdown occurred in 50% of the cells with 0% knockdown in the remaining 50%. Figure 3-4 shows two fluorescence microscope fields from an experiment where DNA-PKcs was measured by immunocytochemistry. The siRNA transfection protocol was similar to that used for the result shown in the figures illustrating western blot analyses, but was from an experiment carried out on another occasion. Panels a and b are the same field of cells from a mock transfected culture showing the cells with a DAPI filter to identify DAPI stained nuclei (a); and with a rhodamine filter to identify nuclei containing measurable DNA-PKcs (b). In this particular experiment, of 156 cells examined, strong DNA-PKcs signals appeared in 85% of the cells, weaker signals in 12%, and no detectable signals in 3%. In cells transfected with DNA-PKcs-targeted siRNA (c and d), it appeared that one cell was virtually unaffected, (normal level of DNA-PKcs) another showed reduced DNA-PKcs, but the rest did not have obviously detectable levels. Scoring numerous other microscope fields from this experiment (322 cells total) indicated an average of about 22% unaffected cells (strong signals comparable to controls mentioned above) or with slightly reduced levels of DNA-PKcs, about 13% had appreciably reduced levels, and the remainder had no visibly detectable levels. Similar results were obtained in several replicate experiments, although the proportion

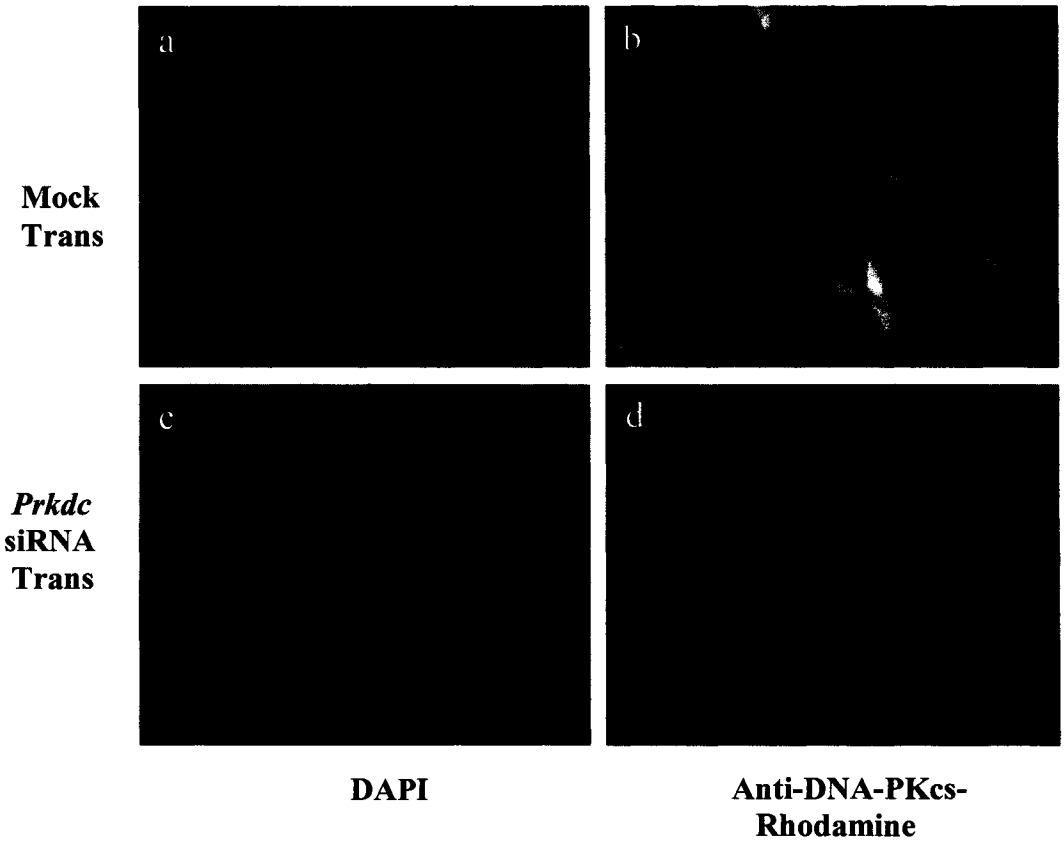


Figure 3-4. Fluorescence microscope images of cells either mock transfected (*Mock Trans*, *a* and *b*, same cells) or transfected twice with siRNA targeted to DNA-PKcs (*c* and *d*, same cells). Cells were stained with DAPI (*blue nuclei*) and Vybrant CFDA SE (*green cytoplasm*) and with rhodamine-labeled mouse anti-DNA-PKcs; photographs were taken with DAPI and FITC filters and images merged (*a* and *c*) or with a rhodamine filter and an FITC filter and merged (*b* and *d*).

unaffected differed. DNA-PKcs is a very abundant protein in human cells (about 50X more abundant per cell than in mouse or hamster cells) and the immunocytochemical detection efficiency range in our experiments from minimum to maximum is not known, nor was a cell by cell quantification by image analysis attempted. For this reason, the comparison of western blot and immunocytochemical analyses in the present study are only semi-quantitative. The immunocytochemistry was important, however for the purpose of indicating heterogeneity among cells, not reflected in western blots. Another unknown for these studies is the quantitative level of DNA-PKcs reduction necessary for radiosensitization.

Figure 3-5 (panels A and B) shows the result of an experiment to test the effect of DNA-PKcs targeted knock-down on the rejoining of broken chromosomes during interphase as measured by inducing premature chromosome condensation (PCC) 4 hours after irradiation. Since the immunocytochemical studies showed that the cells in the population irradiated were not likely to be uniformly reduced in DNA-PKcs, we might expect differences among cells in levels below which radiosensitization occurs, and therefore, a non-uniform radiosensitization. Cytogenetic assays such as those carried out to obtain the results shown in figure 3-6 are capable of detecting such heterogeneity because damage (excess PCC fragments) is measured in each individual cell. Figure 3-5 shows for control (A) and siRNA transfected cells (B) the distribution of cells with various numbers of excess fragments 4 hours after irradiation with 5 Gy of ^{137}Cs gamma rays. Each chromosome break results in an excess fragment. After some time elapses for rejoining, remaining excess fragments can result from either unrejoined breaks, or as is often the case for longer times in normal human cells, from mis-rejoined breaks that

Table 3-1 Excess PCC Fragments Per Cell

Excess Fragments	siRNA		Mock Transfection	
	# of Cells	%	# of Cells	%
0	1	1.087	0	0
1	0	0	1	2
2	1	1.087	1	2
3	1	1.087	3	6
4	0	0	3	6
5	3	3.261	5	10
6	5	5.435	5	10
7	6	6.522	7	14
8	8	8.696	8	16
9	7	7.609	6	12
10	6	6.522	5	10
11	3	3.261	3	6
12	2	2.174	1	2
13	2	2.174	1	2
14	3	3.261	0	0
15	4	4.348	1	2
16	5	5.435	0	0
17	7	7.609	0	0
18	8	8.696	0	0
19	7	7.609	0	0
20	5	5.435	0	0
21	3	3.261	0	0
22	1	1.087	0	0
23	1	1.087	0	0
24	0	0	0	0
25	1	1.087	0	0
26	0	0	0	0
27	1	1.087	0	0
28	1	1.087	0	0
29	0	0	0	0
30	0	0	0	0
TOTAL	92	100	50	100

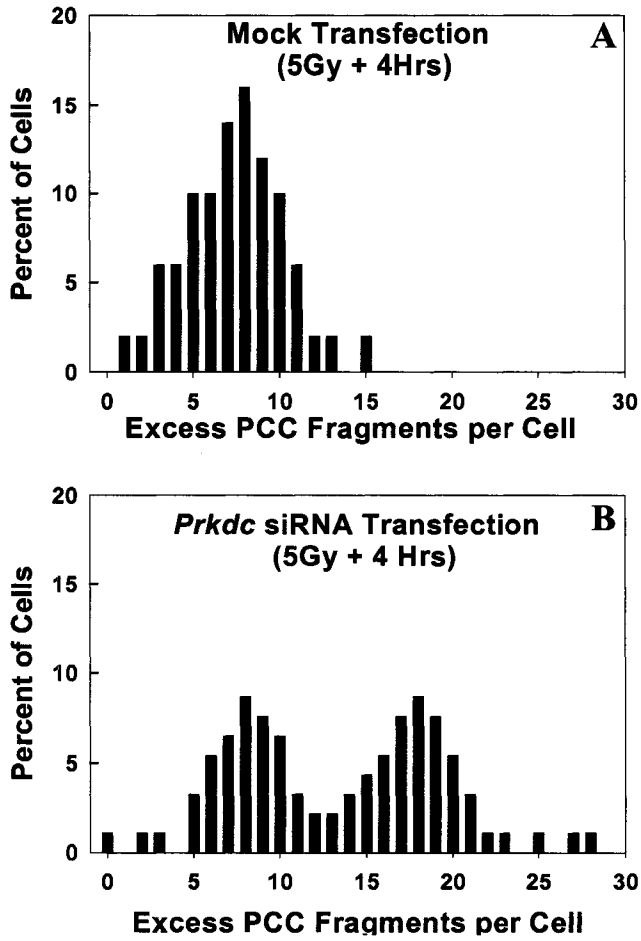


Figure 3-5. Changes in chromosomal radiosensitivity of GM08399 human fibroblasts after two transfections with siRNA targeted to DNA-PKcs mRNA. Excess PCC fragments in interphase G_1 cells 4 h after 5-Gy Cs-137 rays were measured in mock-transfected cells (*A*) or cells transfected with siRNA targeted to DNA-PKcs mRNA (*B*). Approximately 50–60% of the cells apparently suffered sufficient knock-down of DNA-PKcs to impair their ability to rejoin broken chromosomes or to result in excessive incorrect rejoining to yield other excess fragments.

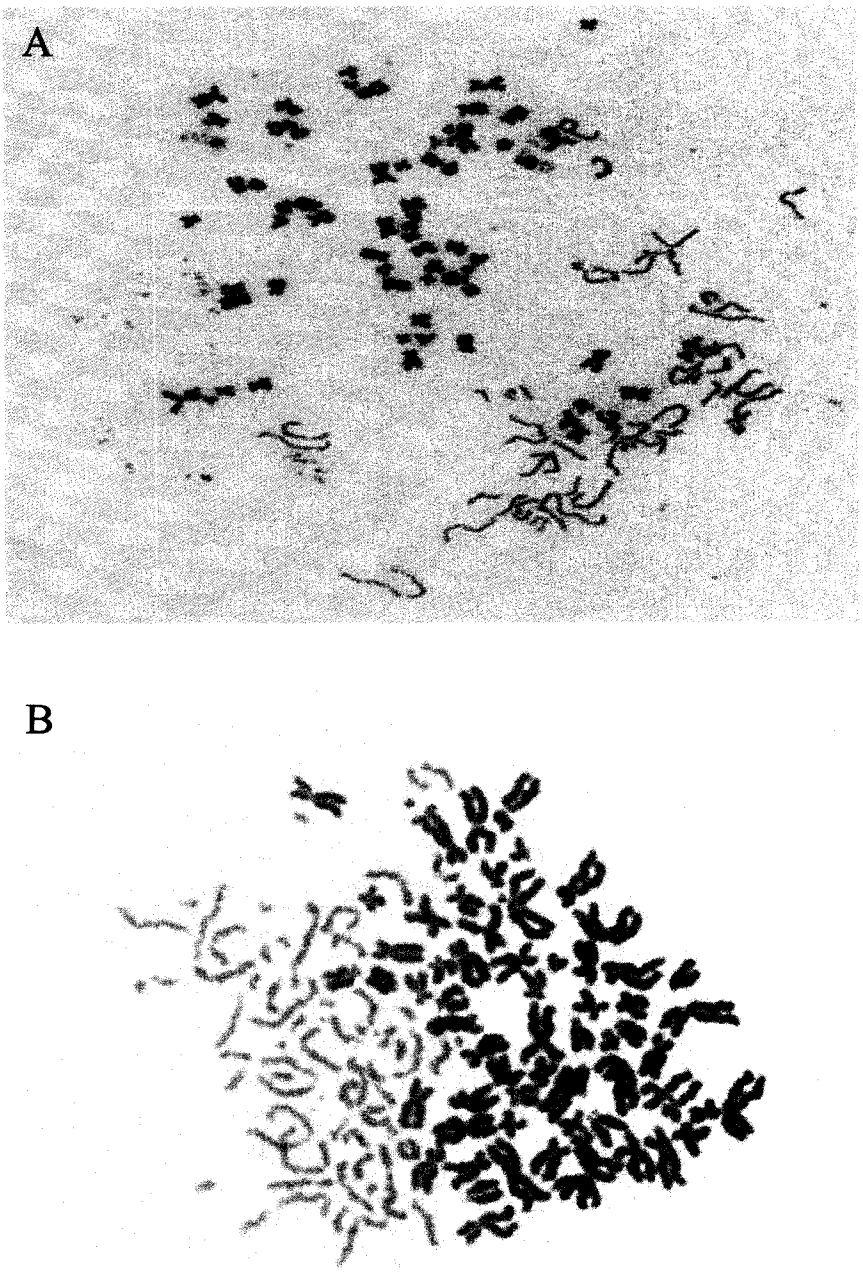


Figure 3-6. The Upper panel shows a PCC from an unirradiated cell. The lower panel is from an irradiated cell that has 18 excess fragments.

result in acentric or centric rings, i.e., asymmetrical intra-arm intra-changes (Cornforth and Bedford 1983b; Cornforth and Bedford 1987) At the shortest time measurement possible after irradiation with the PCC system (~20 min), a dose of 5 Gy results in an average of about 25 to 30 excess PCC fragments per cell, for several other human fibroblast cell strains, and the excess fragment frequency decreases due to rejoining with a half-time of about 1.5 to 1.7 hours (8-10). If these fibroblasts were similar in response, we would expect some 5 or 6 excess fragments per cell at the 4 hour sample time after a dose of 5 Gy. Panel A of figure 3-5 shows that an average of about 8 excess fragments per cell remained; a value not far from the expectation. For cells transfected with DNA-PKcs-targeted siRNA (panel B) the distribution of excess fragments appeared to be bimodal with one peak around 8 and another around 18 excess fragments per cell. These results suggest a sensitization factor of about two-fold (for the subpopulation sensitized) assuming an approximately linear dose response in the 5 Gy dose range for excess fragments 4 hours after irradiation.

The result of an experiment to measure radiosensitivity for induction of chromosomal aberrations measured in the first post-irradiation mitosis, is shown in figure 3-7. We would expect fewer excess acentric fragments than in the interphase PCC experiments because cells were incubated 24 hours after irradiation and are required to progress to mitosis for the assay. For non-transfected cells, there were about 0.8 induced dicentrics (a dicentric is scored as one dicentric plus its associated acentric fragment) and 1.0 deletions per cell (acentric fragments not associated with dicentrics or centric rings). For DNA-PKcs-targeted siRNA transfected cells, we observed about 1.0 induced

Table 3-2 Chromosome-Type Aberrations per Cell with 95 Percent Confidence limits

Treatment	Dose (Gy)	N	Instl. Del.	Term. Del.	Total	Dicent.	Cent. Ring	Total	Grand Total
Prkdc siRNA	0	95	0.032 (0.01, 0.09)	0.073 (0.03, 0.15)	0.116 (0.06, 0.21)	0.010 (0.0002,0.06)	0	0.010 (0.0002,0.06)	0.126 (0.07, 0.22)
	5	83	1.060 (0.85, 1.31)	0.843 (0.66, 1.07)	1.903 (1.63, 2.23)	0.964 (0.77, 1.21)	0.193 (0.11, 0.31)	1.157 (0.95, 1.41)	3.060 (2.70, 3.47)
Mock transfection	0	30	0.133 (0.04, 0.34)	0	0.133 (0.04, 0.34)	0	0	0	0.133 (0.04, 0.34)
	5	103	0.874 (0.71, 1.08)	0.184 (0.11, 0.29)	1.058 (0.87, 1.29)	0.757 (0.60, 0.95)	0.107 (0.05, 0.19)	0.864 (0.70, 1.07)	1.922 (1.67, 2.21)
No Transfectoion	0	30	0.133 (0.04, 0.34)	0	0.133 (0.04,0.34)	0	0	0	0.133 (0.04, 0.34)

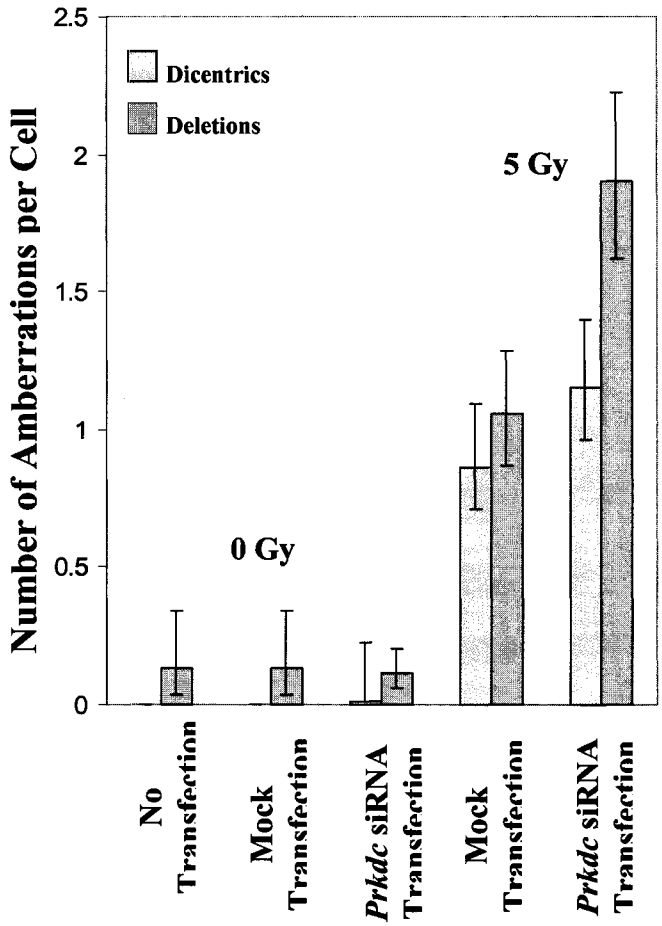


Figure 3-7, the result of an experiment in which chromosome-type aberrations were scored after cells entered their first postirradiation mitosis. Dicentrics (*solid bars*) and deletions (*shaded bars*) were not produced by either mock or siRNA transfections alone. Five Gy of rays increased the dicentric yield, although not significantly more in the siRNA- than in the mock-transfected cells. In contrast, deletions from either unrejoined chromosome breaks or interstitial deletions (asymmetric intra-arm intrachanges) were increased about 2-fold by transfection with siRNA targeted to DNA-PKcs mRNA. Uncertainties shown are 95% confidence limits.

dicentric and 1.9 deletions per cell. This represents a nearly two-fold increase in excess fragments or deletions for the DNA-PKcs-targeted siRNA transfected cells. In this experiment we did not observe the increased frequency of induced chromatid-type aberrations after irradiation of G1 or G0 cells that is usually seen for x-ray sensitive cells with defects in NHEJ (Bahari and Bedford 1989; Cornforth and Bedford 1993; Stackhouse and Bedford 1994; Bedford and Dewey 2002).

The results of an experiment to examine DNA-PKcs-targeted siRNA radiosensitization with respect to cell killing are shown in figures 3-8 and 3-9. In addition to the heterogeneity in radiosensitivity always present for log phase cultures due to cell cycle dependent variation in radiosensitivity, and in spite of some heterogeneity in the proportion of cells with severe or intermediate levels of DNA-PKcs knock-down, there was still a marked radiosensitization of DNA-PKcs-targeted siRNA transfected cells relative to the two control cell populations. As shown in figure 3-8, as expected this was most pronounced in the low dose region (0 to 1 Gy) where survival in mixed heterogeneous cell populations is due to killing of the most sensitive subpopulations. The full survival response over a larger range of doses is shown in figure 3-9. Also shown in figure 3-9 is a theoretical curve for a mixed population of cells consisting of a fraction (20%) of unaffected cells with a radiosensitivity corresponding to curve R (wild-type resistance of untransfected cells), and a fraction (80%) of sensitized cells whose response, curve S, was derived from the slope of the curve sensitized cells in the low dose region between 0 and 1 Gy taken from figure 3-8. This fits the survival estimates well for the anti- *Prkdc* siRNA transfected cells over the entire range of doses. Thus, in spite of what appears as a greater "knock down" of DNA-PKcs from the immunochemical results

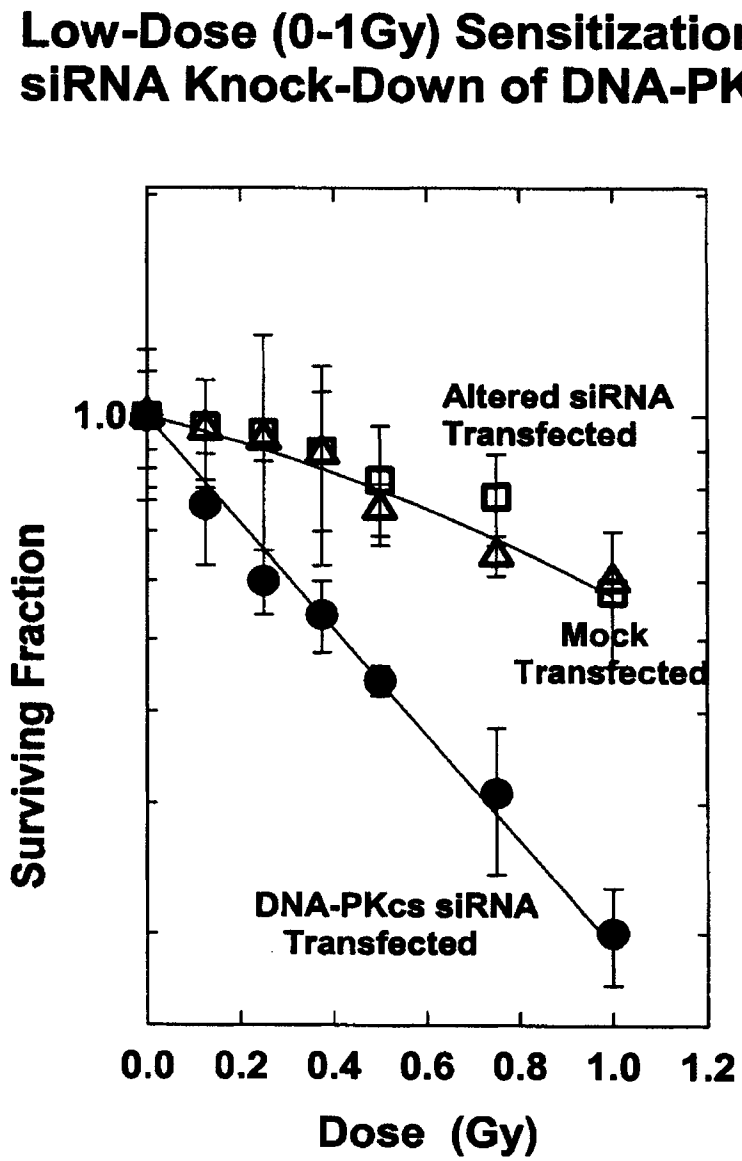


Figure 3-8. Sensitization of GM08399 human fibroblasts with respect to cell killing by transfection with siRNA targeted to DNA-PKcs mRNA. The survival reduction in the low-dose range where the response is due largely to the most sensitive subpopulation of cells.

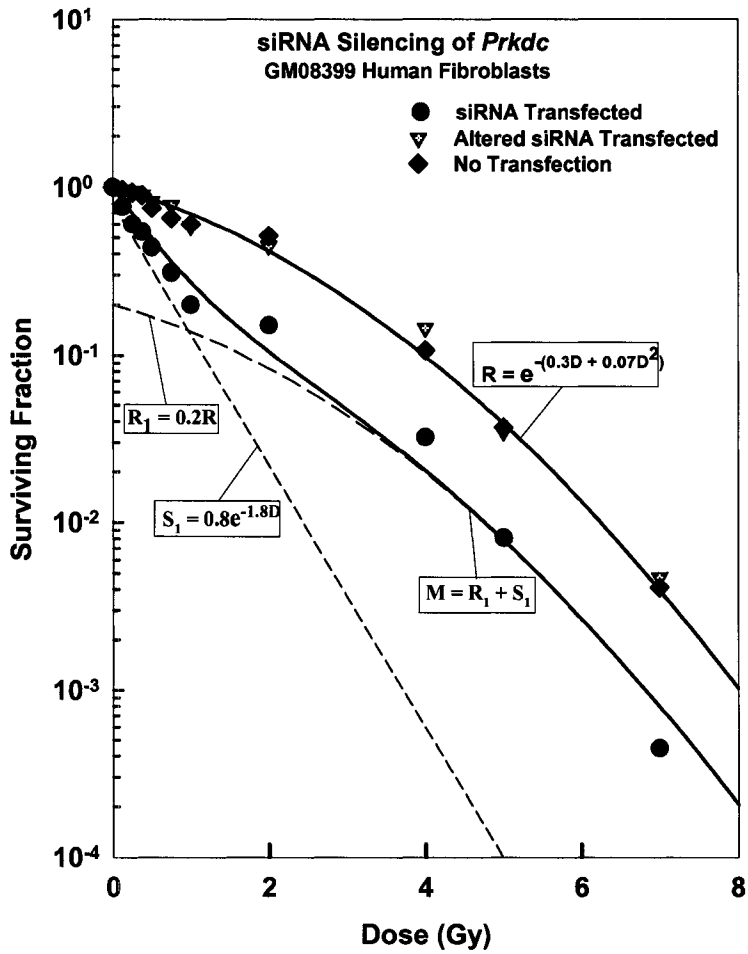


Figure 3-9. Sensitization of GM08399 human fibroblasts with respect to cell killing by transfection with siRNA targeted to DNA-PKcs mRNA. Cell survival was measured by colony formation over a range of doses (0–7 Gy) of Cs-137 rays after transfection with siRNA targeted to DNA-PKcs mRNA (*lower solid curve*), a control siRNA in which the DNA-PKcs siRNA target sequence was altered slightly (*inverted triangles, upper solid curve*), or not transfected (*upper solid curve*). The *lower solid curve* (*M*), which reasonably fits the data, is a composite curve that would be expected from a mixed population of cells consisting of 20% with the sensitivity of the cells that did not experience a DNA-PKcs knock-down (*curve labeled* R_1) and 80% with a sensitivity illustrated by *curve* S_1 estimated from the survival estimates for the sensitized cells in the low-dose (0–0.5 Gy) range. R and M represent surviving fraction, and D represents the doses.

illustrated in figure 3-4, I suggest this result indicates that DNA-PKcs is present in large excess, far above that needed to confer normal radioresistance, and to confer the maximum increase in radiosensitivity possible from a complete lack of DNA-PKcs, a further depletion of this protein may be necessary.

It is also worth noting that one of the "control" cell populations in this experiment was not a "mock" transfection, but instead, cells that were transfected with an siRNA control duplex ($siRNA_c$) in which an A-U base pair was substituted for a U-A base pair in position 3 from the 5' end of the strand of the duplex siRNA equivalent to the mRNA target, and also in which an AC sequence was replaced with a CA sequence in position 12 and 13 from the 5' end in the same strand. Transfection with this $siRNA_c$ duplex did not radiosensitize the cells, nor did it knock down DNA-PKcs as measured in western blots.

Other approaches involving stable expression of interfering RNAs for selective knock down of gene products by introducing vectors that stably integrate and express interfering dsRNA hairpins (Brummelkamp *et al.* 2002; Paddison *et al.* 2002b) may offer some advantages over the transient transfection methods used in the present studies, but there may also be some disadvantages. With a stable expression approach, for example, if sufficient expression is achieved to produce a phenotypic change, and the cellular life-span is not limiting, it should be possible to obtain clonal populations in which there is much less heterogeneity in levels of knock down among cells than is possible with the transient expression knock down. The transient approach, on the other hand may be more appropriate if the long-term knock down is lethal, or for studies involving multiple gene

silencing with little or no restriction on the choice of cells for study so it should be possible, for example to study effects on cells with the same or different genetic backgrounds.

Chapter 4

DNA-PKcs Can Control Expression of ATM

ABSTRACT

A central role of ATM in cellular damage response pathways has been demonstrated, along with a myriad of its interactions with other proteins. To date, however, no reports have appeared establishing direct interactions with DNA-PKcs, a key protein in non-homologous end joining (NHEJ), and by far the major pathway for processing DNA double strand breaks in mammalian cells. By knocking down DNA-PKcs with an siRNA that targets its mRNA, we have shown that ATM protein is also reduced. This is not a cross reactivity of the siRNA with *ATM* mRNA. Quantitative real-time RT-PCR analysis following transfection with anti-*Prkdc** siRNA showed a rapid (hours) decline in its message, but no decline in *ATM* mRNA, until approximately 2 days later, ***after*** the DNA-PKcs protein level had declined. Two different anti-*Prkdc* siRNAs both lowered DNA-PKcs but not other proteins measured. No change in DNA-PKcs occurred when a small base change in the siRNA was applied. A blast search revealed no close matches with other mRNA sequences. Examination of the levels of phosphorylated PKR showed no evidence of an induced interferon response although such a response could be induced with high levels of poly(I)•poly(C). One advantage of the RNAi approach is that interactions occurring ***in vivo*** can be measured in cells of identical genetic background. To our knowledge, this is the first such demonstration of a direct role of DNA-PKcs in controlling expression of ATM, and could link the NHEJ system to other damage responses mediated by ATM.

Introduction

The evolution of molecular mechanisms and systems for repair and rejoining of DNA double strand breaks (DSBs) provided considerable selective advantage for organisms acquiring them. For example, such systems contribute a means for vastly increasing the phenotypic variation among individuals that results from genetic recombination during gamete formation, and allowed the development of an immune system with the diversity necessary to specifically recognize an enormous range of foreign antigens. In addition to the endogenous production of DSBs by various means, they are also produced directly by exposure to medical, industrial or environmental sources of ionizing radiations, and a few chemicals as well. Repair systems further serve to protect cells and the organisms they comprise from excessive DSBs, because unrepaired breaks are usually lethal to cells. Some misrepaired breaks can be mutagenic and some of these can be carcinogenic, so in somatic cells, one way to deal with harmful misrepaired breaks is to signal or invoke an apoptotic or cell death response.

Essentially, there are two main cellular systems for sensing and repairing DSBs that have been extensively studied (eg. reviewed in Jeggo 1998a; Thompson and Schild 2001). One is a system known as non-homologous end joining (NHEJ) which functions to rejoin the two ends of a DSB without reference to whether the correct base sequence is restored after rejoining. This is an error-prone process. The other DSB repair system is based on the processes involving homologous recombination during meiosis, and is known as homology directed repair (HDR). It is inherently an error free process because

it uses an identical homologous sequence on a nearby sister chromatid as a template to insure that the original sequence will be restored after the DSB is repaired.

In the NHEJ system, a DNA dependent protein kinase complex known as DNA-PK first functions to recognize DSB or other double stranded DNA ends such as telomeres. This complex is comprised of the Ku 70/80 heterodimer and a large catalytic subunit DNA-PKcs and binds to DNA ends along with another protein, Artemis. Following some end processing and positioning, the broken ends are placed in a favorable position for rejoining by the XRCC4/Ligase4 complex (Jeggo 1998a; Ma *et al.* 2002). DNA-PKcs is a member of the phosphatidyl-inositol-3-kinase superfamily, and its kinase activity is essential for its function in NHEJ (DeFazio *et al.* 2002). It is also known to phosphorylate some other substrates such as histone H2AX (Burma *et al.* 2001; Park *et al.* 2004). Animals and cells that are defective for DNA-PKcs, such as *scid* mice, are radiosensitive. There is at least one other "alternative end joining" system, but much less is known about this (Dai *et al.* 2003; Wang *et al.* 2003).

For the most part, higher eukaryotic cells rely heavily on this error prone end joining to cope with DSBs. In spite of its error prone nature, perhaps the kinds of errors it produces are better tolerated in organisms with large genomes where a large fraction of sequences are non-coding. In contrast, organisms with highly compact genomes may not tolerate errors and an error free repair system is much more important.

Homology directed repair (HDR) is an error free process and acts on DSBs present after DNA replication to involve the undamaged sister chromatid as a "correct" reference sequence. Cells in the G1 phase of the cell cycle do not participate in HDR, or if they do, it is at levels many orders of magnitude lower (Moynahan and Jasin 1997).

Noncycling cells in G0 cannot carry out HDR at all, because they do not possess measurable levels of rad51, which is a key element in the processes (Yamamoto *et al.* 1996; Flygare *et al.* 1996)

ATM (mutated in Ataxia-Telangiectasia) is a (if not *the*) key protein that, by phosphorylation events or other interactions, mediates signaling that can direct cells toward the HDR response, or to a cell cycle checkpoint that can delay the cell cycle, or to an apoptotic response.

In the process of HDR, very shortly (minutes) after a break is produced, ATM kinase interacts with and phosphorylates some thousands of histone H2AX molecules over a megabase size domain surrounding the DSB (Rogakou *et al.* 1998; Burma *et al.* 2001). This likely serves as an early sensor. Phosphorylation of the NBS1 protein is necessary for its function and this is dependent on ATM kinase, apparently through an interaction with BRCA1. NBS1 is a subunit of the MRN complex (MRE11-RAD50-NBS1) that binds DSBs early after they occur and likely plays a role not only in the recognition, but in the early processing of DNA ends to form single stranded tails that are bound with high affinity by RPA (replication protein A). RPA is also directly phosphorylated by ATM kinase. Next the Rad 51 nucleoprotein filament is formed involving an interaction with BRCA2, and then Rad 54 facilitates transfer of the damaged strand to the homologous region of the nearby sister chromatid so that an error free new strand synthesis can occur and the HDR repair can be completed.

As mentioned above ATM is also very important in signaling cell cycle checkpoint responses. For example, ATM is known to be involved in the phosphorylation of Tp53 through BRCA1 phosphorylation and this can subsequently be

involved in G1, S, and G2 checkpoints. It also phosphorylates FANCD2 protein that is involved in an S phase checkpoint (Taniguchi *et al.* 2002).

Are there any connections between NHEJ and the ATM/HDR network?

There have been many comparisons of the relative contributions of NHEJ and HDR to DSB repair among different organisms such as yeast vs mammals or other vertebrates and during different phases of the cell cycle. In contrast, there has been virtually no evidence of direct connections and protein interactions between the NHEJ system and the ATM-controlled checkpoint and HDR systems. In1993, Allunis-Turner and her colleagues isolated a radiosensitive clone designated M059J from a culture arising from a human glioblastoma biopsy (Allalunis-Turner *et al.* 1993). Other clones from the biopsy (M059K) were normal in radiosensitivity. Later, the genetic defect underlying the hypersensitivity to radiation in the M059J cells was identified as a mutation in the *Prkdc* gene, leading to a virtual absence of DNA-PKcs (Lees-Miller *et al.* 1995). In 1998 Chan noticed that these M059J cells were also deficient in the ATM protein, although RT-PCR showed that *ATM* mRNA was present (Chan *et al.* 1998). The RT-PCR technology available at that time was not amenable to determining the level of mRNA. Some five years later, Tsuchida isolated and sequenced both alleles of the *ATM* in M059J cells and found a different mutation in each allele that would predict a truncation of each of the messages and result in protein degradation (Tsuchida *et al.* 2002). It is still not clear why M059J cells should have different mutations in both alleles of both *ATM* and both alleles of *Prkdc*.

Some further observations on reduced ATM in *scid* mice were made by Dr. Lavin and his colleagues in Brisbane (personal communication). Since our laboratory was using siRNA technologies to knock down DNA-PKcs, and had already reported is successful application for this purpose, conversations with Dr. Lavin led to his suggestion that it might be worth looking at the ATM levels in our samples. A finding of reduced ATM would possibly overcome the serious objection from the previous suggestive observations that the reduced ATM levels were simply a reflection of a natural variation based on different genetic backgrounds, and perhaps other factors. The ATM levels after DNA-PKcs was knocked down following transfection with anti-*Prkdc* siRNA would at the very least be from measurements in identical normal human cells. In addition, with the availability of real time RT PCR, it would be possible in these experiments to follow the levels of both *Prkdc* and *ATM* mRNAs as well as their proteins as a function of time after anti-*Prkdc* siRNA transfections.

Since the RNAi technology is very new, and various known pitfalls have already been reported (Sledz *et al.* 2003; Scacheri *et al.* 2004), it may be worth pointing out here that the controls I included in the experiments described below are in accord with those suggested by the editorial board of *Nature Cell Biology* (Editorial 2003).

To our knowledge, this is the first report showing a direct connection whereby levels of DNA-PKcs, a protein of the NHEJ system can control expression of ATM, and thereby potentially effect, in turn, the vast checkpoint signaling and HDR network controlled by ATM.

Materials and Methods

General aspects of materials and methods used were given in chapter 2, but to maintain continuity in the closer context of the separate aims of chapters 3 and 4, the essential specific methods relating to chapter 4 are summarized again below.

Cells

Three human cell strains or lines and five Chinese hamster-derived cell lines were used for these studies. The subject or test cells for the siRNA knockdown experiments were low passage GM08399 human fibroblast strain described in chapter 3, which was obtained from the NIGMS Coriell Cell Repository. These cells were originally obtained from a skin biopsy from a 19 year old apparently normal female. The cells were grown in alpha MEM containing 15% fetal bovine serum and 50 U/ml of penicillin-G and 50 μg/ml of streptomycin sulfate. Cells were cultured in 6-well tissue culture plates (9.62 cm^2 per well) with 2 ml culture medium per well. When cultures grew to form confluent monolayers the cells were resuspended by trypsin treatment and one-fourth of the cells were reinoculated into each well of a new plate.

For positive and negative control cells to help identify the ATM band on western blots we used two lymphoblastoid-derived cell lines. One designated C3 ABR, was a line derived from a normal human subject and contains normal levels of both ATM and DNA-PKcs. The other line designated L3 are a line derived from a North African Jewish Ataxia-Telangiectasia patient. L3 cells contain normal levels of DNA-PKcs, but fail to produce ATM protein because of a homozygous truncation mutation in both alleles of the gene. The lymphoblastoid cells grow in suspension and were cultured in RPMI 1640

medium with 10% fetal bovine serum and the same antibiotics as for the other cells described in this study.

The hamster cell lines were as follows: CHO-10B2 cells are wild type with respect to radiosensitivity and the expression of DNA-PKcs and were originally obtained from Dr W.C. Dewey, now at the University of California, San Francisco. Irs 20 cells are a radiosensitive mutant derived from CHO-10B2 (Stackhouse and Bedford 1993a; Stackhouse and Bedford 1993b; Stackhouse and Bedford 1994; Lin *et al.* 1997) and are defective in DNA-PKcs due to a mutation that causes substitution of a lysine for a glutamic acid in the fourth residue from the DNA-PKcs C-terminus (Priestley *et al.* 1998). The level of expression of DNA-PKcs protein is low (about 5% to 10% of wild-type levels) but the protein completely lacks kinase activity. Irs20-YAC is an irs20 cell line corrected with a yeast artificial chromosome containing a human DNA-PKcs gene (Priestley *et al.* 1998). The V3/vector and V3/A+ cells were kindly supplied by Dr Susan Lees-Miller, University of Calgary, Alberta, Canada. V3 cells are a radiosensitive DNA-PKcs deficient (no detectable levels) line of AA8-4 CHO cells, and the V3/A+ cells are a stably transfected line corrected with a plasmid containing and expressing wild-type human DNA-PKcs. V3/vector cells are V3 cells containing the same vector but without the DNA-PKcs gene.

siRNAs and Transfections

The siRNAs used to knock down DNA-PKcs were chosen as described by Elbashir (ref) and were synthesized by Dharmacon Research Inc., Lafayette, CO. As we described earlier (Peng *et al.* 2002) two sequences were used. The first (designated

sequence A in the present context) was targeted to a 21 base sequence in *DNA-PKcs* mRNA 293 bases downstream from the start codon and the other (sequence B) was targeted to a 21 base sequence in the kinase domain. The complete sequence of the processed mRNA transcript of *DNA-PKcs* is given in Appendix IV, where the sequences targeted are highlighted. The control sequence used was a modified version of sequence A. The modification involved a transposition of an AC to a CA at positions 12 and 13 from the 5' end of the sense strand, and a substitution of an A to a U at position 3 from the 5' end of the same strand so the double stranded oligo would have an A-U base pair rather than a U-A base pair at this position. These sequences were:

GAUCGCACCUUACUCUGUUdTdT (siRNA sequence A)
dTdTCUAGCGUGGAAUGAGACAA

CUUUAUGGUGGCCAUGGAGdTdT (siRNA sequence B)
dTdTGAAAUACCACCGGUACCUC

GAACGCACCUUCAUCUGUUdTdT (control siRNA)
dTdTCUUGCGUGGAAGUAGACAA

BLAST searches of these sequences against the human mRNA database were carried out to determine whether the chosen sequences had any other potential mRNA targets, especially *ATM* since it was the main subject of the present study. The search returns potential matches of contiguous sequences of 16 bases or more. No such matches were found other than that for *Prkdc* mRNA. Some relatively poor matches were identified for some other non-mammalian species in a wider search. The closest match for either sequence to a sequence in *ATM* mRNA was 8bp. The mRNA sequence for *ATM* is given in Appendix IV.

Oligofectamine Reagent (Invitrogen, Carlsbad, CA) was used for transfecting siRNAs into the GM08399 cells. A standard protocol was developed to obtain a relatively high transfection efficiency and knockdown, by varying the siRNA and Oligofectamine concentrations and cell density. The details of this protocol are given in Chapter 2 and were outlined previously (Peng *et al.* 2002). Briefly, one day before transfection, 1 x 10^5 cells were inoculated into each well of a 6-well plate in 1 ml of alpha MEM medium containing 15% fetal bovine serum and antibiotics. Twelve hours later the medium was removed and replaced with alpha MEM without serum or antibiotics. After another 12 hours the medium was again changed this time using 2 ml of serum and antibiotic free medium. The transfection was carried out 2 to 3 hours later. The Oligonucleotide-Oligofectamine complex was prepared and the transfection was carried out as described in Chapter 2. It is noteworthy that the concentrations of the siRNA tested and reported in the experiments described below are based on the amount of the synthesized siRNA as specified by the supplier (Dharmacon). The material is very expensive, so with the small quantities ordered I did not wish to waste a significant portion in making an independent measurement of the concentration. This is noteworthy because in the experiments described below I estimated the optimal concentration for transfection and *Prkdc* knockdown to be some 15-fold lower than we previously reported (Peng *et al.* 2002). There could be several explanations for this including the possibility that the siRNAs supplied may be a mixture of different length oligos with the sequences being correct but not always the entire sequence being supplied. If the only effective sequence is the 21-mer specified and this is in one case a large fraction of the oligomer supplied, whereas in another case it is a small fraction, the same concentration of total nucleic acid may be the

same for the two samples but the concentration of the specified sequence could be considerably different. Again, this could be determined by running the sample on a gel but not without wasting much of the expensive sample. While the above explanation is only a speculation, the fact remains that a different optimal nominal concentration was found in the experiments reported in this chapter compared to those reported in chapter 3.

As mentioned in chapter 3, we showed that both sequence A and sequence B worked reasonably well separately, but virtually all the experiments were carried out with a combined mixture of the two siRNAs. While in theory this might increase the probably of encountering an "off target" effect, we looked for but did not find any evidence for such an effect and since the combined sequences worked well at the outset of these studies, we continued to use this combination throughout. As specified in the Results and Discussion section, in some instances two transfections were carried out where samples were taken at times beyond two days from the start of the first transfection. In these cases, the cells were subcultured 20 hours after the first transfection and split 1:2 and re-inoculated into new six-well plates. After another day the transfection steps were repeated again.

Protein Extraction and Western Blot Analyses

After various siRNA treatment conditions or time-course protocols, levels of the proteins of interest in cells relative to those in control cells were measured by western blot analysis.

First, culture medium was removed from the wells and after two rinses with PBS, the cells were released from the dish surface by treatment with a solution containing

0.25% trypsin and 0.1% EDTA and resuspended in ice cold alpha MEM medium with 15% fetal bovine serum. The cells were centrifuged and the pellet was resuspended in ice cold PBS, centrifuged again, and the PBS was discarded. For protein extraction 100 µl of Lysis/Extraction buffer (Appendix I) was added and the tubes were vortexed and placed on ice for 10 minutes. The samples were then centrifuged at 10,000 x g for 10 minutes at 4°C and the supernatant (total cell lysate) was collected and the protein concentration was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

An approximately equal amount of total protein (20 to 100 ug) was mixed in 250 µl tubes with 5µl gel loading buffer, the samples were denatured at 95°C for 5 minutes, and were then centrifuged and loaded onto a 6% precast Tris-glycine SDS polyacrylamide gel (Invitrogen, Carlsbad, CA, USA). Protein molecular markers were also loaded and the electrophoresis was carried out at room temperature for 75 minutes with a power supply operating at 125 volts.

After the electrophoresis the proteins were transferred to a nitrocellulose membrane as described in chapter 2.

Immunological Detection of the Proteins

The nitrocellulose membranes were incubated in a blocking buffer (Appendix I) to block non-specific binding of the antibodies, and after rinsing, the primary antibodies were added. For DNA-PKcs protein the antibody was Ab4 (Neomarkers, Fremont, CA, USA) used at a 1:800 dilution. The primary was detected with horseradish peroxidase (HRP) labeled goat anti-mouse secondary antibody (Amersham Pharmarcia Biotech, Piscataway, NJ) diluted 1:2000.

For detection of ATM protein in human cells 1:2500 diluted rabbit anti-ATM (Catalog number NB 100-104) obtained from Novus (Novus, Fremont, CA, USA) was used.

For detection of ATM protein in CHO cells, the rabbit anti-ATM primary antibody APH397 (Serotec, Oxford, UK) 1:1000 diluted or 4BA 1: 2000 diluted was used. The primary antibody 4BA originally described by Dr Martin F. Lavin (Queensland Institute of Medical Research, Brisbane, Australia) was kindly supplied by Dr Susan P. Lees-Miller (University of Calgary, Alberta, Canada).

Horseradish peroxidase conjugated goat anti-rabbit secondary antibodies were used (1:2000 diluted) to detect the ATM primary antibody (Calbiochem/Oncogene, San Diego, CA, USA). The application of the labeled secondary antibody was identical to the procedure described above for DNA-PKcs detection.

Ku 80 and β-Actin

Generally Ku-80 and *β-Actin* proteins were used as the loading controls or standards for relative measurements of the proteins of interest such as DNA-PKcs or ATM. The Ku-80 antibody was mouse anti-Ku80 Ab-2 obtained from Neomarkers (Neomarkers/Lab Vision, Fremont, CA, USA) and the β-actin antibody was mouse anti-β-actin (Catalog number NB 600-501) obtained from Novus (Novus, Fremont, CA, USA).

The secondary antibodies were horseradish peroxidase (HRP) labeled goat anti-mouse secondary antibody (Amersham Pharmarcia Biotech, Piscataway, NJ) diluted 1:2000.

PKR antibodies to test for interferon response

It has been reported that in some instances, especially using certain shRNAs [3] or high concentrations of certain siRNAs that an "interferon response" can be induced. This results in the general shut-down of protein synthesis. To ensure this was not the case for the siRNA treatments described here, I deliberately induced an interferon response as described by others (Conn 2003) and measured this by induction of the appearance of phosphorylated protein kinasePKR which is a key element induced during this response. I then measured the level of phosphorylated PKR in the extracts of siRNA treated cells to determine whether interferon response might be a problem in my studies. To induce the response for measurements of phosphorylated PKR, normal human fibroblasts GM08399 cells were transfected (Oligofectamine) with 1 μM poly(I)·poly(C) (Sigma, St. Louis, MO, USA). Two days after transfection, cells were harvested and Western blotting analysis of phosphorylated PKR was performed after 20 μg of total protein was loaded and separated in a 10% SDS-PAGE gel.

Rabbit anti-phosphorylated PKR antibody (Sigma, St. Louis, MO, USA) diluted 1:1000 was used to detect the induced phosphorylated PKR protein followed by a labeled mouse anti-rabbit secondary antibody as described above for other protein detections.

Detection by Film Densitometry and/or Storm 860 Scanner Image analysis

The labeled secondary antibodies on the membranes were detected in some cases by film densitometry after exposure of x-ray films or by the Storm 860 Scanner detection system. The ECL reagent was used to facilitate detection by x-ray film exposure and film

[3] Short hairpin RNAs (shRNAs) are linear RNAs produced by incorporated vectors containing reverse complementary sequences separated by a short spacer so that a short hairpin dsRNA is produced that acts in the RNAi pathway

densitometry, or the ECL Plus reagent was used for Storm Scanner detection and quantification. A discussion of the restricted exposure ranges for linearity of the film densitometry is presented in Appendix II.

Immunocytochemistry

The antibodies used for detection of both DNA-PKcs and ATM were highly specific as judged from the western blotting results, so useful information from immunocytochemistry could be obtained by simultaneous application of the mouse monoclonal antibody against DNA-PKcs used at a 1:200 dilution and the rabbit monoclonal antibody against ATM used at a 1:2000 dilution, followed by detection with rhodamine anti-mouse or FITC labeled anti-rabbit secondary antibodies. Cells were grown on Lab-Tec chamber slides and immunocytochemistry was carried out 3 days after the second transfection. These transfections were carried out exactly as described for the 6-well plates, with a small scaling factor to adjust for the slightly different culture surface area. Cells were rinsed with PBS and fixed for 10 minutes in 4% paraformaldehyde at 4°C, then rinsed in PBS and were permeabilized to allow penetration of the antibodies by adding 0.2% Triton X-100 for 5 minutes at room temperature. After 3 rinses in PBS, cells were incubated for 45 minutes in 10% goat serum to suppress non-specific binding of antibodies and the cells were then incubated in the primary antibodies, and after rinsing in PBS the bound primary antibodies were detected after incubation with rhodamine labeled goat anti mouse (1:2000) and FITC labeled goat anti-rabbit diluted in a 1.5% blocking serum.

Real Time RT-PCR for Quantification of mRNAs

For the serial detection of mRNAs with time after transfection with *Prkdc* siRNA, samples were taken 4 hours after the first transfection, and then daily until day 8. RNA was extracted using the QIAGEN RNeasy mini kit according the manufacturers instructions. The extracted RNA was eluted into 80 ul of RNase free water and frozen at -80^{0}C until all samples needed for the analysis had been collected. The integrity and size distribution of the RNA was checked by electrophoresis and staining with ethidium bromide to visualize the 28*S* and 18*S* subunits and no evidence of degradation was seen. RNA concentrations were estimated by spectrophotometry and the purity was estimated from the ratio of absorbance readings at 260 nm and 280 nm.

Details of the real time RT PCR are given in chapter 2 and also in Appendix III. Briefly, the probes and primer sets for detection of human *Prkdc, ATM, and alpha tubulin* transcripts were selected from open reading frame regions using Primer Express version 1.5A software (Applied Biosystems, Foster City CA). The TaqMan probes were designed so the predicted melting temperature was at least 10^{0}C higher than that for the primers. Primer and probe sequences were blast searched to ensure unique amplification for the target gene sequences.

The primers and probes used are shown in Table 2-2. In all cases the 5' end of the probes were labeled with the fluorescent dye FAM (6-carboxyfluroscein). The 3' ends were labeled with the quencher BHQ_1 (Blackhole 1) for the *ATM* and *Prkdc* amplifications and with TAMRA (NNN'N"tetramethyl-6-carboxyrhodamine) for the *alpha tubulin*.

The PCR protocols were confirmed with regard to product size and using serial dilutions to generate standard curve. I determined the optimal primer and probe concentrations to give the best efficiency for amplification over a broad range. The optimization was determined for each gene product and this included determination of optimal annealing temperatures. Total RNA was reverse transcribed and PCR reactions were carried out using this cDNA. Details of these optimization conditions are described in chapter 2. From 3 to 5 replicate tubes were prepared for each sample time and these were placed randomly in the sample holder of the iCycler real time PCR machine.

A detailed description of the way the relative changes in *Prkdc* and *ATM* mRNA levels was determined in the various samples taken as a function of time after the start of the anti-*Prkdc* siRNA transfections is presented in chapter 2 with supplementary material given in Appendix III, but the approach was basically as follows: First, for each of the control siRNA transfected samples taken at the various times, the ratio of the amount of amplification of *ATM* (or *Prkdc*) required to reach a given level in the exponential range above the critical threshold level to the amount of amplification of *alpha tubulin* housekeeping gene product to reach that same level was calculated with allowance for the difference in the efficiency of the PCR amplification for the two gene products. Then, the same ratio, also with allowance for the different PCR amplification efficiencies, was calculated for the corresponding anti-*Prkdc* siRNA sample taken at the same time. These ratios are taken to allow for the fact that the absolute quantities of basic sample material may change from one sample time to another (equivalent to loading controls on western blots). The ratio of the relative *ATM* (or *Prkdc*) *to tubulin* for control transfected cells at that sample time was then divided by the ratio of relative *ATM* (or *Prkdc*) *to tubulin* for

the corresponding anti-*Prkdc* transfected cell sample to give the normalized relative change in *ATM* (or *Prkdc*) at that sample time.

I also checked for possible contamination of the samples from genomic DNA by measuring the PCR products following amplification using primers for the *GAPDH* gene located in different but adjacent exons of the genomic DNA. The PCR product from genomic DNA should be about 2000 bp, because a relatively short intron is also located between the primers. No such product was seen, but instead a product of only 200 bp was seen as expected for the cDNA from the processed mRNA.

Results and Discussion

Concentration or Dilution Dependencies of siRNA Knockdown Effectiveness

To avoid off target effects that are not related to the highly specific RNA interference, titration to the lowest possible levels of the siRNA that gave a good response should be used. Another prudent control is to show that two different siRNAs targeting the same mRNA are both effective in knocking down the same mRNA and protein. This was shown in the results presented in chapter 3. Regarding the use of the lowest possible levels, the concentrations we used here and in the previous work (chapter 2 and Peng *et al.* 2002) were in the nanomolar (sub-micromolar) range for this reason. But to illustrate the fact that very low concentrations still provide very effective knockdowns, in two experiments I did examine the concentration or dilution dependence of anti-*Prkdc* siRNA during transfection on the level of DNA-PKcs protein five days after the start of transfection. The results are shown in figure 4-1A and 4-1B. The siRNA material supplied by the manufacturer arrives dry with instructions stating that addition of 1 ml of water will produce a 20 micromolar solution of oligonucleotide. Different quantities (ul) of anti-*Prkdc* siRNA solution were mixed with the recommended OptiMEM medium and Oligofectamine according to the manufacturer's instructions and a fixed quantity was added to the cells for transfection, which proceeded as described in the Materials and Methods section above and in chapter 2. In all the experiments described in this study the "anti-*Prkdc* siRNA" was actually composed of two different siRNAs referred to in the Materials and Methods section as siRNA sequence A and siRNA sequence B, and the concentrations shown in figure 4-1 are the total molar

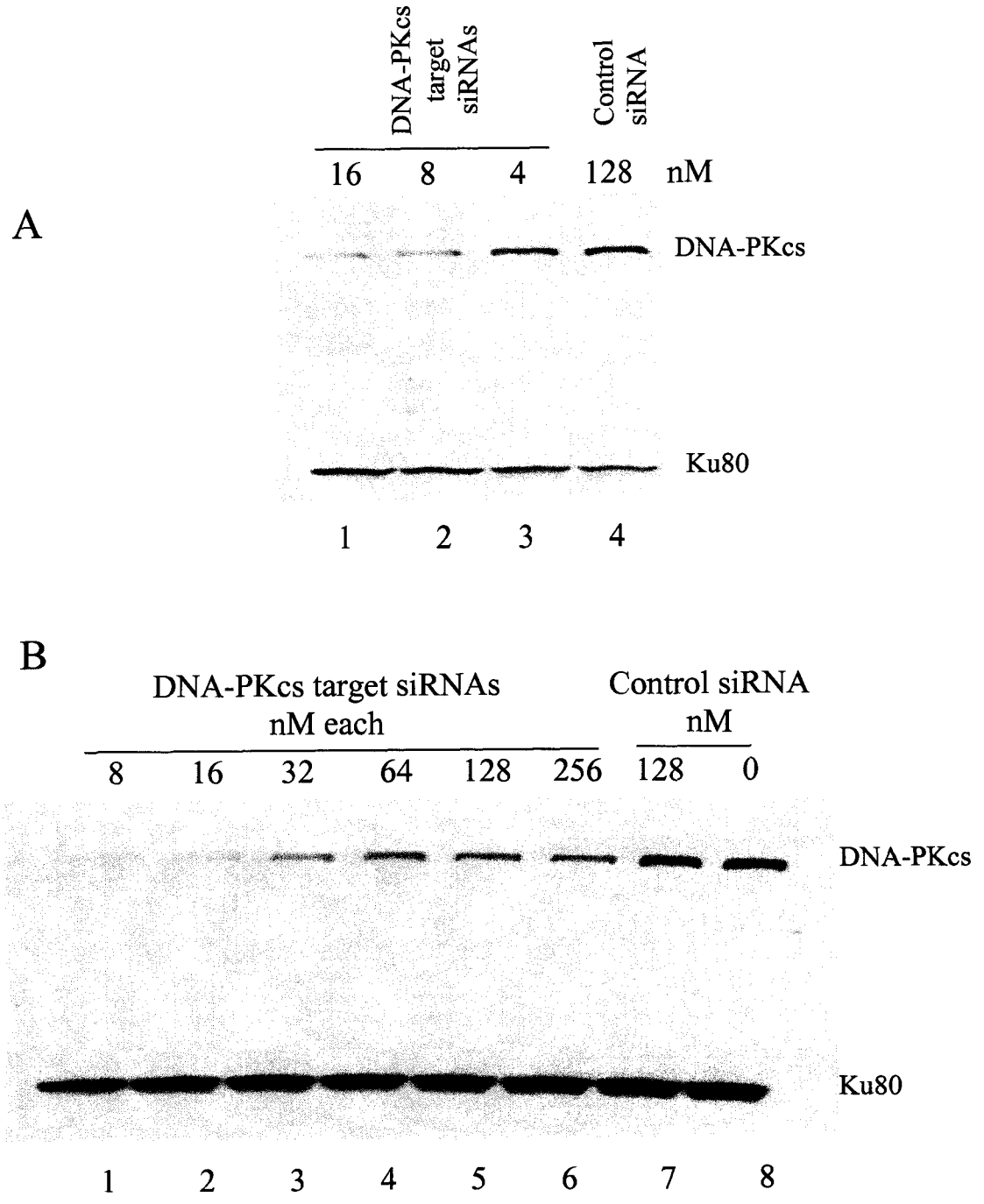


Figure 4-1. Concentration dependency of the inhibition of DNA-PKcs gene expression at the protein level by siRNA. Expression of DNA-PKcs in low-passage normal human fibroblasts (GM08399) after transfection of different concentrations of siRNA targeted to DNA-PKcs mRNA. The concentrations used were 4, 8, 16, 32, 64, 128 and 256nM of the DNA-PKcs targeting siRNAs, 128nm control siRNA and mock transfection without siRNA.

concentrations based on the above information from the supplier. Figure 4-1A lanes 1, 2, and 3 show western blots for DNA-PKcs when siRNA concentrations during transfections were 16nM, 8nM, and 4nM respectively. Lane 4 was from a control siRNA transfection. Ku80 was used as a loading control. The effectiveness was greatest for the 16nM and 8nM concentrations (lanes 1 and 2) with lesser knockdowns at the lower concentration of 4nM. An apparently reverse concentration effectiveness was seen in the second experiment shown in figure 4-1B where the 8nM (lanel) and 16nM (lane 2) concentrations were again more effective than the higher concentrations of 32nM, 64nM, 128nM, and 256nM, all of which appeared to show perhaps about 50% knock down with no concentration or dilution dependence in this range. Since this experiment did not include a parallel immunocytochemical analysis, I do not know whether there were simply more cells that were not successfully transfected and had no knockdown at all for these higher concentrations that, from the western blots, appear to show less knockdown. From these experiments I chose to use the 16nM concentration of the siRNAs in the remainder of the study since, for whatever reason, the knockdown was better. The supplier claims our observation of an apparent reverse concentration effect is not unusual in their experience, and is likely the result of small differences in the oligofectamine to siRNA ratios during transfection. The series of experiments reported for this study started with a new batch of siRNAs from the supplier, and the apparent high effectiveness at some 10-fold lower concentration compared to the studies reported in chapter 3 may be due either to erroneous estimates of the amounts of siRNAs actually provided by the supplier, or to some as yet unknown change in effectiveness of transfections or oligofectamine over the course of time from the first to the second set of experiments.

Another safeguard experiment was carried out to determine whether the anti-*Prkdc* siRNA transfections might be causing off target effects through the induction of an interferon response that results in a generalized reduction in protein synthesis. For this, cells were transfected with a poly(I)·poly(C) double stranded oligonucleotide that is a classical inducer of the interferon response using the same protocol used for the siRNA transfections. The induction of this response is accompanied by a large increase in the phosphorylated form of protein kinase R (PKR). Figure 4-2 shows a western blot from extracts of cells 5 days after transfection with poly(I)·poly(C) (lane 1) and at the same time after transfections with anti-*Prkdc* siRNA (lane 2) or with no transfection. The antibody against phosphorylated PKR revealed a band at the expected molecular weight for the sample transfected with polyIpolyC but no detectable band for the anti-*Prkdc* siRNA transfected cells or the control untransfected cells.

Levels of ATM in Cells Following anti-Prkdc siRNA Knockdown

Figure 4-3 shows a western blot of samples for measurement of both DNA-PKcs and ATM proteins 5 days after the start of transfections of low passage normal human fibroblast cultures with anti-*Prkdc* siRNA (lane 3), a control siRNA (lane 4) with a slightly modified sequence (see above), and a control sample from untransfected cells (lane 5). Lanes 1 and 2 are controls from two different untransfected lymphoblastoid cell lines, one derived from a patient with Ataxia-Telangiectasia (L3 $ATM^{-/-}$; lane 1), and the other from a normal individual (C3 ABR $ATM^{+/+}$; lane 2). Antibodies against Ku 80

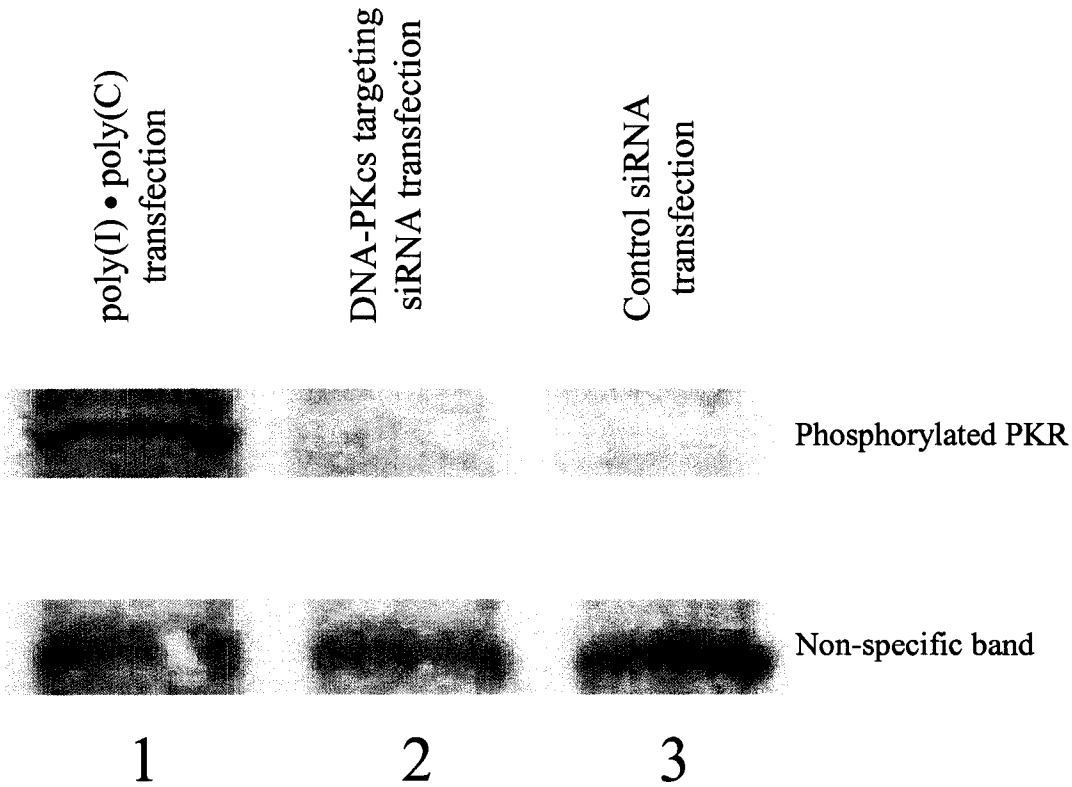


Figure 4-2. Western blot analysis, detecting phsophorylated PKR in low passage normal human fibroblast cultures five days after the start of an DNA-PKcs-targeted siRNA (lane 2) and control siRNA (lane 3) transfection. And 2 days after one transfection of high concentration (1μM) of poly(I)·poly(C) to induce the interferon response (lane 1). A non-specific band is used as a loading control.

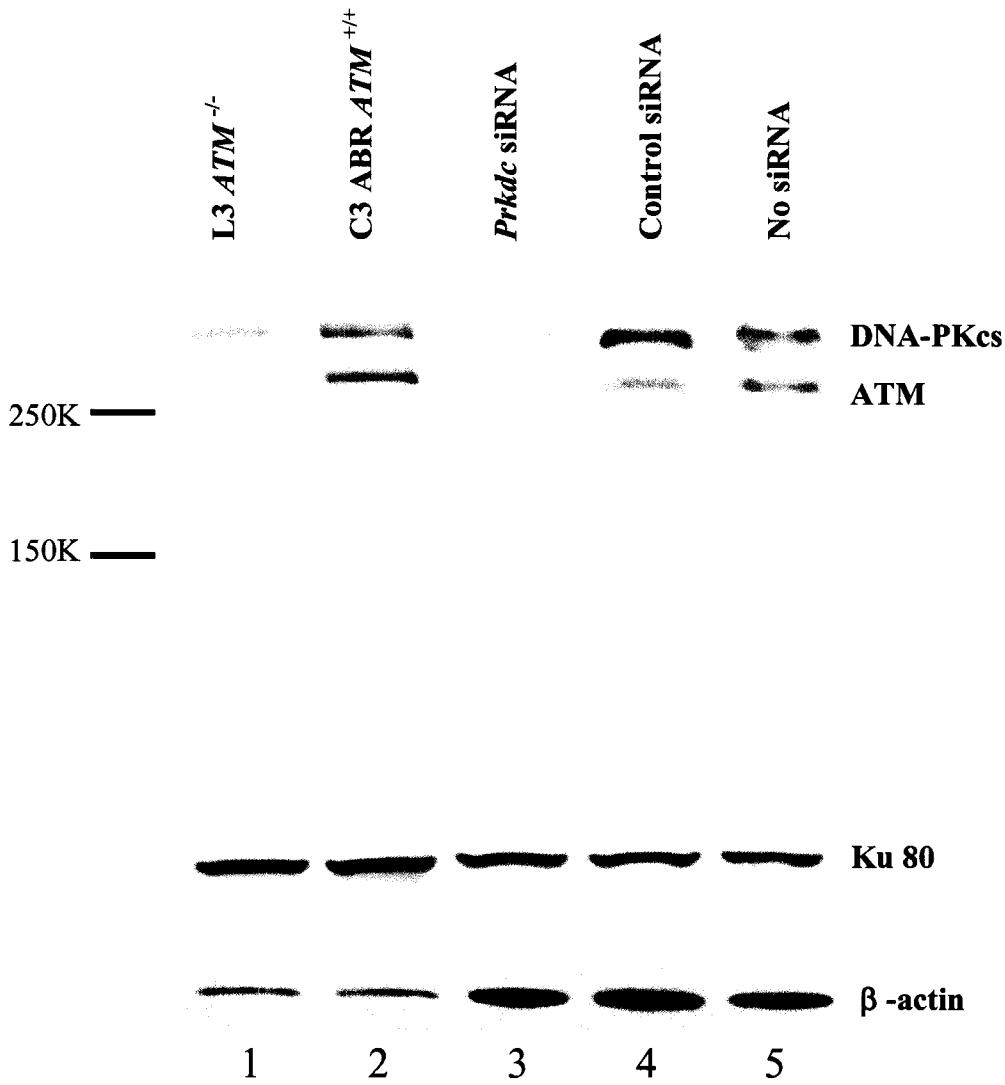


Figure 4-3. Western blot analysis, simultaneously detecting DNA-PKcs, ATM, and Ku80 (loading control) in low passage normal human fibroblast cultures (lanes 3-5) five days after the start of an anti-*Prkdc*- siRNA knock-down protocol. Transfections were at day 0 and day 2, with the assay carried out at day 5 (see: Y. Peng, et. al., Cancer Res. 62, 6400-6404, 2002). Lane 3 shows that a markedly reduced ATM accompanied the *Prkdc*-targeted knock down. Lane 4 is from a control in which the targeting siRNA was altered by 2 bases. Lane 5 was from a non-treated control. Lanes 1 and 2 were from completely different cell lines that were of lymphoblast origin; lane 1 from an *ATM* (-/-) homozygote and lane 2 from an *ATM* (+/+) homozygote, and show that there is no cross-reactivity of the antibodies.

and β-actin served as loading controls, and the lymphoblastoid cell lines helped to identify the ATM band in the human fibroblast test cells. The anti-*Prkdc* siRNA transfection clearly reduced the levels of both DNA-PKcs and ATM in the sample taken 5 days after transfection.

Figure 4-4 shows the result of an experiment to measure DNA-PKcs and ATM proteins immunocytochemically, on a cell by cell basis. Five days after transfections, the low passage normal human fibroblast (GM08399) cells were prepared and stained as described previously. The left upper and lower panels show fluorescence microscope fields of cells stained with DAPI to stain nuclei. The top panels all show the same microscope field for cells transfected with anti-*Prkdc* siRNA, and the bottom panels are also from the same field of cells but in this case the cells were transfected with the control siRNA. The middle panels show the immunocytochemical detection of DNA-PKcs (red) with the excitation and emission filters set for detection of the rhodamine labeled secondary antibody against the primary anti-DNA-PKcs antibody. In the lower middle panel, for control siRNA transfections, all the cells were labeled. Most of the cells in the upper middle panel from the anti-*Prkdc* siRNA transfected cells did show knockdown of DNA-PKcs, but two cells appear to be completely unaffected. If some cells in a culture do not become transfected by the procedure, it is not surprising that western blots can never show 100% knockdown, and in fact for intermediate levels of knockdown, without immunocytochemistry it is impossible to accurately estimate the effectiveness of the siRNA transfection. In this case, the fact that a few cells were not successfully transfected was (fortuitously) an advantage, because when the same field of cells was examined for detection of green fluorescence to detect ATM in the right hand

Figure 4-4. Illustrates the simultaneous immunocytochemical detection of DNA-PKcs and ATM in the same fields of cells with the same anti-*Prkdc* siRNA transfection protocol used in the experiment illustrated in figure 4-1. GM08399 cells were fixed with paraformaldehyde, permeabalized with Triton X-100, then immunostained with mouse monoclonal antibody to DNA-PKcs and rabbit monoclonal antibody to ATM. After incubation with appropriate Rhodamine- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, fluorescent images were captured on a fluorescent microscope. The same cells that were successfully transfected and had reduced DNA-PKcs, also had reduced ATM protein.

panels, the upper panel showed a 1 to 1 correspondence between the cells that did or did not experience knockdown DNA-PKcs (middle panel) and ATM (right panel). In other words, in cells from the same culture where DNA-PKcs was knocked down, ATM was also knocked down, and where DNA-PKcs was not knocked down, neither was ATM.

Figure 4-5 shows quantitative measurements of both DNA-PKcs and ATM protein from western blots (panel A), and of *Prkdc and ATM* mRNAs measured by real-time RT PCR (panel B) as a function of time after the start of transfections with anti-*Prkdc* siRNA. The *Prkdc* mRNA (panel B) was markedly reduced at the earliest sampling time, 4 hours after transfection and remained at a level about 35% of controls until shortly after the second transfection when there appeared to be a further reduction to about 10 to 15% of the initial value. The level of DNA-PKcs protein remained approximately at control or initial levels 1 day after transfection, but dropped to about 40% of the initial level after 2 days, and there was a further drop to around 15% to 20% in the samples taken at 3, 3.7, 4, and 5 days. For *ATM* mRNA the levels were largely unaffected and remained fairly constant for about 2 days, and only after the DNA-PKcs levels had dropped, did the *ATM* mRNA begin to drop as shown from the sample at around 2.7 days. The *ATM* mRNA level eventually dropped to around 25% of initial levels. (Judging from the immunocytochemistry result the samples for protein and mRNA analysis are presumably a mixture of transfected and some untransfected cells.) Only after the ATM mRNA levels dropped did the ATM protein levels begin to drop. The ATM level was reduced to some 20% to 30% of initial levels after around 5 days. For later sampling times, 6, 7, and 8 days after the start of the transfections the mRNA and protein levels return to pre-transfection levels. The transfections are only transient and

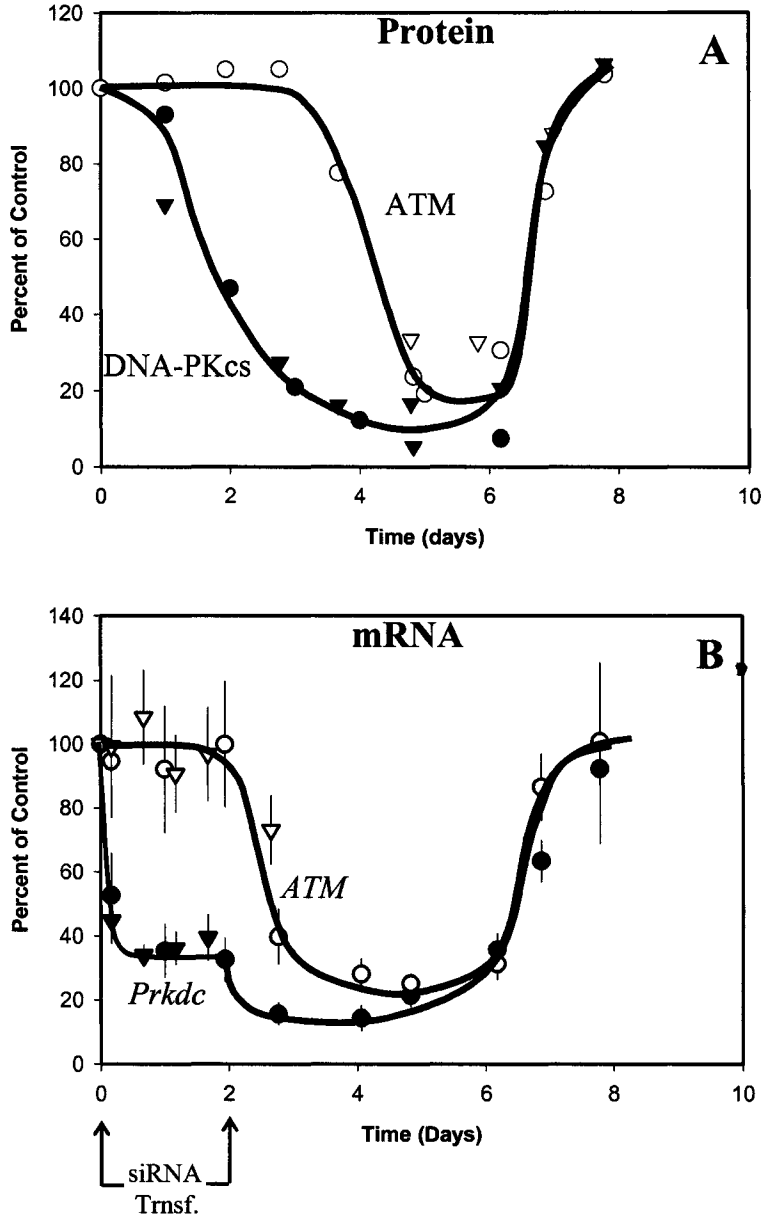


Figure 4-5 summarizes the results of some experiments designed to answer the question of whether the siRNA might also be directly affecting the *ATM* mRNA. Panel A shows a time-course series of measurements of DNA-PKcs from western blots after *Prkdc*-targeted siRNA transfections, as well as the ATM protein levels. Panel B shows quantitative real-time RT-PCR measurements of *Prkdc* and *ATM* mRNA levels as a function of time after the start of the *Prkdc*-targeted siRNA transfection protocol. While the *Prkdc* mRNA dropped fairly rapidly (hours) after transfections, clearly there was no effect on the *ATM* mRNA until much later (days), and not until ***after*** the DNA-PKcs protein level (panel A) had already dropped. After about 6 to 7 days the levels returned to pre-transfection levels.

the cells are dividing throughout the sampling except for growth delays resulting from the transfections at day 0 and day 2. This time course experiment also serves to show that the anti-*Prkdc* siRNA is unlikely to exert an effect on *ATM* mRNA as a collateral off target effect since no effect was seen on the ATM protein or its message until more that 2 days after transfection.

At this stage, it is not clear whether DNA-PKcs exerts its effect on ATM mRNA directly or indirectly on the *ATM* promoter to reduce transcription, or through some post-transcriptional mechanism, perhaps involving pre-mRNA processing.

Regarding so-called off target or non-specific effects or pitfalls that have sometimes been reported with the use of RNAi in general or siRNA knockdowns in particular, the following summary of evidence argues strongly that such confounding effects are not operating here. First, no other specific proteins measured in the anti-*Prkdc* siRNA transfected cells (Ku 80, β-actin, laminA/C) were shown to be affected aside from DNA-PKcs and ATM (see below). Second, the anti-*Prkdc* siRNA did not induce a detectable interferon response. Third, two different siRNA sequences targeting the same *Prkdc* mRNA were both effective in knocking down DNA-PKcs. Fourth, the concentrations of anti-*Prkdc* siRNA for effective knockdown of DNA-PKcs were in the nanomolar range. Fifth, the closest match found of the 21 base siRNA target sequences in *Prkdc* mRNA to similar sequences in *ATM* mRNA was an 8 base sequence in *ATM*, and no off target siRNA knock down with a sequence match closer that 15 bases has ever been reported (Elbashir *et al.* 2001c). Sixth, a control siRNA sequence with two small base sequence modifications had no effect whatever on the levels of any proteins measured including DNA-PKcs and ATM. Lastly, the anti-*Prkdc* siRNA knocked down

the *Prkdc* mRNA very shortly after transfection, but the *ATM* mRNA was unaffected for at least 2 days and decreased only after the DNA-PKcs protein levels decreased. These criteria more than meet the recommended quality standards published in an editorial commentary recently in *Nature Cell Biology* (Editorial 2003).

ATM in Other Cells with Known Defects in DNA-PKcs

Since there are several radiosensitive Prkdc mutant Chinese hamster cell lines, it was of interest to determine whether there might also be an accompanying ATM deficit in these cells. In particular, there is one mutant cell line, V3, that has very little if any DNA-PKcs and another, irs-20 isolated some years ago in this laboratory (Stackhouse and Bedford 1993a; Stackhouse and Bedford 1993b; Stackhouse and Bedford 1994; Lin *et al.* 1997; Priestley *et al.* 1998) with about 5 to 10% of normal levels of a mutant DNA-PKcs that is kinase-inactive. Plasmid or YAC corrected clonal lines of these mutant cells that are of normal wild-type radiosensitivity were also available. Different antibodies for detecting the rodent ATM were used but this antibody also detected the human ATM so the control cells (L3 *ATM*-/- and C3 ABR *ATM*+/+ human lymphoblastoid cell lines) could also be used. It is worth noting that in general, rodent cells constitutively express only some 2% to 5% of the levels of DNA-PKcs seen in human cells.

Figure 4-6 shows a western blot for DNA-PKcs and ATM for seven different cell lines. β-actin was used as a loading control in all cases. The amounts of total protein loaded are shown for each lane. For detection of ATM and the loading control β-actin all lanes were loaded with 70ug of total protein, but there were very different quantities of total protein loaded for the DNA-PKcs determinations. Lanes 1 and 2, respectively, were

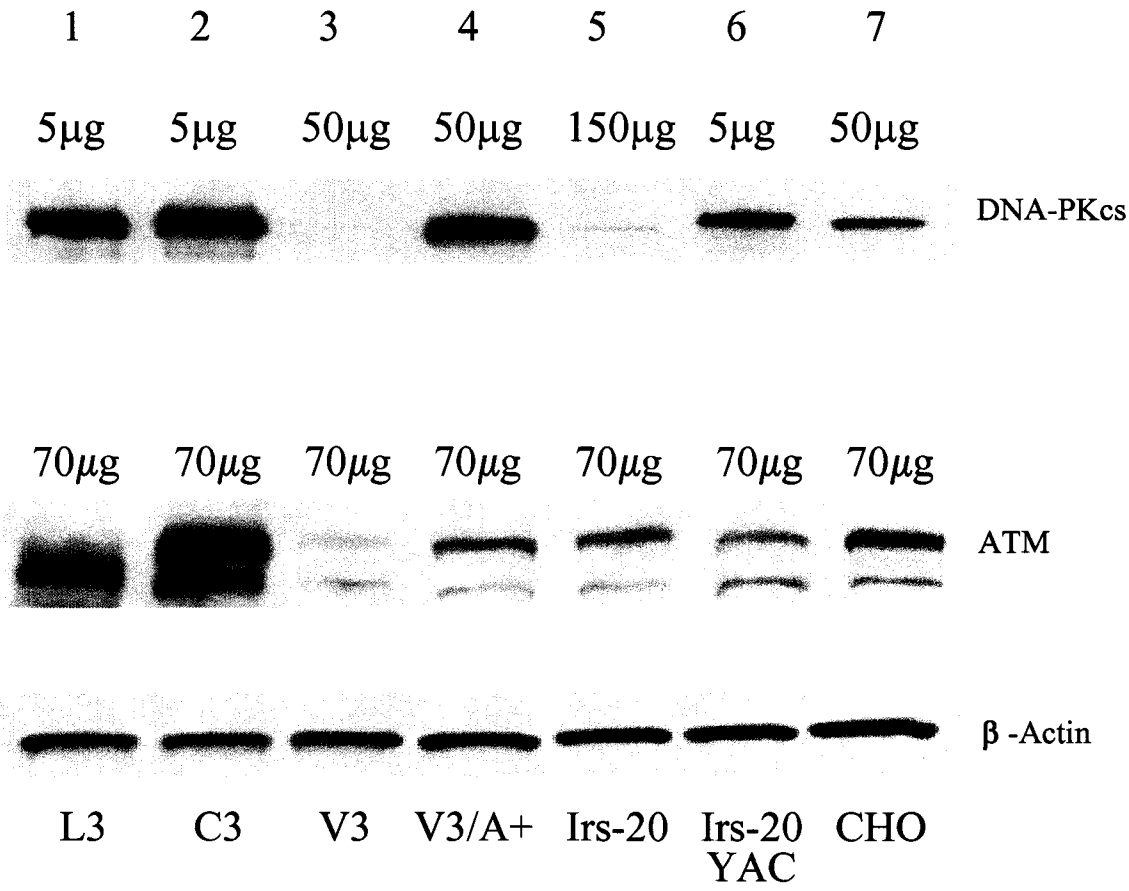


Figure 4-6. Western blot analysis of ATM levels in different DNA-PKcs mutant hamster cells, and the corresponding human DNA-PKcs corrected cells. Lanes 1 and 2 are from extracts of human cells. Lane 1 is from *ATM-/-* cells and lane 2 from *ATM+/+* cells. V3 hamster cells and the empty vector controls (V3, lane 3) do not have measurable DNA-PKcs but have a reduced ATM level. Corrected V3 cells (V3/A+, lane 4), which have a plasmid containing human DNA-PKcs, contain normal or above normal levels of DNA-PKcs as well as normal levels of ATM. Irs-20 cells (lane 5) have a point mutation in the catalytic subunit of DNA-PKcs and have about 5% to 10% of normal levels of the kinase defective mutant DNA-PKcs protein, but a normal level of ATM. The irs-20 cells that have a YAC containing human DNA-PKcs (lane 6), also have higher than normal levels of DNA-PKcs, but normal levels of ATM. Lane 7 is from wild-type CHO-10B2 cells. Beta actin was used as a loading control. Loading was the same in all cases (70ug) for the ATM samples, but different quantities of total protein were loaded for the DNA-PKcs samples to adjust for different levels normally present in the different cell lines.

from extracts of human L3 ATM-/- and human C3 ABR ATM+/+ cells. For DNA-PKcs westerns using the human cells, only 5ug of protein was loaded. The ATM antibody detects a nonspecific band of lower molecular weight as seen in from the result following detection with this particular anti-ATM antibody. The same non-specific band is present in both the *ATM*-/- and *ATM*+/+ cells (lanes 1 and 2) but the ATM band of course is absent in the *ATM*-/- cells (lane 1). Lane 3 and lane 4 are from extracts of either V3 (with an empty vector) or V3/A+ containing a vector with Prkdc that corrects the cells to wild type radiosensitivity. There was no detectable DNA-PKcs in the V3 cells but there was a large amount (but still 10-fold less than human cells) in the V3/A+ corrected cells. There was a reduced but not largely reduced level of ATM in the V3 cells (lane 3 middle panel), and in the corrected V3/A+ cells there was a significantly increased level of ATM (lane 4) relative to the uncorrected V3 cells (lane 3). Lanes 5 and 6 were from extracts of irs-20 *Prkdc* mutant cells that contain a reduced quantity of a mutant DNA-PKcs protein with no kinase activity (lane 5) and from a YAC corrected clone (containing human *Prkdc*) of irs-20 cells (lane 6). Although the amount of DNA-PKcs mutant protein in irs-20 cells (lane 5) is only about 5% of the level of wild-type DNA-PKcs in wild-type parental CHO cells (lane7), the level of ATM in the irs-20 cells is approximately the same as that in the wild-type cells. The irs-20 cells corrected with the human *Prkdc*-containing YAC had a large abundance of DNA-PKcs, at least 20 to 30-fold more than that in the wild-type cells, but this did not alter the level of ATM relative to wild-type cells.

In the case of these various mutant cell lines and the corrected clonal derivatives, all cells in a given cell line were presumably the same so that there would not be a

problem of different proportions of successfully transfected cells. If in the case of the siRNA transfected human cells the effect of DNA-PKcs knock down is even greater as might be expected comparing the western blot data and the immunocytochemistry, then the question arises as to why there is not as dramatic a correlation between the amounts of DNA-PKcs in the various mutant and wild-type CHO cells as seen for the human cells. There are at least two possible explanations that might be involved in this apparent discrepancy. First, the constitutive levels of expression of DNA-PKcs are some 20 to 50-fold higher in human compared to CHO cells, and at least for these human cells, the ATM level (figure 4-6 middle panel) is perhaps 3 to 5-fold higher than in wild-type CHO cells. Starting at this higher ATM level in human cells, an 80 to 90% relative reduction in ATM may be easier to achieve with a 90% reduction in DNA-PKcs, than with a starting basal level of ATM that is some 3-fold lower in CHO but with a DNA-PKcs level that is already 20 to 50-fold lower than for human cells. Another possibility might be that the efficiencies of the process whereby DNA-PKcs controls ATM mRNA transcription or processing to a mature message may be less efficient in CHO than in human cells.

Whatever the explanation, it seems clear that DNA-PK can control the expression of ATM in human cells and DNA-PKcs deficiencies could have consequences on processes connected to the network of damage response pathways controlled by ATM that have not been recognized previously.

Ahn, J. Y., Schwarz, J. K., Piwnica-Worms, H., and Canman, C. E. (2000) Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. *Cancer Research* **60,** 5934-5936.

Allalunis-Turner, M. J., Barron, G. M., Day, R. S., Dobler, K. D., and Mirzayans, R. (1993) Isolation of 2 Cell-Lines from A Human-Malignant Glioma Specimen Differing in Sensitivity to Radiation and Chemotherapeutic Drugs. *Radiation Research* **134,** 349-354.

Andegeko, Y., Moyal, L., Mittelman, L., Tsarfaty, I., Shiloh, Y., and Rotman, G. (2001) Nuclear retention of ATM at sites of DNA double strand breaks. *Journal of Biological Chemistry* **276,** 38224-38230.

Araki, R., Fujimori, A., Hamatani, K., Mita, K., Saito, T., Mori, M., Fukumura, R., Morimyo, M., Muto, M., Itoh, M., Tatsumi, K., and Abe, M. (1997) Nonsense mutation at Tyr-4046 in the DNA-dependent protein kinase catalytic subunit of severe combined immune deficiency mice. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 2438-2443.

Bahari, I. B. and Bedford, J. S. (1989) Chromosomal radiosensitivity and its implications the radiosensitivity of Chinese hamster XR-1 mutant cells. *Sains Malasiana* **18,** 65-80.

Bailey, S. M., Cornforth, M. N., Kurimasa, A., Chen, D. J., and Goodwin, E. H. (2001) Strand-specific postreplicative processing of mammalian telomeres. *Science* **293,** 2462-2465.

Bailey, S. M., Meyne, J., Chen, D. J., Kurimasa, A., Li, G. C., Lehnert, B. E., and Goodwin, E. H. (1999) DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 14899-14904.

Bakkenist, C. J. and Kastan, M. B. (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421,** 499-506.

Banin, S., Moyal, L., Shieh, S. Y., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998) Enhanced phosphorylation of p53 by ATN in response to DNA damage. *Science* **281,** 1674-1677.

Baskaran, R., Wood, L. D., Whitaker, L. L., Canman, C. E., Morgan, S. E., Xu, Y., Barlow, C., Baltimore, D., WynshawBoris, A., Kastan, M. B., and Wang, J. Y. J. (1997) Ataxia telangiectasia mutant protein activates c-Abl tyrosine kinase in response to ionizing radiation. *Nature* **387,** 516-519.

Bassing, C. H., Chua, K. F., Sekiguchi, J., Suh, H., Whitlow, S. R., Fleming, J. C., Monroe, B. C., Ciccone, D. N., Yan, C., Vlasakova, K., Livingston, D. M., Ferguson, D. O., Scully, R., and Alt, F. W. (2002a) Increased ionizing radiation sensitivity and

genomic instability in the absence of histone H2AX. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 8173-8178.

Bassing, C. H., Swat, W., and Alt, F. W. (2002b) The mechanism and regulation of chromosomal V(D)J recombination. *Cell* **109,** S45-S55.

Baumann, P. and West, S. C. (1997) The human Rad51 protein: polarity of strand transfer and stimulation by hRP-A. *Embo Journal* **16,** 5198-5206.

Beadle, G. W. and Tatum, E. L. (1941) Genetic control of biochemical reactions in Neurosora. *Proceedings of the National Academy of Sciences of the United States of America* **27,** 499-506.

Beadle, G. W. and Tatum, E. L. (1945) Neurospora .2. Methods of Producing and Detecting Mutations Concerned with Nutritional Requirements. *American Journal of Botany* **32,** 678-686.

Bedford, J. S. and Dewey, W. C. (2002) Historical and current highlights in radiation biology: Has anything important been learned by irradiating cells? *Radiation Research* **158,** 251-291.

Bedford, J. S. and Goodhead, D. T. (1989) Breakage of Human Interphase Chromosomes by Alpha-Particles and X-Rays. *International Journal of Radiation Biology* **55,** 211-216.

Bedford, J. S. and Mitchell, J. B. (1977) Chromosome Condensation and Radiation-Induced G2 Arrest Studied by Induction of Premature Chromosome Condensation Following Cell-Fusion. *Radiation Research* **70,** 641.

Bedford, J. S., Mitchell, J. B., Griggs, H. G., and Bender, M. A. (1978) Radiation-Induced Cellular Reproductive Death and Chromosome-Aberrations. *Radiation Research* **76,** 573-586.

Bender, M. A., Bedford, J. S., and Mitchell, J. B. (1973) Mechanisms of Chromosomal Aberration Production .2. Aberrations Induced by 5-Bromodeoxyuridine and Visible Light. *Mutation Research* **20,** 403-416.

Bender, M. A., Griggs, H. G., and Bedford, J. S. (1974) Mechanisms of Chromosomal Aberration Production .3. Chemicals and Ionizing-Radiation. *Mutation Research* **23,** 197-212.

Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409,** 363-366.

Bevington, P. R. (1969) Propagation of errors. In: *Data Reduction and Error Analysis for the Physical Sciences*, pp. 56-65. McGraw-Hill: New York.

Boder, E. and Sedgwick, R. P. (1958) Ataxia-Telangiectasia - A Familial Syndrome of Progressive Cerebellar Ataxia, Oculocutaneous Telangiectasia and Frequent Pulmonary Infection. *Pediatrics* **21,** 526-554.

Borrelli, M. J., Mackey, M. A., and Dewey, W. C. (1987) A Method for Freezing Synchronous Mitotic and G1 Cells. *Experimental Cell Research* **170,** 363-368.

Bostock, C. J., Prescott, D. M., and KIRKPATR.JB (1971) Evaluation of Double Thymidine Block for Synchronizing Mammalian Cells at G1-S Border. *Experimental Cell Research* **68,** 163-&.

Boultwood, J. (2001) Ataxia telangiectasia gene mutations in leukaemia and lymphoma. *Journal of Clinical Pathology* **54,** 512-516.

Bridge, A. J., Pebernard, S., Ducraux, A., Nicoulaz, A. L., and Iggo, R. (2003) Induction of an interferon response by RNAi vectors in mammalian cells. *Nature Genetics* **34,** 263-264.

Brown, K. D., Ziv, Y., Sadanandan, S. N., Chessa, L., Collins, F. S., Shiloh, Y., and Tagle, D. A. (1997) The ataxia-telangiectasia gene product, a constitutively expressed nuclear protein that is not up-regulated following genome damage. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 1840-1845.

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296,** 550-553.

Brush, G. S., Anderson, C. W., and Kelly, T. J. (1994) The Dna-Activated Protein-Kinase Is Required for the Phosphorylation of Replication Protein-A During Simian-Virus-40 Dna-Replication. *Proceedings of the National Academy of Sciences of the United States of America* **91,** 12520-12524.

Burma, S., Chen, B. P., Murphy, M., Kurimasa, A., and Chen, D. J. (2001) ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *Journal of Biological Chemistry* **276,** 42462-42467.

Canman, C. E. and Lim, D. S. (1998) The role of ATM in DNA damage responses and cancer. *Oncogene* **17,** 3301-3308.

Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science* **281,** 1677-1679.

Caplen, N. J., Parrish, S., Imani, F., Fire, A., and Morgan, R. A. (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 9742-9747.

Chan, D. W., Chen, B. P. C., Prithivirajsingh, S., Kurimasa, A., Story, M. D., Qin, J., and Chen, D. J. (2002) Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks. *Genes & Development* **16,** 2333-2338.

Chan, D. W., Gately, D. P., Urban, S., Galloway, A. M., Lees-Miller, S. P., Yen, T., and Allalunis-Turner, J. (1998) Lack of correlation between ATM protein expression and tumour cell radiosensitivity. *International Journal of Radiation Biology* **74,** 217-224.

Chen, F. Q., Nastasi, A., Shen, Z. Y., Brenneman, M., Crissman, H., and Chen, D. J. (1997) Cell cycle-dependent protein expression of mammalian homologs of yeast DNA double-strand break repair genes Rad51 and Rad52. *Mutation Research-Dna Repair* **384,** 205-211.

Chen, G. and Lee, E. Y. H. P. (1996) The product of the ATM gene is a 370-kDa nuclear phosphoprotein. *Journal of Biological Chemistry* **271,** 33693-33697.

Chen, P., Peng, C., Luff, J., Spring, K., Watters, D., Bottle, S., Furuya, S., and Lavin, M. F. (2003) Oxidative stress is responsible for deficient survival and dendritogenesis in Purkinje neurons from ataxia-telangiectasia mutated mutant mice. *Journal of Neuroscience* **23,** 11453-11460.

Cleaver, J. E. (1968) Defective Repair Replication of Dna in Xeroderma Pigmentosum. *Nature* **218,** 652-&.

Cogoni, C., Irelan, J. T., Schumacher, M., Schmidhauser, T. J., Selker, E. U., and Macino, G. (1996) Transgene silencing of the al-1 gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. *Embo Journal* **15,** 3153-3163.

Cogoni, C., Romano, N., and Macino, G. (1994) Suppression of Gene-Expression by Homologous Transgenes. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **65,** 205-209.

Conn, G. L. (2003) Expression of active RNA-activated protein kinase (PKR) in bacteria. *Biotechniques* **35,** 682-+.

Cornforth, M. N. and Bedford, J. S. (1983a) High-Resolution Measurement of Breaks in Prematurely Condensed Chromosomes by Differential Staining. *Chromosoma* **88,** 315-318.

Cornforth, M. N. and Bedford, J. S. (1983b) X-Ray-Induced Breakage and Rejoining of Human Interphase Chromosomes. *Science* **222,** 1141-1143.

Cornforth, M. N. and Bedford, J. S. (1985) On the Nature of A Defect in Cells from Individuals with Ataxia-Telangiectasia. *Science* **227,** 1589-1591.

Cornforth, M. N. and Bedford, J. S. (1987) A Quantitative Comparison of Potentially Lethal Damage Repair and the Rejoining of Interphase Chromosome Breaks in Low Passage Normal Human-Fibroblasts. *Radiation Research* **111,** 385-405.

Cornforth, M. N. and Bedford, J. S. (1993) Ionizing-Radiation Damage and Its Early Development in Chromosomes. *Advances in Radiation Biology, Vol 17* **17,** 423-496.

Cortez, D., Wang, Y., Qin, J., and Elledge, S. J. (1999) Requirement of ATM-dependent phosphorylation of BRCA1 in the DNA damage response to double-strand breaks. *Science* **286,** 1162-1166.

Dai, Y., Kysela, B., Hanakahi, L. A., Manolis, K., Riballo, E., Stumm, M., Harville, T. O., West, S. C., Oettinger, M. A., and Jeggo, P. A. (2003) Nonhomologous end joining and V(D)J recombination require an additional factor. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 2462-2467.

DeFazio, L. G., Stansel, R. M., Griffith, J. D., and Chu, G. (2002) Synapsis of DNA ends by DNA-dependent protein kinase. *Embo Journal* **21,** 3192-3200.

Der, S. D., Yang, Y. L., Weissmann, C., and Williams, B. R. G. (1997) A double-stranded RNA-activated protein kinase-dependent pathway mediating stress-induced apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 3279-3283.

Ding, Q., Reddy, Y. V. R., Wang, W., Woods, T., Douglas, P., Ramsden, D. A., Lees-Miller, S. P., and Meek, K. (2003) Autophosphorylation of the catalytic subunit of the DNA-dependent protein kinase is required for efficient end processing during DNA double-strand break repair. *Molecular and Cellular Biology* **23,** 5836-5848.

Dykxhoorn, D. M., Novina, C. D., and Sharp, P. A. (2003) Killing the messenger: Short RNAs that silence gene expression. *Nature Reviews Molecular Cell Biology* **4,** 457-467.

Editorial (2003) Whither RNAi. *Nature Cell Biology* **5,** 489-490.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411,** 494-498.

Elbashir, S. M., Harborth, J., Weber, K., and Tuschl, T. (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* **26,** 199-213.

Elbashir, S. M., Lendeckel, W., and Tuschl, T. (2001b) RNA interference is mediated by 21-and 22-nucleotide RNAs. *Genes & Development* **15,** 188-200.

Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001c) Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *Embo Journal* **20,** 6877-6888.

Elson, A., Wang, Y. Q., Daugherty, C. J., Morton, C. C., Zhou, F., CamposTorres, J., and Leder, P. (1996) Pleiotropic defects in ataxia-telangiectasia protein-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 13084-13089.

Essers, J., Hendriks, R. W., Swagemakers, S. M. A., Troelstra, C., deWit, J., Bootsma, D., Hoeijmakers, J. H. J., and Kanaar, R. (1997) Disruption of mouse RAD54 reduces ionizing radiation resistance. *Cell* **89,** 195-204.

Evans, J. W., Liu, X. F., Kirchgessner, C. U., and Brown, J. M. (1996) Induction and repair of chromosome aberrations in scid cells measured by premature chromosome condensation. *Radiation Research* **145,** 39-46.

Fire, A., Xu, S. Q., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391,** 806-811.

Flygare, J., Benson, F., and Hellgren, D. (1996) Expression of the human RAD51 gene during the cell cycle in primary human peripheral blood lymphocytes. *Biochimica et Biophysica Acta-Molecular Cell Research* **1312,** 231-236.

Foray, N., Marot, D., Randrianarison, V., Dalla Venezia, N., Picard, D., Perricaudet, M., Favaudon, V., and Jeggo, P. (2002) Constitutive association of BRCA1 and c-Abl and its ATM-dependent disruption after irradiation. *Molecular and Cellular Biology* **22,** 4020-4032.

Fournier, C., Brons, S., and Tauchor-Scholz, G. (2003) Quantification of chemiluminescent signals using photon-sensitive films or a CCD camera. *Biotechniques* **35,** 284-+.

Gatei, M., Scott, S. P., Filippovitch, I., Soronika, N., Lavin, M. F., Weber, B., and Khanna, K. K. (2000) Role for ATM in DNA damage-induced phosphorylation of BRCA1. *Cancer Research* **60,** 3299-+.

Gately, D. P., Hittle, J. C., Chan, G. K. T., and Yen, T. J. (1998) Characterization of ATM expression, localization, and associated DNA-dependent protein kinase activity. *Molecular Biology of the Cell* **9,** 2361-2374.

Gatti, R. A., Berkel, I., Boder, E., Braedt, G., Charmley, P., Concannon, P., Ersoy, F., Foroud, T., Jaspers, N. G. J., Lange, K., Lathrop, G. M., Leppert, M., Nakamura, Y., Oconnell, P., Paterson, M., Salser, W., Sanal, O., Silver, J., Sparkes, R. S., Susi, E., Weeks, D. E., Wei, S., White, R., and Yoder, F. (1988) Localization of An Ataxia-Telangiectasia Gene to Chromosome 11Q22-23. *Nature* **336,** 577-580.

Gellert, M., Hesse, J. E., Hiom, K., Melek, M., Modesti, M., Paull, T. T., Ramsden, D. A., and van Gent, D. C. (1999) V(D)J recombination: Links to transposition and double-strand break repair. *Cold Spring Harbor Symposia on Quantitative Biology* **64,** 161-167.

Goodspeed, T. H. and Olson, A. R. (1928) The production of variation in Nicotiana species by x-ray treatment of sex cells. *Proceedings of the National Academy of Sciences of the United States of America* **14,** 66-68.

Guo, S. and Kemphues, K. J. (1995) Par-1, A Gene Required for Establishing Polarity in C-Elegans Embryos, Encodes A Putative Ser/Thr Kinase That Is Asymmetrically Distributed. *Cell* **81,** 611-620.

Haber, J. E. (2000) Partners and pathways - repairing a double-strand break. *Trends in Genetics* **16,** 259-264.

Hamilton, A. J. and Baulcombe, D. C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286,** 950-952.

Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* **404,** 293-296.

Hammond, S. M., Caudy, A. A., and Hannon, G. J. (2001) Post-transcriptional gene silencing by double-stranded RNA. *Nature Reviews Genetics* **2,** 110-119.

Hannon, G. J. (2002) RNA interference. *Nature* **418,** 244-251.

Harnden, D. G. (1994) The Nature of Ataxia-Telangiectasia - Problems and Perspectives. *International Journal of Radiation Biology* **66,** S13-S19.

Hartley, K. O., Gell, D., Smith, G. C. M., Zhang, H., Divecha, N., Connelly, M. A., Admon, A., LeesMiller, S. P., Anderson, C. W., and Jackson, S. P. (1995) Dna-Dependent Protein-Kinase Catalytic Subunit - A Relative of Phosphatidylinositol 3-Kinase and the Ataxia-Telangiectasia Gene-Product. *Cell* **82,** 849-856.

Helleday, T. (2003) Pathways for mitotic homologous recombination in mammalian cells. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* **532,** 103-115.

Herzog, K. H., Chong, M. J., Kapsetaki, M., Morgan, J. I., and McKinnon, P. J. (1998) Requirement for Atm in ionizing radiation-induced cell death in the developing central nervous system. *Science* **280,** 1089-1091.

Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J., and Mak, T. W. (2000) DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science* **287,** 1824-1827.

Hoeijmakers, J. H. J. (2001) Genome maintenance mechanisms for preventing cancer. *Nature* **411,** 366-374.

Holen, T., Amarzguioui, M., Wiiger, M. T., Babaie, E., and Prydz, H. (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. *Nucleic Acids Research* **30,** 1757-1766.

Hunter, T. (1995) When Is A Lipid Kinase Not A Lipid Kinase - When It Is A Protein-Kinase. *Cell* **83,** 1-4.

Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P. S. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotechnology* **21,** 635-637.

Jeggo, P. A. (1998a) DNA breakage and repair. *Advances in Genetics, Vol 38* **38,** 185-218.

Jeggo, P. A. (1998b) Identification of genes involved in repair of DNA double-strand breaks in mammalian cells. *Radiation Research* **150,** S80-S91.

Jeggo, P. A. and Kemp, L. M. (1983) X-Ray-Sensitive Mutants of Chinese-Hamster Ovary Cell-Line - Isolation and Cross-Sensitivity to Other Dna-Damaging Agents. *Mutation Research* **112,** 313-327.

Johnson, N. and Kotz, S. (1969) Discrete Distributions. pp. 96-98. Wiley: New York.

Johnson, R. T. and Rao, P. N. (1970) Mammalian Cell Fusion - Induction of Premature Chromosome Condensation in Interphase Nuclei. *Nature* **226,** 717-&.

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. (1991) Participation of P53 Protein in the Cellular-Response to Dna Damage. *Cancer Research* **51,** 6304-6311.

Kawasaki, H., Suyama, E., Iyo, M., and Taira, K. (2003) siRNAs generated by recombinant human Dicer induce specific and significant but target site-independent gene silencing in human cells. *Nucleic Acids Research* **31,** 981-987.

Kelner, A. (1949) Effect of Visible Light on the Recovery of Streptomyces Griseus Conidia from Ultraviolet Irradiation Injury. *Proceedings of the National Academy of Sciences of the United States of America* **35,** 73-79.

Kennerdell, J. R. and Carthew, R. W. (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* **95,** 1017-1026.

Kennerdell, J. R. and Carthew, R. W. (2000) Heritable gene silencing in Drosophila using double-stranded RNA. *Nature Biotechnology* **18,** 896-898.

Ketting, R. F., Fischer, S. E. J., Bernstein, E., Sijen, T., Hannon, G. J., and Plasterk, R. H. A. (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C-elegans. *Genes & Development* **15,** 2654-2659.

Khabar, K. S. A., Siddiqui, Y. M., al Zoghaibi, F., al Haj, L., Dhalla, M., Zhou, A. M., Dong, B. H., Whitmore, M., Paranjape, J., Al Ahdal, M. N., Al Mohanna, F., Williams, B. R. G., and Silverman, R. H. (2003) RNase L mediates transient control of the interferon response through modulation of the double-stranded RNA-dependent protein kinase PKR. *Journal of Biological Chemistry* **278,** 20124-20132.

Khanna, K. K. (2000) Cancer risk and the ATM gene: A continuing debate. *Journal of the National Cancer Institute* **92,** 795-802.

Khosravi, R., Maya, R., Gottlieb, T., Oren, M., Shiloh, Y., and Shkedy, D. (1999) Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 14973-14977.

Kirchgessner, C. U., Patil, C. K., Evans, J. W., Cuomo, C. A., Fried, L. M., Carter, T., Oettinger, M. A., and Brown, J. M. (1995) Dna-Dependent Kinase (P350) As A Candidate Gene for the Murine Scid Defect. *Science* **267,** 1178-1183.

Kozlov, S., Gueven, N., Keating, K., Ramsay, J., and Lavin, M. F. (2003) ATP activates ataxia-telangiectasia mutated (ATM) in vitro - Importance of autophosphorylation. *Journal of Biological Chemistry* **278,** 9309-9317.

Kumar, R., Conklin, D. S., and Mittal, V. (2003) High-throughput selection of effective RNAi probes for gene silencing. *Genome Research* **13,** 2333-2340.

Labhart, P. (1997) mRNA encoding the catalytic subunit of DNA-dependent protein kinase is widely expressed in Xenopus cells. *Gene* **203,** 235-240.

Ladenburger, E. M., Fackelmayer, F. O., Hameister, H., and Knippers, R. (1997) MCM4 and PRKDC, human genes encoding proteins MCM4 and DNA-PKcs, are close neighbours located on chromosome 8q12->q13. *Cytogenetics and Cell Genetics* **77,** 268-270.

Lavin, M. F. (1999) ATM: the product of the gene mutated in ataxia-telangiectasia. *International Journal of Biochemistry & Cell Biology* **31,** 735-740.

Lavin, M. F., Khanna, K. K., Beamish, H., Spring, K., Watters, D., and Shiloh, Y. (1995) Relationship of the Ataxia-Telangiectasia Protein Atm to Phosphoinositide 3-Kinase. *Trends in Biochemical Sciences* **20,** 382-383.

Lavin, M. F. and Shiloh, Y. (1997) The genetic defect in ataxia-telangiectasia. *Annual Review of Immunology* **15,** 177-202.

Leber, R., Wise, T. W., Mizuta, R., and Meek, K. (1998) The XRCC4 gene product is a target for and interacts with the DNA-dependent protein kinase. *Journal of Biological Chemistry* **273,** 1794-1801.

Lee, S. E., Mitchell, R. A., Cheng, A., and Hendrickson, E. A. (1997) Evidence for DNA-PK-dependent and -independent DNA double-strand break repair pathways in mammalian cells as a function of the cell cycle. *Molecular and Cellular Biology* **17,** 1425-1433.

Lees-Miller, S. P., Godbout, R., Chan, D. W., Weinfeld, M., Day, R. S., Barron, G. M., and Allalunisturner, J. (1995) Absence of P350 Subunit of Dna-Activated Protein-Kinase from A Radiosensitive Human Cell-Line. *Science* **267,** 1183-1185.

Lees-Miller, S. P. and Meek, K. (2003) Repair of DNA double strand breaks by non-homologous end joining. *Biochimie* **85,** 1161-1173.

LeesMiller, S. P., Sakaguchi, K., Ullrich, S. J., Appella, E., and Anderson, C. W. (1992) Human Dna-Activated Protein-Kinase Phosphorylates Serine-15 and Serine-37 in the Amino-Terminal Transactivation Domain of Human P53. *Molecular and Cellular Biology* **12,** 5041-5049.

Liang, F., Han, M. G., Romanienko, P. J., and Jasin, M. (1998) Homology-directed repair is a major double-strand break repair pathway in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **95,** 5172-5177.

Lim, D. S. and Hasty, P. (1996) A mutation in mouse rad51 results in an early embryonic lethal that is suppressed by a mutation in p53. *Molecular and Cellular Biology* **16,** 7133-7143.

Lim, D. S., Kim, S. T., Xu, B., Maser, R. S., Lin, J. Y., Petrini, J. H. J., and Kastan, M. B. (2000a) ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature* **404,** 613-+.

Lim, D. S., Vogel, H., Willerford, D. M., Sands, A. T., Platt, K. A., and Hasty, P. (2000b) Analysis of ku80-mutant mice and cells with deficient levels of p53. *Molecular and Cellular Biology* **20,** 3772-3780.

Lin, J. Y. D., MuhlmannDiaz, M. C., Stackhouse, M. A., Robinson, J. F., Taccioli, G. E., Chen, D. J., and Bedford, J. S. (1997) An ionizing radiation-sensitive CHO mutant cell line: irs-20 .4. Genetic complementation, V(D)J recombination and the scid phenotype. *Radiation Research* **147,** 166-171.

Little, J. B. and Nagasawa, H. (1985) Effect of Confluent Holding on Potentially Lethal Damage Repair, Cell-Cycle Progression, and Chromosomal-Aberrations in Human Normal and Ataxia-Telangiectasia Fibroblasts. *Radiation Research* **101,** 81-93.

Liu, C. M., Liu, D. P., Dong, W. J., and Liang, C. C. (2004) Retrovirus vector-mediated stable gene silencing in human cell. *Biochemical and Biophysical Research Communications* **313,** 716-720.

Liu, N., Lamerdin, J. E., Tebbs, R. S., Schild, D., Tucker, J. D., Shen, M. R., Brookman, K. W., Siciliano, M. J., Walter, C. A., Fan, W. F., Narayana, L. S., Zhou, Z. Q.,

Adamson, A. W., Sorensen, K. J., Chen, D. J., Jones, N. J., and Thompson, L. H. (1998) XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages. *Molecular Cell* **1,** 783-793.

Luo, G. B., Yao, M. S., Bender, C. F., Mills, M., Bladl, A. R., Bradley, A., and Petrini, J. H. J. (1999) Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 7376-7381.

Ma, Y. M., Pannicke, U., Schwarz, K., and Lieber, M. R. (2002) Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell* **108,** 781-794.

Matsuoka, S., Rotman, G., Ogawa, A., Shiloh, Y., Tamai, K., and Elledge, S. J. (2000) Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **97,** 10389-10394.

Mcgrath, R. A. and Williams, R. W. (1966) Reconstruction in Vivo of Irradiated Escherichia Coli Deoxyribonucleic Acid Rejoining of Broken Pieces. *Nature* **212,** 534-&.

McManus, M. T., Petersen, C. P., Haines, B. B., Chen, J. Z., and Sharp, P. A. (2002) Gene silencing using micro-RNA designed hairpins. *Rna-A Publication of the Rna Society* **8,** 842-850.

McManus, M. T. and Sharp, P. A. (2002) Gene silencing in mammals by small interfering RNAs. *Nature Reviews Genetics* **3,** 737-747.

Meyn, M. S. (1995) Ataxia-Telangiectasia and Cellular-Responses to Dna-Damage. *Cancer Research* **55,** 5991-6001.

Meyn, M. S. (1997) Chromosome instability syndromes: Lessons for carcinogenesis. *Genetic Instability and Tumorigenesis* **221,** 71-148.

Meyn, M. S., Strasfeld, L., and Allen, C. (1994) Testing the Role of P53 in the Expression of Genetic Instability and Apoptosis in Ataxia-Telangiectasia. *International Journal of Radiation Biology* **66,** S141-S149.

Miller, V. M., Xia, H. B., Marrs, G. L., Gouvion, C. M., Lee, G., Davidson, B. L., and Paulson, H. L. (2003) Allele-specific silencing of dominant disease genes. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 7195-7200.

Morrison, C., Sonoda, E., Takao, N., Shinohara, A., Yamamoto, K., and Takeda, S. (2000) The controlling role of ATM in homologous recombinational repair of DNA damage. *Embo Journal* **19,** 463-471.

Moss, E. G. and Taylor, J. M. (2003) Small-interfering RNAs in the radar of the interferon system. *Nature Cell Biology* **5,** 771-772.

Moynahan, M. E. and Jasin, M. (1997) Loss of heterozygosity induced by a chromosomal double-strand break. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 8988-8993.

MuhlmannDiaz, M. C. and Bedford, J. S. (1994) Breakage of Human-Chromosome-4, Human-Chromosome-19 and Human-Chromosome-y in G(0) Cells Immediately After Exposure to Gamma-Rays. *International Journal of Radiation Biology* **65,** 165-173.

Muller, H. J. (1927) Artificial transmutation of the gene. *Science* **66,** 84-87.

Muller, P. Y., Janovjak, H., Miserez, A. R., and Dobbie, Z. (2002) Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques* **32,** 1372-+.

Myers, J. W., Jones, J. T., Meyer, T., and Ferrell, J. E. (2003) Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing. *Nature Biotechnology* **21,** 324-328.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990) Introduction of A Chimeric Chalcone Synthase Gene Into Petunia Results in Reversible Co-Suppression of Homologous Genes in Trans. *Plant Cell* **2,** 279-289.

Nelson, P., Kiriakidou, M., Sharma, A., Maniataki, E., and Mourelatos, Z. (2003) The microRNA world: small is mighty. *Trends in Biochemical Sciences* **28,** 534-540.

Olive, P. L. (1998) The role of DNA single- and double-strand breaks in cell killing by ionizing radiation. *Radiation Research* **150,** S42-S51.

Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J., and Conklin, D. S. (2002a) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes & Development* **16,** 948-958.

Paddison, P. J., Caudy, A. A., and Hannon, G. J. (2002b) Stable suppression of gene expression by RNAi in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 1443-1448.

Painter, R. B. and Young, B. R. (1980) Radiosensitivity in Ataxia-Telangiectasia - A New Explanation. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **77,** 7315-7317.

Pandita, T. K., Lieberman, H. B., Lim, D. S., Dhar, S., Zheng, W., Taya, Y., and Kastan, M. B. (2000) Ionizing radiation activates the ATM kinase throughout the cell cycle. *Oncogene* **19,** 1386-1391.

Park, E., Chan, D. W., Oettinger, M. A., and Kwon, J. (2004) DNA-PK is activated by nucleosomes and phosphorylates H2AX within the nucleosomes in an acetylation-dependent manner. *Nucleic Acids Research* **31,** 6819-6827.

Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellert, M., and Bonner, W. M. (2000) A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Current Biology* **10,** 886-895.

Pecker, I., Avraham, K. B., Gilbert, D. J., Savitsky, K., Rotman, G., Harnik, R., Fukao, T., Schrock, E., Hirotsune, S., Tagle, D. A., Collins, F. S., WynshawBoris, A., Ried, T., Copeland, N. G., Jenkins, N. A., Shiloh, Y., and Ziv, Y. (1996) Identification and chromosomal localization of Atm, the mouse homolog of the ataxia-telangiectasia gene. *Genomics* **35,** 39-45.

Peng, Y. L., Zhang, Q. M., Nagasawa, H., Okayasu, R., Liber, H. L., and Bedford, J. S. (2002) Silencing expression of the catalytic subunit of DNA-dependent protein kinase by small interfering RNA sensitizes human cells for radiation-induced chromosome damage, cell killing, and mutation. *Cancer Research* **62,** 6400-6404.

Peterson, S. R., Kurimasa, A., Oshimura, M., Dynan, W. S., Bradbury, E. M., and Chen, D. J. (1995) Loss of the Catalytic Subunit of the Dna-Dependent Protein-Kinase in Dna Double-Strand-Break-Repair Mutant Mammalian-Cells. *Proceedings of the National Academy of Sciences of the United States of America* **92,** 3171-3174.

Petrini, J. H. J. and Stracker, T. H. (2003) The cellular response to DNA double-strand breaks: defining the sensors and mediators. *Trends in Cell Biology* **13,** 458-462.

Pfeiffer, P., Goedecke, W., and Obe, G. (2000) Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations. *Mutagenesis* **15,** 289-302.

Pittman, D. L. and Schimenti, J. C. (2000) Midgestation lethality in mice deficient for the RecA-related gene, Rad51d/Rad51l3. *Genesis* **26,** 167-173.

Poltoratsky, V. P., Shi, X. Y., York, J. D., Lieber, M. R., and Carter, T. H. (1995) Human Dna-Activated Protein-Kinase (Dna-Pk) Is Homologous to Phosphatidylinositol Kinases. *Journal of Immunology* **155,** 4529-4533.

Powell, S. N., Willers, H., and Xia, F. (2002) BRCA2 keeps Rad51 in line: High-fidelity homologous recombination prevents breast and ovarian cancer? *Molecular Cell* **10,** 1262-1263.

Priestley, A., Beamish, H. J., Gell, D., Amatucci, A. G., Muhlmann-Diaz, M. C., Singleton, B. K., Smith, G. C. M., Blunt, T., Schalkwyk, L. C., Bedford, J. S., Jackson, S. P., Jeggo, P. A., and Taccioli, G. E. (1998) Molecular and biochemical characterisation of DNA-dependent protein kinase-defective rodent mutant irs-20. *Nucleic Acids Research* **26,** 1965-1973.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S., and Khvorova, A. (2004) Rational siRNA design for RNA interference. *Nature Biotechnology*.

Rijkers, T., Van den Ouweland, J., Morolli, B., Rolink, A. G., Baarends, W. M., Van Sloun, P. P. H., Lohman, P. H. M., and Pastink, A. (1998) Targeted inactivation of mouse

RAD52 reduces homologous recombination but not resistance to ionizing radiation. *Molecular and Cellular Biology* **18,** 6423-6429.

Rogakou, E. P., Nieves-Niera, W., Boon, C., Pommier, Y., and Bonner, W. M. (1998) Histone H2AX serine-139 phosphorylation is induced by the introduction of the initial breaks into DNA as a result of the apoptotic endonuclease. *Molecular Biology of the Cell* **9,** 109A.

Romano, N. and Macino, G. (1992) Quelling - Transient Inactivation of Gene-Expression in Neurospora-Crassa by Transformation with Homologous Sequences. *Molecular Microbiology* **6,** 3343-3353.

Rotman, G. and Shiloh, Y. (1999) ATM: A mediator of multiple responses to genotoxic stress. *Oncogene* **18,** 6135-6144.

Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L. L., Kopinja, J., Zhang, M. D., McManus, M. T., Gertler, F. B., Scott, M. L., and Van Parijs, L. (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nature Genetics* **33,** 401-406.

Sapkota, G. P., Deak, M., Kieloch, A., Morrice, N., Goodarzi, A. A., Smythe, C., Shiloh, Y., Lees-Miller, S. P., and Alessi, D. R. (2002) Ionizing radiation induces ataxia telangiectasia mutated kinase (ATM)-mediated phosphorylation of LKB1/STK11 at Thr-366. *Biochemical Journal* **368,** 507-516.

Savage, J. R. K. (1976) Classification and Relationships of Induced Chromosomal Structural-Changes. *Journal of Medical Genetics* **13,** 103-122.

Savitsky, K., BarShira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T., Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S. R., Simmons, A., Clines, G. A., Sartiel, A., Gatti, R. A., Chessa, L., Sanal, O., Lavin, M. F., Jaspers, N. G. J., Malcolm, A., Taylor, R., Arlett, C. F., Miki, T., Weissman, S. M., Lovett, M., Collins, F. S., and Shiloh, Y. (1995a) A Single Ataxia-Telangiectasia Gene with A Product Similar to Pi-3 Kinase. *Science* **268,** 1749-1753.

Savitsky, K., Sfez, S., Tagle, D. A., Ziv, Y., Sartiel, A., Collins, F. S., Shiloh, Y., and Rotman, G. (1995b) The Complete Sequence of the Coding Region of the Atm Gene Reveals Similarity to Cell-Cycle Regulators in Different Species. *Human Molecular Genetics* **4,** 2025-2032.

Savitsky, K., Ziv, Y., BarShira, A., Gilad, S., Tagle, D. A., Smith, S., Uziel, T., Sfez, S., Nahmias, J., Sartiel, A., Eddy, R. L., Shows, T. B., Collins, F. S., Shiloh, Y., and Rotman, G. (1996) A human gene (DDX10) encoding a putative DEAD-box RNA helicase at 11q22-q23. *Genomics* **33,** 199-206.

Sax K. (1938) Chromosome Aberrations induced by x-rays. *Genetics* **23,** 494-516.

Scacheri, P. C., Rozenblatt-Rosen, O., Caplen, N. J., Wolfsberg, T. G., Umayam, L., Lee, J. C., Hughes, C. M., Shanmugam, K. S., Bhattacharjee, A., Meyerson, M., and Collins, F. S. (2004) Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 1892-1897.

Schultz, L. B., Chehab, N. H., Malikzay, A., and Halazonetis, T. D. (2000) p53 Binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. *Journal of Cell Biology* **151,** 1381-1390.

Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D. N., and Fesik, S. W. (2003) Specificity of short interfering RNA determined through gene expression signatures. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 6347-6352.

Setlow, R. B., Carrier, W. L., and Swenson, P. A. (1963) Thymine Dimers and Inhibition of Dna Synthesis by Ultraviolet Irradiation of Cells. *Science* **142,** 1464-&.

Shafman, T., Khanna, K. K., Kedar, P., Spring, K., Kozlov, S., Yen, T., Hobson, K., Gatei, M., Zhang, N., Watters, D., Egerton, M., Shiloh, Y., Kharbanda, S., Kufe, D., and Lavin, M. F. (1997) Interaction between ATM protein and c-Abl in response to DNA damage. *Nature* **387,** 520-523.

Sharp, P. A. (2001) RNA interference - 2001. *Genes & Development* **15,** 485-490.

Shin, E. K., Perryman, L. E., and Meek, K. (1997) A kinase-negative mutation of DNA-PKCS in equine SCID results in defective coding and signal joint formation. *Journal of Immunology* **158,** 3565-3569.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K. L., Parrish, S., Timmons, L., Plasterk, R. H. A., and Fire, A. (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107,** 465-476.

Sipley, J. D., Menninger, J. C., Hartley, K. O., Ward, D. C., Jackson, S. P., and Anderson, C. W. (1995) Gene for the Catalytic Subunit of the Human Dna-Activated Protein-Kinase Maps to the Site of the Xrcc7 Gene on Chromosome-8. *Proceedings of the National Academy of Sciences of the United States of America* **92,** 7515-7519.

Sledz, C. A., Holko, M., de Veer, M. J., Silverman, R. H., and Williams, B. R. G. (2003) Activation of the interferon system by short-interfering RNAs. *Nature Cell Biology* **5,** 834-839.

Smith, G. C. M., Cary, R. B., Lakin, N. D., Hann, B. C., Teo, S. H., Chen, D. J., and Jackson, S. P. (1999) Purification and DNA binding properties of the ataxia-telangiectasia gene product ATM. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 11134-11139.

Smith, G. C. M. and Jackson, S. P. (1999) The DNA-dependent protein kinase. *Genes & Development* **13,** 916-934.

Song, Q. Z., LeesMiller, S. P., Kumar, S., Zhang, N., Chan, D. W., Smith, G. C. M., Jackson, S. P., Alnemri, E. S., Litwack, G., Khanna, K. K., and Lavin, M. F. (1996) DNA-dependent protein kinase catalytic subunit: A target for an ICE-like protease in apoptosis. *Embo Journal* **15,** 3238-3246.

Sonoda, E., Sasaki, M. S., Buerstedde, J. M., Bezzubova, O., Shinohara, A., Ogawa, H., Takata, M., Yamaguchi-Iwai, Y., and Takeda, S. (1998) Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. *Embo Journal* **17,** 598-608.

Stackhouse, M. A. and Bedford, J. S. (1993a) An Ionizing Radiation-Sensitive Mutant of Cho Cells - Irs-20 .1. Isolation and Initial Characterization. *Radiation Research* **136,** 241-249.

Stackhouse, M. A. and Bedford, J. S. (1993b) An Ionizing Radiation-Sensitive Mutant of Cho Cells - Irs-20 .2. Dose-Rate Effects and Cellular-Recovery Processes. *Radiation Research* **136,** 250-254.

Stackhouse, M. A. and Bedford, J. S. (1994) An Ionizing Radiation-Sensitive Mutant of Cho Cells, Irs-20 .3. Chromosome-Aberrations, Dna Breaks and Mitotic Delay. *International Journal of Radiation Biology* **65,** 571-582.

Stadler, L. J. (1928a) Genetic effects of x-rays in maize. *Proceedings of the National Academy of Sciences of the United States of America* **14,** 69-75.

Stadler, L. J. (1928b) Mutations in barley induced by x-rays and radium. *Science* **68,** 186-187.

Stamato, T. D. and Waldren, C. A. (1977) Isolation of Uv-Sensitive Variants of Cho-K1 by Nylon Cloth Replica Plating. *Somatic Cell Genetics* **3,** 431-440.

Sung, P., Trujillo, K. M., and Van Komen, S. (2000) Recombination factors of Saccharomyces cerevisiae. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* **451,** 257-275.

Swift, M., Reitnauer, P. J., Morrell, D., and Chase, C. L. (1987) Breast and Other Cancers in Families with Ataxia-Telangiectasia. *New England Journal of Medicine* **316,** 1289-1294.

Tabara, H., Grishok, A., and Mello, C. C. (1998) RNAi in C-elegans: Soaking in the genome sequence. *Science* **282,** 430-431.

Takata, M., Sasaki, M. S., Tachiiri, S., Fukushima, T., Sonoda, E., Schild, D., Thompson, L. H., and Takeda, S. (2001) Chromosome instability and defective recombinational repair in knockout mutants of the five Rad51 paralogs. *Molecular and Cellular Biology* **21,** 2858-2866.

Taniguchi, T., Garcia-Higuera, I., Xu, B., Andreassen, P. R., Gregory, R. C., Kim, S. T., Lane, W. S., Kastan, M. B., and D'Andrea, A. D. (2002) Convergence of the Fanconi anemia and ataxia telangiectasia signaling pathways. *Cell* **109,** 459-472.

Taylor, A. M. R., Harcourt, S. A., Lehmann, A. R., Stevens, S., and Bridges, B. A. (1975) Ataxia Telangiectasia - Human Mutation with Abnormal Radiation Sensitivity. *Nature* **258,** 427-429.

Thacker, J. (1989) Inherited Sensitivity to X-Rays in Man. *Bioessays* **11,** 58-62.

Theodosiou, A., Smith, A., Gillieron, C., Arkinstall, S., and Ashworth, A. (1999) MKP5, a new member of the MAP kinase phosphatase family, which selectively dephosphorylates stress-activated kinases. *Oncogene* **18,** 6981-6988.

Thompson, L. H. and Schild, D. (2001) Homologous recombinational repair of DNA ensures mammalian chromosome stability. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* **477,** 131-153.

Tijsterman, M., Ketting, R. F., and Plasterk, R. H. A. (2002) The genetics of RNA silencing. *Annual Review of Genetics* **36,** 489-519.

Timmons, L., Court, D. L., and Fire, A. (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. *Gene* **263,** 103-112.

Timmons, L. and Fire, A. (1998) Specific interference by ingested dsRNA. *Nature* **395,** 854.

Tsuchida, R., Yamada, T., Takagi, M., Shimada, A., Ishioka, C., Katsuki, Y., Igarashi, T., Chessa, L., Delia, D., Teraoka, H., and Mizutani, S. (2002) Detection of ATM gene mutation in human glioma cell line M059J by a rapid frameshift/stop codon assay in yeast. *Radiation Research* **158,** 195-201.

Uziel, T., Savitsky, K., Platzer, M., Ziv, Y., Helbitz, T., Nehls, M., Boehm, T., Rosenthal, A., Shiloh, Y., and Rotman, G. (1996) Genomic organization of the ATM gene. *Genomics* **33,** 317-320.

Valerie, K. and Povirk, L. F. (2003) Regulation and mechanisms of mammalian double-strand break repair. *Oncogene* **22,** 5792-5812.

Vickers, T. A., Koo, S., Bennett, C. F., Crooke, S. T., Dean, N. M., and Baker, B. F. (2003) Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents - A comparative analysis. *Journal of Biological Chemistry* **278,** 7108-7118.

Wang, H. C., Perrault, A. R., Takeda, Y., Qin, W., Wang, H. Y., and Iliakis, G. (2003) Biochemical evidence for Ku-independent backup pathways of NHEJ. *Nucleic Acids Research* **31,** 5377-5388.

Waterman, M. J. F., Stavridi, E. S., Waterman, J. L. F., and Halazonetis, T. D. (1998) ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. *Nature Genetics* **19,** 175-178.

Woo, R. A., Jack, M. T., Xu, Y., Burma, S., Chen, D. J., and Lee, P. W. K. (2002) DNA damage-induced apoptosis requires the DNA-dependent protein kinase, and is mediated by the latent population of p53. *Embo Journal* **21,** 3000-3008.

Xia, Y. R., Welch, C. L., Warden, C. H., Lange, E., Fukao, T., Lusis, A. J., and Gatti, R. A. (1996) Assignment of the mouse ataxia-telangiectasia gene (Atm) to mouse Chromosome 9. *Mammalian Genome* **7,** 554-555.

Xiao, Y. H. and Weaver, D. T. (1997) Conditional gene targeted deletion by Cre recombinase demonstrates the requirement for the double-strand break repair Mre11 protein in murine embryonic stem cells. *Nucleic Acids Research* **25,** 2985-2991.

Xu, B., Kim, S. T., Lim, D. S., and Kastan, M. B. (2002) Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation. *Molecular and Cellular Biology* **22,** 1049-1059.

Yamamoto, A., Taki, T., Yagi, H., Habu, T., Yoshida, K., Yoshimura, Y., Yamamoto, K., Matsushiro, A., Nishimune, Y., and Morita, T. (1996) Cell cycle dependent expression of the mouse Rad51 gene in proliferating cells. *Molecular & General Genetics* **251,** 1-12.

Yoshihara, T., Ishida, M., Kinomura, A., Katsura, M., Tsuruga, T., Tashiro, S., Asahara, T., and Miyagawa, K. (2004) XRCC3 deficiency results in a defect in recombination and increased endoreduplication in human cells. *Embo Journal* **23,** 670-680.

Yoshinari, K., Miyagishi, M., and Taira, K. (2004) Effects on RNAi of the tight structure, sequence and position of the targeted region. *Nucleic Acids Research* **32,** 691-699.

Zamore, P. D., Tuschl, T., Sharp, P. A., and Bartel, D. P. (2000) RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101,** 25-33.

Zdzienicka, M. Z., Vanderschans, G. P., Natarajan, A. T., Thompson, L. H., Neuteboom, I., and Simons, J. W. I. M. (1992) A Chinese-Hamster Ovary Cell Mutant (Em-C-11) with Sensitivity to Simple Alkylating-Agents and A Very High-Level of Sister Chromatid Exchanges. *Mutagenesis* **7,** 265-269.

Zhao, S., Weng, Y. C., Yuan, S. S. F., Lin, Y. T., Hsu, H. C., Lin, S. C. J., Gerbino, E., Song, M. H., Zdzienicka, M. Z., Gatti, R. A., Shay, J. W., Ziv, Y., Shiloh, Y., and Lee, E. Y. H. P. (2000) Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. *Nature* **405,** 473-477.

Zhu, J., Petersen, S., Tessarollo, L., and Nussenzweig, A. (2001) Targeted disruption of the Nijmegen breakage syndrome gene NBS1 leads to early embryonic lethality in mice. *Current Biology* **11,** 105-109.

Appendices

Appendix I: **Composition of Solutions**

Appendix II: **Quantification of Proteins on Western Blots**

Appendix III: **Real-Time RT-PCR**

Appendix IV: **Human DNA-PKcs, ATM and α-Tubulin mRNA Sequences from GenBank**

Appendix 1: Solutions

This appendix includes all recipes and solutions used in material methods. All reagents are prepared using distilled, deionized water.

Phosphate buffered saline (Dubecoo's PBS), pH 7.2

NaCl 8g/L
KCl 0.2g/L
Na_2HPO_4 1.15g/L
KH_2PO_4 0.2g/L

Hypotonic solution

KCl 0.075M

Solutions for analysis of protein by western blotting

Lysis / Extraction Buffer

50 mM Tris-HCl pH 7.5
150 mM NaCl,
2 mM EDTA
2 mM EGTA
25 mM NaF
25 mM β-glycerophosphate
0.2% Triton X-100,
0.3% NP-40

and freshly add 0.1mM sodium vanadate (100X stock soluction), 0.1mM PMSF (phenylmethylsulfonyl fluoride) (1000X stock solution), 5ug/ml Leupepsin (Sigma, St. Louis, MO, USA), 5ug/ml Aprotinin (Sigma, St. Louis, MO, USA) and Complete™ Mini protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IL, USA).

5X SDS Gel-loading Buffer

250 mM Tris·HCl (pH 6.8)
500 mM DTT (dithiothreitol)
10% SDS
0.5% bromophenol blue
10 % glycerol

DTT should be added just before the buffer is used from 1M stock solution.

5X Running Buffer, pH 8.3

125mM Tris
960mM Glycine
5g/L SDS

10X Transfer Buffer, pH 8.3

125mM Tris
960mM Glycine

to prepare 1000 ml 1X buffer, mix 700 ml H_2O, 100 ml 10X transfer buffer, and 200 ml methanol.

5X TBST, pH 8.0

125 mM Tris
625 mM NaCl
0.125% Tween 20

5X TBS, pH 8.0

125 mM Tris
625 mM NaCl

Blocking Buffer

7% dry milk in 1X TBST.

4% Paraformaldehyde Solution.

Paraformaldehyde fumes are dangerous, and should be kept in the fume hood whenever possible.

Add 4 gm paraformaldehyde in 45 ml water. Heat to 65-70°C and stir 15 minutes in the fume hood. Then 1 or 2 drops of 1N NaOH solution were added. When the solution was clear, water was added to bring the volume to 50 ml and. then another 50 ml of 2X PBS was added to give a final volume of 100ml of 1X PBS containing 4 gm of paraformaldehyde.

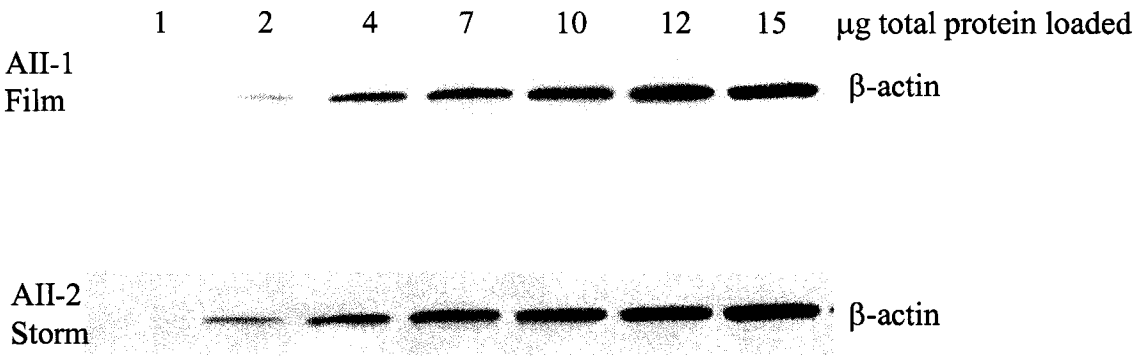
Appendix II: Quantification of Proteins on Western Blots

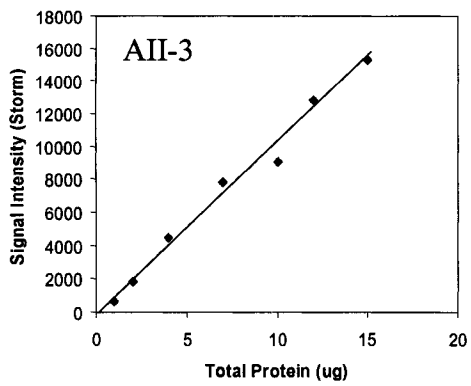
Historically, protein levels from western blots were detected from x-ray film densities, but in more recent times more sophisticated image analysis instruments have come into increasing use that measure, for example, photons emitted by chemiluminescent probes bound to the detecting antibody. The latter approach has the advantage of exhibiting a linear increase in signal as a function of the amount of protein over a very wide range of protein levels. Film densitometry generally shows very clean and low background levels, and for this reason is asthetically pleasing from the point of view of illustrating a semiquantitative result. The reason for this is, in fact, its drawback. There is a nonlinearity of film density response at both the low and high exposure ends of the exposure range. At the low exposure end there is essentially a threshold level where there is no measureable increase in film density for very small exposures, and thus, low background films with a very high contrast between the band being detected and the surrounding areas of the film are easily achieved. There is also a saturation of the film density in the high exposure range and this result in the narrower range of linearity (eg, see review and discussion by Fournier, Brons and Taucher-Scholz, *BioTechniques 35*: 284-290, 2003). This does not mean that film densitometry cannot be quantitative if care is taken to work within the linear range. Film densitometry has been readily available to our laboratory from the outset of this project, and I began utilizing the Storm 860 scanner imaging system (Amersham Pharmarcia Biotech, Piscataway, NJ) for some of the more critical determinations of protein quantities later in the project. In fact, in Chapter 4 where ATM and DNA-PKcs were measured as a function of time after anti-Prkdc siRNA transfections. Both film densitometry and Storm scanner imaging were carried out on several overlapping sample time points, and very similar estimates of protein levels relative to those in untreated controls, were obtained with both being first normalized to internal β-actin controls. To accomplish this, however care was taken to insure the film exposures of western blots yielded film densities lying within the linear range.

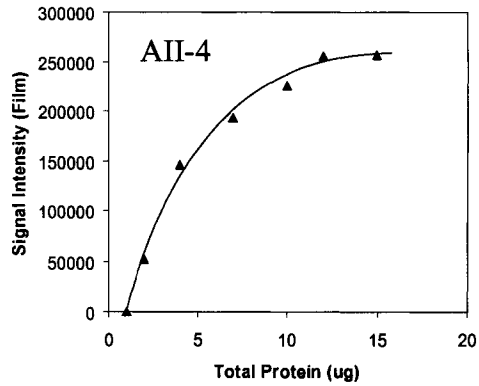
The following illustrates the comparison I made of the film exposure density vs β-actin protein quantities on a western blot and the Storm scanner signal intensity detected vs β-actin protein quantities on the same western blot. Figure AII-1 shows the western blot with detection of β-actin after an x-ray film exposure. Lanes 1, 2, 3, 4, 5, 6, and 7, respectively, had 1μg, 2ug, 4μg, 7μg, 10μg, 12μg, and 15μg of total protein loaded. The print of the image from the Storm scanner with the same western blot is shown in figure AII-2. The film density readings vs protein loaded is plotted in figure AII-3. The response appears to have a small threshold as the 1μg sample gave a reading of zero film density, but the response was fairly linear to between 5 or 6 μg. For a protein level of 7 μg the film density gave a reading that was about 85% of the expected reading from extrapolation of the linear region, but for 10 μg of protein the film reading was only 57% of the expected reading. Clearly readings above the film density corresponding to the 7μg protein loading would give unacceptable errors. The signal intensity readings from the Storm scanner (ECL + chemiluminescence detection of labeled anti-actin antibody) using the same western blot are shown in figure AII-4. The readings are essentially linear over this entire range. Figure AII-5 plots the Storm scanner signal vs the film density

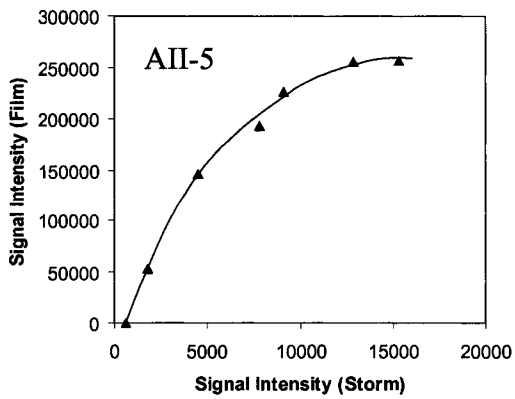
signal and shows the nonlinearity due to the nonlinearity of the film density, especially at the high film density readings.

In carrying out the quantitative measurements of DNA-PKcs and ATM proteins in Chapter 4 (Figure 4-5). There were overlapping measurements some using Storm scanning and some using film densitometry, but care was taken to work within the linear range of film densitometry.









Appendix III: Real-Time RT-PCR

Figure AIII-1 illustrates the difference in efficiencies of PCR for the different mRNAs targeted. There are several lines in each group and these reflect samples taken at different times after transfection of the cells. Though the scale of this particular plot does not allow resolution of individual lines, each line in a group actually represents serial measurements on a given sample after each cycle of PCR (plotted on the abscissa) of the amount of a fluorescent probe released during that cycle. As a result of this release the probe accumulates each cycle in proportion to the amount of DNA produced in that PCR cycle. The fact that the different mRNAs targeted display different slopes, threshold, and saturation levels underscores the importance of determining the efficiencies of each mRNA studied.

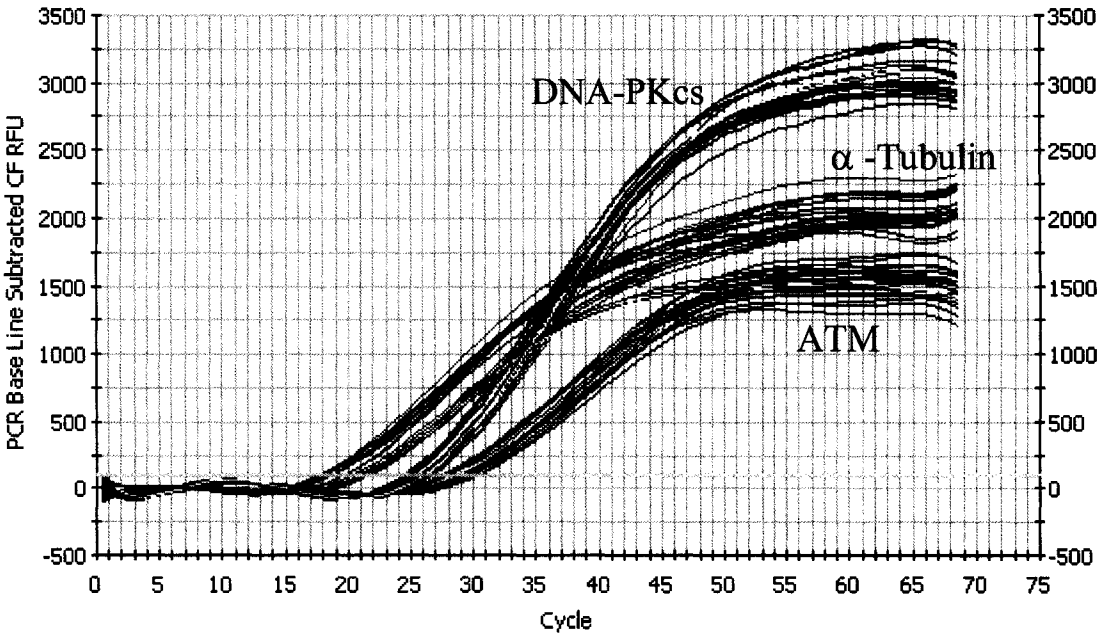


Figure AIII-1 An example of Real-time RT-PCR amplification plots of DNA-PKcs, ATM and α -Tubulin. An increase in fluorescence was plotted on the *y*-axis (ΔRn) and cycle number on the *x*-axis.

To check that the product of the PCR amplifications yield a product that is expected from the known mRNA sequence targetes and the primers used, it is necessary to measure their length by agarose gel electrophoresis as illustrated in figure AIII-2.

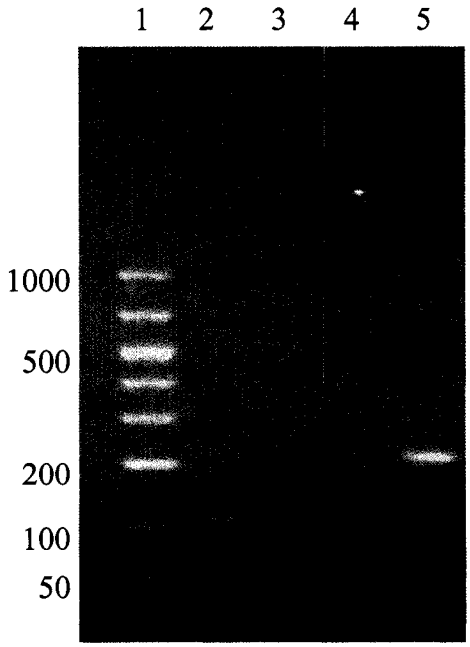


Figure AIII-2 Agarose gel electrophoresis of PCR products. PCR primers were tested for target specifcity by electrophoresis analysis of PCR products obtained from 25µl PCR reactions and visualized by UV spectrophotometry with ethidium Bromide. cDNA was reverse transcribed and amplified from total RNA sample extraced from GM08399 cells. The PCR product sizes for the primer pairs were confirmed by using a 100bp molecular weight marker as a reference (lane 1).The DNA-PKcs (lane 2), ATM (Lane 3), and α-Tubulin (lane 4) correspond to the specific target products. Lane 5 is a PCR product of the GAPDH gene using primers located in two exons flanking a 2000bp intron to show the samples are free of genomic DNA in the extracted mRNA. The product sizes are of the expected size.

There were 3 to 5 relpicate samples prepared for each real time RT PCR run since there are occasional variations in sample to sample replicates. It is also necessay to determine the PCR efficiency for each target mRNA. The generation of standard curves to determine efficiencies for the reaction are illustrated in figures AIII-3 through AIII-5 below. Starting material was obtained by carrying out a reverse transcription reaction from a sample of purified RNA and using this to carry out real time PCR with the chosen primers and probe for each different mRNA target (which is now a cDNA). To obtain the calibration curve, serial dilutions were made of the reverse transcribed procuct, and a real time PCR sample was set up and run with each of the different gene-specific primers and probes. Figure AIII-3a was for *Prkdc*, and shows the yield of product as a function of PCR cycle number for a 100-fold range of serial dilutions. From this, as shown in figure AIII-3b, the number of cycles to reach a given C_T critical concentration levels in the linear range was plotted against the log of the dilution used. The most concentrated (undiluted) sample was arbitrarily chosen as a unit of 1.0, so right hand extremity of the abscissa is 0 (the log of 1.0 is 0) and the further dilutions range to the 100-fold dilution (log of 10^{-2} is -2) on the far left of the scale. The greater the dilution, and the poorer is the efficiency, the more cycles of PCR are required to achieve the C_T value. The efficiency is calculated from the slopes of the calibration curves. The effieiency is $E = 10^{-(1/slope)} - 1$. For the Prkdc the efficiency was 96.6%.

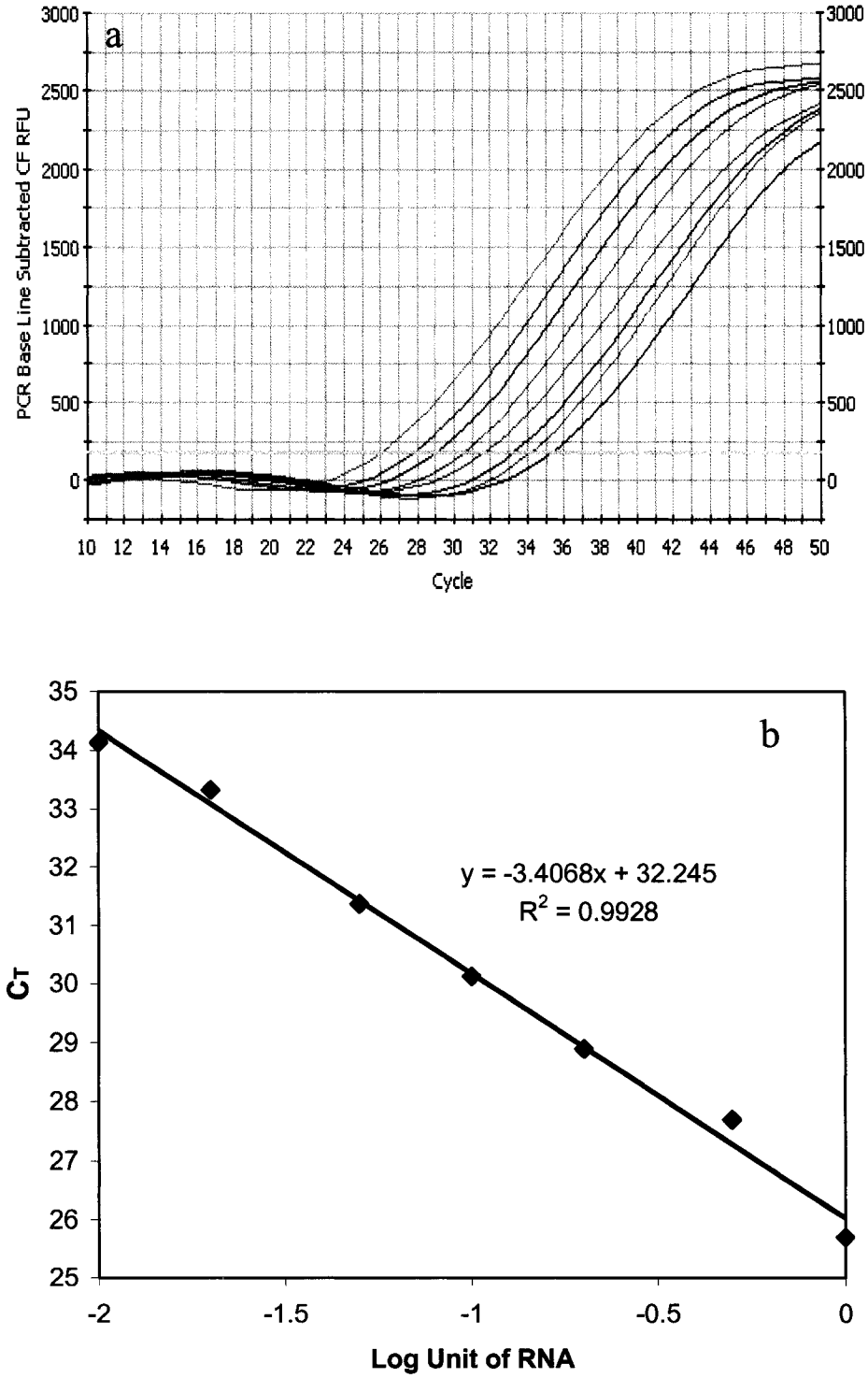


Figure AIII-3. Standard curve generated for the real-time RT-PCR of *Prkdc* from a total cDNA dilution series.

Figure AIII-4a and AIII-4b correspond to the same calibration for *ATM* where the efficiency, E, was calculated to be 49%.

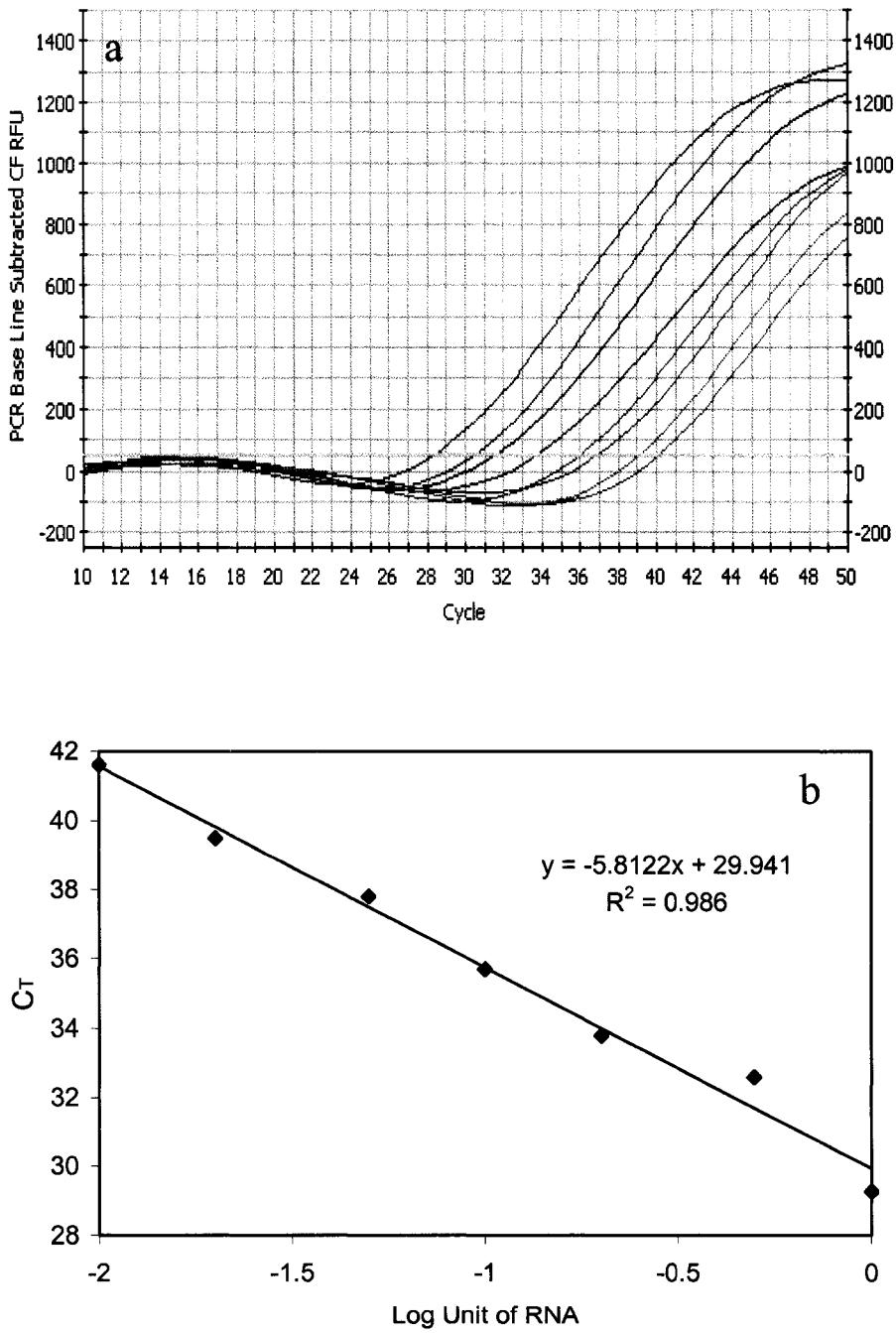


Figure AIII-4 Standard curve generated for the real-time RT-PCR of *ATM* from a total cDNA dilution series.

Likewise, Figure AIII-5a and AIII-5b correspond to the calibration for *α-Tubulin* which was the gene used for normalizations. The efficiency is 90%.

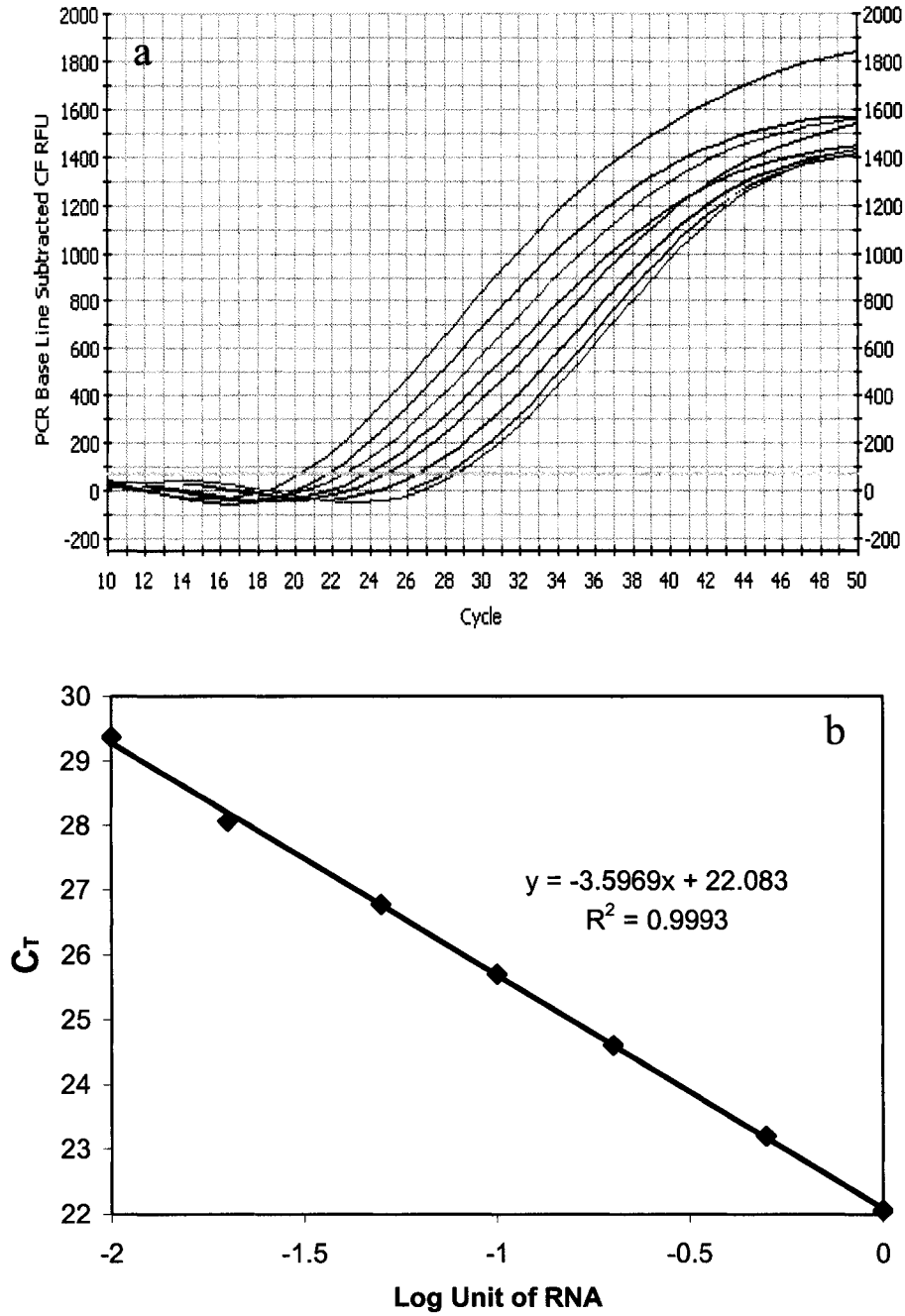


Figure AIII-5 Standard curve generated for the real-time RT-PCR of *α-Tubulin* from a total cDNA dilution series.

Appendix IV: Human DNA-PKcs, ATM and α-Tubulin mRNA Sequences from GenBank

DNA-PKcs mRNA: (cDNA sequence):

The start and stop codons and the sequences targeted for degredation by siRNAs are highlighted in red, and the sequences chosen to design the primers and fluorescent probe are underlined.

```
LOCUS       NM_006904              13509 bp    mRNA    linear   PRI 20-DEC-2003
DEFINITION  Homo sapiens protein kinase, DNA-activated, catalytic polypeptide
            (PRKDC,mRNA.
ACCESSION   NM_006904
VERSION     NM_006904.6  GI:31340617

ORIGIN
        1 ggggcatttc cgggtccggg ccgagcgggc gcacgcgcgg gagcgggact cggcggcatg
       61 gcgggctccg gagccggtgt gcgttgctcc ctgctgcggc tgcaggagac cttgtccgct
      121 gcggaccgct gcggtgctgc cctggccggt catcaactga tccgcggcct ggggcaggaa
      181 tgcgtcctga gcagcagccc cgcggtgctg gcattacaga catctttagt tttttccaga
      241 gatttcggtt tgcttgtatt tgtccggaag tcactcaaca gtattgaatt tcgtgaatgt
      301 agagaagaaa tcctaaagtt tttatgtatt ttcttagaaa aaatgggcca gaagatcgca
      361 ccttactctg ttgaaattaa gaacacttgt accagtgttt atacaaaaga tagagctgct
      421 aaatgtaaaa ttccagccct ggaccttctt attaagttac ttcagacttt tagaagttct
      481 agactcatgg atgaatttaa aattggagaa ttatttagta aattctatgg agaacttgca
      541 ttgaaaaaaa aaataccaga tacagtttta gaaaaagtat atgagctcct aggattattg
      601 ggtgaagttc atcctagtga gatgataaat aatgcagaaa acctgttccg cgcttttctg
      661 ggtgaactta agacccagat gacatcagca gtaagagagc ccaaactacc tgttctggca
      721 ggatgtctga aggggttgtc ctcacttctg tgcaacttca ctaagtccat ggaagaagat
      781 ccccagactt caagggagat ttttaatttt gtactaaagg caattcgtcc tcagattgat
      841 ctgaagagat atgctgtgcc ctcagctggc ttgcgcctat ttgccctgca tgcatctcag
      901 tttagcacct gccttctgga caactacgtg tctctatttg aagtcttgtt aaagtggtgt
      961 gcccacacaa atgtagaatt gaaaaaagct gcactttcag ccctggaatc ctttctgaaa
     1021 caggtttcta atatggtggc gaaaaatgca gaaatgcata aaaataaact gcagtacttt
     1081 atggagcagt tttatggaat catcagaaat gtggattcga acaacaagga gttatctatt
     1141 gctatccgtg gatatggact ttttgcagga ccgtgcaagg ttataaacgc aaaagatgtt
     1201 gacttcatgt acgttgagct cattcagcgc tgcaagcaga tgttcctcac ccagacagac
     1261 actggtgacg accgtgttta tcagatgcca agcttcctcc agtctgttgc aagcgtcttg
     1321 ctgtaccttg acacagttcc tgaggtgtat actccagttc tggagcacct cgtggtgatg
     1381 cagatagaca gtttcccaca gtacagtcca aaaatgcagc tggtgtgttg cagagccata
     1441 gtgaaggtgt tcctagcttt ggcagcaaaa gggccagttc tcaggaattg cattagtact
     1501 gtggtgcatc agggtttaat cagaatatgt tctaaaccag tggtccttcc aaagggccct
     1561 gagtctgaat ctgaagacca ccgtgcttca ggggaagtca gaactggcaa atggaaggtg
     1621 cccacataca aagactacgt ggatctcttc agacatctcc tgagctctga ccagatgatg
     1681 gattctattt tagcagatga agcatttttc tctgtgaatt cctccagtga aagtctgaat
     1741 catttacttt atgatgaatt tgtaaaatcc gttttgaaga ttgttgagaa attggatctt
     1801 acacttgaaa tacagactgt tggggaacaa gagaatggag atgaggcgcc tggtgtttgg
     1861 atgatcccaa cttcagatcc agcggctaac ttgcatccag ctaaacctaa agatttttcg
     1921 gctttcatta acctggtgga attttgcaga gagattctcc ctgagaaaca agcagaattt
     1981 tttgaaccat gggtgtactc attttcatat gaattaattt tgcaatctac aaggttgccc
     2041 ctcatcagtg gtttctacaa attgctttct attacagtaa gaaatgccaa gaaaataaaa
     2101 tatttcgagg gagttagtcc aaagagtctg aaacactctc ctgaagaccc agaaaagtat
     2161 tcttgctttg ctttatttgt gaaatttggc aaagaggtgg cagttaaaat gaagcagtac
     2221 aaagatgaac ttttggcctc ttgtttgacc tttcttctgt ccttgccaca caacatcatt
     2281 gaactcgatg ttagagccta cgttcctgca ctgcagatgg ctttcaaact gggcctgagc
     2341 tatacccccc tggcagaagt aggcctgaat gctctagaag aatggtcaat ttatattgac
     2401 agacatgtaa tgcagcctta ttacaaagac attctcccct gcctggatgg atacctgaag
     2461 acttcagcct tgtcagatga gaccaagaat aactgggaag tgtcagctct ttctcgggct
     2521 gcccagaaag gatttaataa agtggtgtta aagcatctga agaagacaaa gaacctttca
     2581 tcaaacgaag caatatcctt agaagaaata agaattagag tagtacaaat gcttggatct
     2641 ctaggaggac aaataaacaa aaatcttctg acagtcacgt cctcagatga gatgatgaag
```

2701 agctatgtgg cctgggacag agagaagcgg ctgagctttg cagtgccctt tagagagatg
2761 aaacctgtca ttttcctgga tgtgttcctg cctcgagtca cagaattagc gctcacagcc
2821 agtgacagac aaactaaagt tgcagcctgt gaacttttac atagcatggt tatgtttatg
2881 ttgggcaaag ccacgcagat gccagaaggg ggacagggag ccccacccat gtaccagctc
2941 tataagcgga cgtttcctgt gctgcttcga cttgcgtgtg atgttgatca ggtgacaagg
3001 caactgtatg agccactagt tatgcagctg attcactggt tcactaacaa caagaaattt
3061 gaaagtcagg atactgttgc cttactagaa gctatattgg atggaattgt ggaccctgtt
3121 gacagtactt taagagattt ttgtggtcgg tgtattcgag aattccttaa atggtccatt
3181 aagcaaataa caccacagca gcaggagaag agtccagtaa acaccaaatc gcttttcaag
3241 cgactttata gccttgcgct tcaccccaat gctttcaaga ggctgggagc atcacttgcc
3301 tttaataata tctacaggga attcagggaa gaagagtctc tggtggaaca gtttgtgttt
3361 gaagccttgg tgatatacat ggagagtctg gccttagcac atgcagatga gaagtcctta
3421 ggtacaattc aacagtgttg tgatgccatt gatcacctat gccgcatcat tgaaaagaag
3481 catgtttctt taaataaagc aaagaaacga cgtttgccgc gaggatttcc accttccgca
3541 tcattgtgtt tattggatct ggtcaagtgg cttttagctc attgtgggag gccccagaca
3601 gaatgtcgac acaaatccat tgaactcttt tataaattcg ttcctttatt gccaggcaac
3661 agatccccta atttgtggct gaaagatgtt ctcaaggaag aaggtgtctc ttttctcatc
3721 aacacctttg agggggtgg ctgtggccag ccctcgggca tcctggccca gcccaccctc
3781 ttgtaccttc gggggccatt cagcctgcag gccacgctat gctggctgga cctgctcctg
3841 gccgcgttgg agtgctacaa cacgttcatt ggcgagagaa ctgtaggagc gctccaggtc
3901 ctaggtactg aagcccagtc ttcacttttg aaagcagtgg ctttcttctt agaaagcatt
3961 gccatgcatg acattatagc agcagaaaag tgctttggca ctggggcagc aggtaacaga
4021 acaagcccac aagagggaga aaggtacaac tacagcaaat gcaccgttgt ggtccggatt
4081 atggagttta ccacgactct gctaaacacc tccccggaag gatggaagct cctgaagaag
4141 gacttgtgta atacacacct gatgagagtc ctggtgcaga cgctgtgtga gcccgcaagc
4201 ataggtttca acatcggaga cgtccaggtt atggctcatc ttcctgatgt ttgtgtgaat
4261 ctgatgaaag ctctaaagat gtccccatac aaagatatcc tagagaccca tctgagagag
4321 aaaataacag cacagagcat tgaggagctt tgtgccgtca acttgtatgg ccctgacgcg
4381 caagtggaca ggagcaggct ggctgctgtt gtgtctgcct gtaaacagct tcacagagct
4441 gggcttctgc ataatatatt accgtctcag tccacagatt tgcatcattc tgttggcaca
4501 gaacttcttt ccctggttta taaaggcatt gcccctggag atgagagaca gtgtctgcct
4561 tctctagacc tcagttgtaa gcagctggcc agcggacttc tggagttagc ctttgctttt
4621 ggaggactgt gtgagcgcct tgtgagtctt ctcctgaacc cagcggtgct gtccacggcg
4681 tccttgggca gctcacaggg cagcgtcatc cacttctccc atggggagta tttctatagc
4741 ttgttctcag aaacgatcaa cacggaatta ttgaaaaatc tggatcttgc tgtattggag
4801 ctcatgcagt cttcagtgga taataccaaa atggtgagtg ccgttttgaa cggcatgtta
4861 gaccagagct tcagggagcg agcaaaccag aaacaccaag gactgaaact tgcgactaca
4921 attctgcaac actggaagaa gtgtgattca tggtgggcca aagattcccc tctcgaaact
4981 aaaatggcag tgctggcctt actggcaaaa attttacaga ttgattcatc tgtatctttt
5041 aatacaagtc atggttcatt ccctgaagtc tttacaacat atattagtct acttgctgac
5101 acaaagctgg atctacattt aaagggccaa gctgtcactc ttcttccatt cttcaccagc
5161 ctcactggag gcagtctgga ggaacttaga cgtgttctgg agcagctcat cgttgctcac
5221 ttccccatgc agtccaggga atttcctcca ggaactccgc ggttcaataa ttatgtggac
5281 tgcatgaaaa agtttctaga tgcattggaa ttatctcaaa gccctatgtt gttggaattg
5341 atgacagaag ttctttgtcg ggaacagcag catgtcatgg aagaattatt tcaatccagt
5401 ttcaggagga ttgccagaag gggttcatgt gtcacacaag taggccttct ggaaagcgtg
5461 tatgaaatgt tcaggaagga tgacccccgc ctaagtttca cacgccagtc ctttgtggac
5521 cgctccctcc tcactctgct gtggcactgt agcctggatg ctttgagaga attcttcagc
5581 acaattgtgg tggatgccat tgatgtgttg aagtccaggt ttacaaagct aaatgaatct
5641 acctttgata ctcaaatcac caagaagatg ggctactata agattctaga cgtgatgtat
5701 tctcgccttc ccaaagatga tgttcatgct aaggaatcaa aaattaatca agttttccat
5761 ggctcgtgta ttacagaagg aaatgaactt acaaagacat tgattaaatt gtgctacgat
5821 gcatttacag agaacatggc aggagagaat cagctgctgg agaggagaag actttaccat
5881 tgtgcagcat acaactgcgc catatctgtc atctgctgtg tcttcaatga gttaaaattt
5941 taccaaggtt ttctgtttag tgaaaaacca gaaaagaact tgcttatttt tgaaaatctg
6001 atcgacctga agcgccgcta taattttcct gtagaagttg aggttcctat ggaaagaaag
6061 aaaaagtaca ttgaaattag gaaagaagcc agagaagcag caaatgggga ttcagatggt
6121 ccttcctata tgtcttccct gtcatatttg gcagacagta ccctgagtga ggaaatgagt
6181 caatttgatt tctcaaccgg agttcagagc tattcataca gctcccaaga ccctagacct
6241 gccactggtc gttttcggag acgggagcag cgggacccca cggtgcatga tgatgtgctg
6301 gagctggaga tggacgagct caatcggcat gagtgcatgg cgcccctgac ggccctggtc
6361 aagcacatgc acagaagcct gggcccgcct caaggagaag aggattcagt gccaagagat
6421 cttccttctt ggatgaaatt cctccatggc aaactgggaa atccaatagt accattaaat
6481 atccgtctct tcttagccaa gcttgttatt aatacagaag aggtctttcg cccttacgcg
6541 aagcactggc ttagcccctt gctgcagctg gctgcttctg aaaacaatgg aggagaagga
6601 attcactaca tggtggttga gatagtggcc actattcttt catggacagg cttggccact
6661 ccaacagggg tccctaaaga tgaagtgtta gcaaatcgat tgcttaattt cctaatgaaa
6721 catgtctttc atccaaaaag agctgtgttt agacacaacc ttgaaattat aaagaccctt
6781 gtcgagtgct ggaaggattg tttatccatc ccttataggt taatatttga aaagttttcc
6841 ggtaaagatc ctaattctaa agacaactca gtagggattc aattgctagg catcgtgatg
6901 gccaatgacc tgcctcccta tgacccacag tgtggcatcc agagtagcga atacttccag

```
 6961 gctttggtga ataatatgtc ctttgtaaga tataaagaag tgtatgccgc tgcagcagaa
 7021 gttctaggac ttatacttcg atatgttatg gagagaaaaa acatactgga ggagtctctg
 7081 tgtgaactgg ttgcgaaaca attgaagcaa catcagaata ctatggagga caagtttatt
 7141 gtgtgcttga acaaagtgac caagagcttc cctcctcttg cagacaggtt catgaatgct
 7201 gtgttctttc tgctgccaaa atttcatgga gtgttgaaaa cactctgtct ggaggtggta
 7261 ctttgtcgtg tggagggaat gacagagctg tacttccagt taaagagcaa ggacttcgtt
 7321 caagtcatga gacatagaga tgatgaaaga caaaaagtat gtttggacat aatttataag
 7381 atgatgccaa agttaaaacc agtagaactc cgagaacttc tgaaccccgt tgtggaattc
 7441 gtttcccatc cttctacaac atgtagggaa caaatgtata atattctcat gtggattcat
 7501 gataattaca gagatccaga aagtgagaca gataatgact cccaggaaat atttaagttg
 7561 gcaaaagatg tgctgattca aggattgatc gatgagaacc ctggacttca attaattatt
 7621 cgaaatttct ggagccatga aactaggtta ccttcaaata ccttggaccg gttgctggca
 7681 ctaaattcct tatattctcc taagatagaa gtgcactttt taagtttagc aacaaatttt
 7741 ctgctcgaaa tgaccagcat gagcccagat tatccaaacc ccatgttcga gcatcctctg
 7801 tcagaatgcg aatttcagga atataccatt gattctgatt ggcgtttccg aagtactgtt
 7861 ctcactccga tgtttgtgga gacccaggcc tcccagggca ctctccagac ccgtacccag
 7921 gaagggtccc tctcagctcg ctggccagtg gcagggcaga taagggccac ccagcagcag
 7981 catgacttca cactgacaca gactgcagat ggaagaagct catttgattg gctgaccggg
 8041 agcagcactg acccgctggt cgaccacacc agtccctcat ctgactcctt gctgtttgcc
 8101 cacaagagga gtgaaaggtt acagagagca cccttgaagt cagtggggcc tgattttggg
 8161 aaaaaaaggc tgggccttcc aggggacgag gtggataaca aagtgaaagg tgcggccggc
 8221 cggacggacc tactacgact gcgcagacgg tttatgaggg accaggagaa gctcagtttg
 8281 atgtatgcca gaaaaggcgt tgctgagcaa aaacgagaga aggaaatcaa gagtgagtta
 8341 aaaatgaagc aggatgccca ggtcgttctg tacagaagct accggcacgg agaccttcct
 8401 gacattcaga tcaagcacag cagcctcatc accccgttac aggccgtggc ccagagggac
 8461 ccaataattg caaaacagct ctttagcagc ttgttttctg gaattttgaa agagatggat
 8521 aaatttaaga cactgtctga aaaaaacaac atcactcaaa agttgcttca agacttcaat
 8581 cgttttctta ataccacctt ctctttcttt ccacccttg tctcttgtat tcaggacatt
 8641 agctgtcagc acgcagccct gctgagcctc gacccagcgg ctgttagcgc tggttgcctg
 8701 gccagcctac agcagcccgt gggcatccgc ctgctagagg aggctctgct ccgcctgctg
 8761 cctgctgagc tgcctgccaa gcgagtccgt gggaaggccc gcctccctcc tgatgtcctc
 8821 agatgggtgg agcttgctaa gctgtataga tcaattggag aatacgacgt cctccgtggg
 8881 atttttacca gtgagatagg aacaaagcaa atcactcaga gtgcattatt agcagaagcc
 8941 agaagtgatt attctgaagc tgctaagcag tatgatgagg ctctcaataa acaagactgg
 9001 gtagatggtg agcccacaga agccgagaag gatttttggg aacttgcatc ccttgactgt
 9061 tacaaccacc ttgctgagtg gaaatcactt gaatactgtt ctacagccag tatagacagt
 9121 gagaaccccc cagacctaaa taaaatctgg agtgaaccat tttatcagga aacatatcta
 9181 ccttacatga tccgcagcaa gctgaagctg ctgctccagg gagaggctga ccagtccctg
 9241 ctgacattta ttgacaaagc tatgcacggg gagctccaga aggcgattct agagcttcat
 9301 tacagtcaag agctgagtct gctttacctc ctgcaagatg atgttgacag agccaaatat
 9361 tacattcaaa atggcattca gagttttatg cagaattatt ctagtattga tgtcctctta
 9421 caccaaagta gactcaccaa attgcagtct gtacaggctt taacagaaat tcaggagttc
 9481 atcagcttta taagcaaaca aggcaattta tcatctcaag ttccccttaa gagacttctg
 9541 aacacctgga caaacagata tccagatgct aaaatggacc caatgaacat ctgggatgac
 9601 atcatcacaa atcgatgttt ctttctcagc aaaatagagg agaagcttac ccctcttcca
 9661 gaagataata gtatgaatgt ggatcaagat ggagacccca gtgacaggat ggaagtgcaa
 9721 gagcaggaag aagatatcag ctccctgatc aggagttgca agttttccat gaaaatgaag
 9781 atgatagaca gtgcccggaa gcagaacaat ttctcacttg ctatgaaact actgaaggag
 9841 ctgcataaag agtcaaaaac cagagacgat tggctggtga gctgggtgca gagctactgc
 9901 cgcctgagcc actgccggag ccggtcccag ggctgctctg agcaggtgct cactgtgctg
 9961 aaaacagtct ctttgttgga tgagaacaac gtgtcaagct acttaagcaa aaatattctg
10021 gctttccgtg accagaacat tctcttgggt acaacttaca ggatcatagc gaatgctctc
10081 agcagtgagc cagcctgcct tgctgaaatc gaggaggaca aggctagaag aatcttagag
10141 ctttctggat ccagttcaga ggattcagag aaggtgatcg cgggtctgta ccagagagca
10201 ttccagcacc tctctgaggc tgtgcaggcg gctgaggagg aggcccagcc tccctcctgg
10261 agctgtgggc ctgcagctgg ggtgattgat gcttacatga cgctggcaga tttctgtgac
10321 caacagctgc gcaaggagga agagaatgca tcagttattg attctgcaga actgcaggcg
10381 tatccagcac ttgtggtgga gaaaatgttg aaagctttaa aattaaattc caatgaagcc
10441 agattgaagt ttcctagatt acttcagatt atagaacggt atccagagga gactttgagc
10501 ctcatgacaa aagagatctc ttccgttccc tgctggcagt tcatcagctg gatcagccac
10561 atggtggcct tactggacaa agaccaagcc gttgctgttc agcactctgt ggaagaaatc
10621 actgataact acccgcaggc tattgtttat cccttcatca taagcagcga aagctattcc
10681 ttcaaggata cttctactgg tcataagaat aaggagtttg tggcaaggat taaaagtaag
10741 ttggatcaag gaggagtgat tcaagatttt attaatgcct tagatcagct ctctaatcct
10801 gaactgctct ttaaggattg gagcaatgat gtaagagctg aactagcaaa aacccctgta
10861 aataaaaaaa acattgaaaa aatgtatgaa agaatgtatg cagccttggg tgacccaaag
10921 gctccaggcc tgggggcctt tagaaggaag tttattcaga cttttggaaa agaatttgat
10981 aaacattttg ggaaaggagg ttctaaacta ctgagaatga agctcagtga cttcaacgac
11041 attaccaaca tgctactttt aaaaatgaac aaagactcaa agccccctgg gaatctgaaa
11101 gaatgttcac cctggatgag cgacttcaaa gtggagttcc tgagaaatga gctggagatt
11161 cccggtcagt atgacggtag gggaaagcca ttgccagagt accacgtgcg aatcgccggg
```

11221 tttgatgagc gggtgacagt catggcgtct ctgcgaaggc ccaagcgcat catcatccgt
11281 ggccatgacg agagggaaca ccctttcctg gtgaagggtg gcgaggacct gcggcaggac
11341 cagcgcgtgg agcagctctt ccaggtcatg aatgggatcc tggcccaaga ctccgcctgc
11401 agccagaggg ccctgcagct gaggacctat agcgttgtgc ccatgacctc caggttagga
11461 ttaattgagt ggcttgaaaa tactgttacc ttgaaggacc ttcttttgaa caccatgtcc
11521 caagaggaga aggcggctta cctgagtgat cccagggcac cgccgtgtga atataaagat
11581 tggctgacaa aaatgtcagg aaaacatgat gttggagctt acatgctaat gtataagggc
11641 gctaatcgta ctgaaacagt cacgtctttt agaaaacgag aaagtaaagt gcctgctgat
11701 ctcttaaagc gggccttcgt gaggatgagt acaagccctg aggctttcct ggcgctccgc
11761 tcccacttcg ccagctctca cgctctgata tgcatcagcc actggatcct cgggattgga
11821 gacagacatc tgaacaactt tatggtggcc atggagactg gcggcgtgat cgggatcgac
11881 tttgggcatg cgtttggatc cgctacacag tttctgccag tccctgagtt gatgcctttt
11941 cggctaactc gccagtttat caatctgatg ttaccaatga aagaaacggg ccttatgtac
12001 agcatcatgg tacacgcact ccgggccttc cgctcagacc ctggcctgct caccaacacc
12061 atggatgtgt ttgtcaagga gccctccttt gattggaaaa attttgaaca gaaaatgctg
12121 aaaaaaggag ggtcatggat tcaagaaata aatgttgctg aaaaaaattg gtacccccga
12181 cagaaaatat gttacgctaa gagaaagtta gcaggtgcca atccagcagt cattacttgt
12241 gatgagctac tcctgggtca tgagaaggcc cctgccttca gagactatgt ggctgtggca
12301 cgaggaagca aagatcacaa cattcgtgcc caagaaccag agagtgggct ttcagaagag
12361 actcaagtga agtgcctgat ggaccaggca acagacccca acatccttgg cagaacctgg
12421 gaaggatggg agccctggat gtgaggtctg tgggagtctg cagatagaaa gcattacatt
12481 gtttaaagaa tctactatac tttggttggc agcattccat gagctgattt tcctgaaaca
12541 ctaaagagaa atgtcttttg tgctacagtt tcgtagcatg agtttaaatc aagattatga
12601 tgagtaaatg tgtatgggtt aaatcaaaga taaggttata gtaacatcaa agattaggtg
12661 aggtttatag aaagatagat atccaggctt accaaagtat taagtcaaga atataatatg
12721 tgatcagctt tcaaagcatt tacaagtgct gcaagttagt gaaacagctg tctccgtaaa
12781 tggaggaaat gtggggaagc cttggaatgc ccttctggtt ctggcacatt ggaaagcaca
12841 ctcagaaggc ttcatcacca agattttggg agagtaaagc taagtatagt tgatgtaaca
12901 ttgtagaagc agcataggaa caataagaac aataggtaaa gctataatta tggcttatat
12961 ttagaaatga ctgcatttga tattttagga tatttttcta ggtttttcc tttcatttta
13021 ttctcttcta gttttgacat tttatgatag atttgctctc tagaaggaaa cgtctttatt
13081 taggagggca aaaattttgg tcatagcatt cacttttgct attccaatct acaactggaa
13141 gatacataaa agtgctttgc attgaatttg ggataacttc aaaaatccca tggttgttgt
13201 tagggatagt actaagcatt tcagttccag gagaataaaa gaaattccta tttgaaatga
13261 attcctcatt tggaggaaaa aaagcatgca ttctagcaca acaagatgaa attatggaat
13321 acaaaagtgg ctccttccca tgtgcagtcc ctgtcccccc ccgccagtcc tccacaccca
13381 aactgtttct gattggcttt tagctttttg ttgttttttt ttttccttct aacacttgta
13441 tttggaggct cttctgtgat tttgagaagt atactcttga gtgtttaata aagttttttt
13501 ccaaaagta

ATM mRNA: (cDNA sequence):

The start and stop codons are highlighted in red, and the sequences chosen to design the primers and fluorescent probe for real time RT PCR are highlighted in blue.

```
LOCUS       NM_000051               9886 bp    mRNA    linear   PRI 20-DEC-2003
DEFINITION  Homo sapiens ataxia telangiectasia mutated (includes
            complementation groups A, C and D) (ATM), transcript variant 1,
            mRNA.
ACCESSION   NM_000051
VERSION     NM_000051.2  GI:20336202

        1 gcgagaggag tcgggatctg cgctgcagcc accgccgcgg ttgatactac tttgaccttc
       61 cgagtgcagt gaggcataca tcacaatttg gaattatgca ttggtttatc aatttacttg
      121 tttattgtca ccctgctgcc cagatatgac ttcatgagga cagtgatgtg tgttctgaaa
      181 ttgtgaacca tgagtctagt acttaatgat ctgcttatct gctgccgtca actagaacat
      241 gatagagcta cagaacgaaa gaaagaagtt gagaaattta agcgcctgat tcgagatcct
      301 gaaacaatta aacatctaga tcggcattca gattccaaac aaggaaaata tttgaattgg
      361 gatgctgttt ttagattttt acagaaatat attcagaaag aaacagaatg tctgagaata
      421 gcaaaaccaa atgtatcagc ctcaacacaa gcctccaggc agaaaaagat gcaggaaatc
      481 agtagtttgg tcaaatactt catcaaatgt gcaaacagaa gagcacctag gctaaaatgt
      541 caagaactct taaattatat catggataca gtgaaagatt catctaatgg tgctatttac
      601 ggagctgatt gtagcaacat actactcaaa gacattcttt ctgtgagaaa atactggtgt
      661 gaaatatctc agcaacagtg gttagaattg ttctctgtgt acttcaggct ctatctgaaa
      721 ccttcacaag atgttcatag agttttagtg gctagaataa ttcatgctgt taccaaagga
      781 tgctgttctc agactgacgg attaaattcc aaatttttgg actttttttc caaggctatt
      841 cagtgtgcga gacaagaaaa gagctcttca ggtctaaatc atatcttagc agctcttact
      901 atcttcctca agactttggc tgtcaacttt cgaattcgag tgtgtgaatt aggagatgaa
      961 attcttccca ctttgcttta tatttggact caacataggc ttaatgattc tttaaaagaa
     1021 gtcattattg aattatttca actgcaaatt tatatccatc atccgaaagg agccaaaacc
     1081 caagaaaaag gtgcttatga atcaacaaaa tggagaagta ttttatacaa cttatatgat
     1141 ctgctagtga atgagataag tcatatagga agtagaggaa agtattcttc aggatttcgt
     1201 aatattgccg tcaaagaaaa tttgattgaa ttgatggcag atatctgtca ccaggttttt
     1261 aatgaagata ccagatcctt ggagatttct caatcttaca ctactacaca aagagaatct
     1321 agtgattaca gtgtcccttg caaaaggaag aaaatagaac taggctggga agtaataaaa
     1381 gatcaccttc agaagtcaca gaatgatttt gatcttgtgc cttggctaca gattgcaacc
     1441 caattaatat caaagtatcc tgcaagttta cctaactgtg agctgtctcc attactgatg
     1501 atactatctc agcttctacc ccaacagcga catggggaac gtacaccata tgtgttacga
     1561 tgccttacgg aagttgcatt gtgtcaagac aagaggtcaa acctagaaag ctcacaaaag
     1621 tcagatttat taaaactctg gaataaaatt tggtgtatta cctttcgtgg tataagttct
     1681 gagcaaatac aagctgaaaa ctttggctta cttggagcca taattcaggg tagtttagtt
     1741 gaggttgaca gagaattctg gaagttattt actgggtcag cctgcagacc ttcatgtcct
     1801 gcagtatgct gtttgacttt ggcactgacc accagtatag ttccaggagc ggtaaaaatg
     1861 ggaatagagc aaaatatgtg tgaagtaaat agaagctttt ctttaaagga atcaataatg
     1921 aaatggctct tattctatca gttagagggt gacttagaaa atagcacaga agtgcctcca
     1981 attcttcaca gtaattttcc tcatcttgta ctggagaaaa ttcttgtgag tctcactatg
     2041 aaaaactgta aagctgcaat gaatttttc caaagcgtgc cagaatgtga acaccaccaa
     2101 aaagataaag aagaactttc attctcagaa gtagaagaac tatttcttca gacaactttt
     2161 gacaagatgg acttttaac cattgtgaga gaatgtggta tagaaaagca ccagtccagt
     2221 attggcttct ctgtccacca gaatctcaag gaatcactgg atcgctgtct tctgggatta
     2281 tcagaacagc ttctgaataa ttactcatct gagattacaa attcagaaac tcttgtccgg
     2341 tgttcacgtc ttttggtggg tgtccttggc tgctactgtt acatgggtgt aatagctgaa
     2401 gaggaagcat ataagtcaga attattccag aaagccaact ctctaatgca atgtgcagga
     2461 gaaagtatca ctctgtttaa aaataagaca aatgaggaat tcagaattgg ttccttgaga
     2521 aatatgatgc agctatgtac acgttgcttg agcaactgta ccaagaagag tccaaataag
     2581 attgcatctg gctttttcct gcgattgtta acatcaaagc taatgaatga cattgcagat
     2641 atttgtaaaa gtttagcatc cttcatcaaa aagccatttg accgtggaga agtagaatca
     2701 atggaagatg atactaatgg aaatctaatg gaggtggagg atcagtcatc catgaatcta
     2761 tttaacgatt accctgatag tagtgttagt gatgcaaacg aacctggaga gagccaaagt
     2821 accataggtg ccattaatcc tttagctgaa gaatatctgt caaagcaaga tctacttttc
     2881 ttagacatgc tcaagttctt gtgtttgtgt gtaactactg ctcagaccaa tactgtgtcc
```

2941 tttagggcag ctgatattcg gaggaaattg ttaatgttaa ttgattctag cacgctagaa
3001 cctaccaaat ccctccacct gcatatgtat ctaatgcttt taaaggagct tcctggagaa
3061 gagtacccct tgccaatgga agatgttctt gaacttctga aaccactatc caatgtgtgt
3121 tctttgtatc gtcgtgacca agatgtttgt aaaactattt taaaccatgt ccttcatgta
3181 gtgaaaaacc taggtcaaag caatatggac tctgagaaca caagggatgc tcaaggacag
3241 tttcttacag taattggagc attttggcat ctaacaaagg agaggaaata tatattctct
3301 gtaagaatgg ccctagtaaa ttgccttaaa actttgcttg aggctgatcc ttattcaaaa
3361 tgggccattc ttaatgtaat gggaaaagac tttcctgtaa atgaagtatt tacacaattt
3421 cttgctgaca atcatcacca agttcgcatg ttggctgcag agtcaatcaa tagattgttc
3481 caggacacga agggagattc ttccaggtta ctgaaagcac ttcctttgaa gcttcagcaa
3541 acagcttttg aaaatgcata cttgaaagct caggaaggaa tgagagaaat gtcccatagt
3601 gctgagaacc ctgaaacttt ggatgaaatt tataatagaa aatctgtttt actgacgttg
3661 atagctgtgg ttttatcctg tagccctatc tgcgaaaaac aggctttgtt tgccctgtgt
3721 aaatctgtga aagagaatgg attagaacct caccttgtga aaaaggtttt agagaaagtt
3781 tctgaaactt ttggatatag acgtttagaa gactttatgg catctcattt agattatctg
3841 gttttggaat ggctaaatct tcaagatact gaatacaact tatcttcttt tccttttatt
3901 ttattaaact acacaaatat tgaggatttc tatagatctt gttataaggt tttgattcca
3961 catctggtga ttagaagtca ttttgatgag gtgaagtcca ttgctaatca gattcaagag
4021 gactggaaaa gtcttctaac agactgcttt ccaaagattc ttgtaaatat tcttccttat
4081 tttgcctatg agggtaccag agacagtggg atggcacagc aaagagagac tgctaccaag
4141 gtctatgata tgcttaaaag tgaaaactta ttgggaaaac agattgatca cttattcatt
4201 agtaatttac cagagattgt ggtggagtta ttgatgacgt tacatgagcc agcaaattct
4261 agtgccagtc agagcactga cctctgtgac ttttcagggg atttggatcc tgctcctaat
4321 ccacctcatt ttccatcgca tgtgattaaa gcaacatttg cctatatcag caattgtcat
4381 aaaaccaagt taaaaagcat tttagaaatt ctttccaaaa gccctgattc ctatcagaaa
4441 attcttcttg ccatatgtga gcaagcagct gaaacaaata atgtttataa gaagcacaga
4501 attcttaaaa tatatcacct gtttgttagt ttattactga aagatataaa aagtggctta
4561 ggaggagctt gggcctttgt tcttcgagac gttatttata ctttgattca ctatatcaac
4621 caaaggcctt cttgtatcat ggatgtgtca ttacgtagct tctccctttg ttgtgactta
4681 ttaagtcagg tttgccagac agccgtgact tactgtaagg atgctctaga aaaccatctt
4741 catgttattg ttggtacact tatacccctt gtgtatgagc aggtggaggt tcagaaacag
4801 gtattggact tgttgaaata cttagtgata gataacaagg ataatgaaaa cctctatatc
4861 acgattaagc ttttagatcc ttttcctgac catgttgttt ttaaggattt gcgtattact
4921 cagcaaaaaa tcaaatacag tagaggaccc ttttcactct tggaggaaat taaccatttt
4981 ctctcagtaa gtgtttatga tgcacttcca ttgacaagac ttgaaggact aaaggatctt
5041 cgaagacaac tggaactaca taaagatcag atggtggaca ttatgagagc ttctcaggat
5101 aatccgcaag atgggattat ggtgaaacta gttgtcaatt tgttgcagtt atccaagatg
5161 gcaataaacc acactggtga aaaagaagtt ctagaggctg ttggaagctg cttgggagaa
5221 gtgggtccta tagatttctc taccatagct atacaacata gtaaagatgc atcttatacc
5281 aaggccctta agttatttga agataaagaa cttcagtgga ccttcataat gctgacctac
5341 ctgaataaca cactggtaga agattgtgtc aaagttcgat cagcagctgt tacctgtttg
5401 aaaaacattt tagccacaaa gactggacat agtttctggg agatttataa gatgacaaca
5461 gatccaatgc tggcctatct acagcctttt agaacatcaa gaaaaaagtt tttagaagta
5521 cccagatttg acaaagaaaa ccctttttgaa ggcctggatg atataaatct gtggattcct
5581 ctaagtgaaa atcatgacat ttggataaag acactgactt gtgcttttt ggacagtgga
5641 ggcacaaaat gtgaaattct tcaattatta aagccaatgt gtgaagtgaa aactgacttt
5701 tgtcagactg tacttccata cttgattcat gatattttac tccaagatac aaatgaatca
5761 tggagaaatc tgctttctac acatgttcag ggatttttca ccagctgtct tcgacacttc
5821 tcgcaaacga gccgatccac aacccctgca aacttggatt cagagtcaga gcacttttc
5881 cgatgctgtt tggataaaaa atcacaaaga acaatgcttg ctgttgtgga ctacatgaga
5941 agacaaaaga gaccttcttc aggaacaatt tttaatgatg ctttctggct ggatttaaat
6001 tatctagaag ttgccaaggt agctcagtct tgtgctgctc actttacagc tttactctat
6061 gcagaaatct atgcagataa gaaaagtatg gatgatcaag agaaaagaag tcttgcattt
6121 gaagaaggaa gccagagtac aactatttct agcttgagtg aaaaaagtaa agaagaaact
6181 ggaataagtt tacaggatct tctcttagaa atctacagaa gtatagggga gccagatagt
6241 ttgtatggct gtggtggagg gaagatgtta caacccatta ctagactacg aacatatgaa
6301 cacgaagcaa tgtggggcaa agccctagta acatatgacc tcgaaacagc aatcccctca
6361 tcaacacgcc aggcaggaat cattcaggcc ttgcagaatt tgggactctg ccatattctt
6421 tccgtctatt taaaaggatt ggattatgaa aataaagact ggtgtcctga actagaagaa
6481 cttcattacc aagcagcatg gaggaatatg cagtgggacc attgcacttc cgtcagcaaa
6541 gaagtagaag gaaccagtta ccatgaatca ttgtacaatg ctctacaatc tctaagagac
6601 agagaattct ctacatttta tgaaagtctc aaatatgcca gagtaaaaga agtggaagag
6661 atgtgtaagc gcagccttga gtctgtgtat tcgctctatc ccacacttag caggttgcag

6721 gccattggag agctggaaag cattggggag cttttctcaa gatcagtcac acatagacaa
6781 ctctctgaag tatatattaa gtggcagaaa cactcccagc ttctcaagga cagtgatttt
6841 agttttcagg agcctatcat ggctctacgc acagtcattt tggagatcct gatggaaaag
6901 gaaatggaca actcacaaag agaatgtatt aaggacattc tcaccaaaca ccttgtagaa
6961 ctctctatac tggccagaac tttcaagaac actcagctcc ctgaaagggc aatatttcaa
7021 attaaacagt acaattcagt tagctgtgga gtctctgagt ggcagctgga agaagcacaa
7081 gtattctggg caaaaaagga gcagagtctt gccctgagta ttctcaagca aatgatcaag
7141 aagttggatg ccagctgtgc agcgaacaat cccagcctaa aacttacata cacagaatgt
7201 ctgagggttt gtggcaactg gttagcagaa acgtgcttag aaaatcctgc ggtcatcatg
7261 cagacctatc tagaaaaggc agtagaagtt gctggaaatt atgatggaga aagtagtgat
7321 gagctaagaa atggaaaaat gaaggcattt ctctcattag cccggttttc agatactcaa
7381 taccaaagaa ttgaaaacta catgaaatca tcggaatttg aaaacaagca agctctcctg
7441 aaaagagcca aagaggaagt aggtctcctt agggaacata aaattcagac aaacagatac
7501 acagtaaagg ttcagcgaga gctggagttg gatgaattag ccctgcgtgc actgaaagag
7561 gatcgtaaac gcttcttatg taaagcagtt gaaaattata tcaactgctt attaagtgga
7621 gaagaacatg atatgtgggt attccggctt tgttccctct ggcttgaaaa ttctggagtt
7681 tctgaagtca atggcatgat gaagagagac ggaatgaaga ttccaacata taaatttttg
7741 cctcttatgt accaattggc tgctagaatg gggaccaaga tgatgggagg cctaggattt
7801 catgaagtcc tcaataatct aatctctaga atttcaatgg atcaccccca tcacactttg
7861 tttattatac tggccttagc aaatgcaaac agagatgaat ttctgactaa accagaggta
7921 gccagaagaa gcagaataac taaaaatgtg cctaaacaaa gctctcagct tgatgaggat
7981 cgaacagagg ctgcaaatag aataatatgt actatcagaa gtaggagacc tcagatggtc
8041 agaagtgttg aggcactttg tgatgcttat attatattag caaacttaga tgccactcag
8101 tggaagactc agagaaaagg cataaatatt ccagcagacc agccaattac taaacttaag
8161 aatttagaag atgttgttgt ccctactatg gaaattaagg tggaccacac aggagaatat
8221 ggaaatctgg tgactataca gtcatttaaa gcagaatttc gcttagcagg aggtgtaaat
8281 ttaccaaaaa taatagattg tgtaggttcc gatggcaagg agaggagaca gcttgttaag
8341 ggccgtgatg acctgagaca agatgctgtc atgcaacagg tcttccagat gtgtaataca
8401 ttactgcaga gaaacacgga aactaggaag aggaaattaa ctatctgtac ttataaggtg
8461 gttcccctct ctcagcgaag tggtgttctt gaatggtgca caggaactgt ccccattggt
8521 gaatttcttg ttaacaatga agatggtgct cataaaagat acaggccaaa tgatttcagt
8581 gcctttcagt gccaaaagaa aatgatggag gtgcaaaaaa agtcttttga agagaaatat
8641 gaagtcttca tggatgtttg ccaaaatttt caaccagttt tccgttactt ctgcatggaa
8701 aaattcttgg atccagctat ttggtttgag aagcgattgg cttatacgcg cagtgtagct
8761 acttcttcta ttgttggtta catacttgga cttggtgata gacatgtaca gaatatcttg
8821 ataaatgagc agtcagcaga acttgtacat atagatctag gtgttgcttt tgaacagggc
8881 aaaatccttc ctactcctga gacagttcct tttagactca ccagagatat tgtggatggc
8941 atgggcatta cgggtgttga aggtgtcttc agaagatgct gtgagaaaac catggaagtg
9001 atgagaaact ctcaggaaac tctgttaacc attgtagagg tccttctata tgatccactc
9061 tttgactgga ccatgaatcc tttgaaagct ttgtatttac agcagaggcc ggaagatgaa
9121 actgagcttc accctactct gaatgcagat gaccaagaat gcaaacgaaa tctcagtgat
9181 attgaccaga gtttcaacaa agtagctgaa cgtgtcttaa tgagactaca agagaaactg
9241 aaaggagtgg aagaaggcac tgtgctcagt gttggtggac aagtgaattt gctcatacag
9301 caggccatag accccaaaaa tctcagccga cttttcccag gatggaaagc ttgggtgtga
9361 tcttcagtat atgaattacc ctttcattca gcctttagaa attatatttt agcctttatt
9421 tttaacctgc caacatactt taagtaggga ttaatattta agtgaactat tgtgggtttt
9481 tttgaatgtt ggttttaata cttgatttaa tcaccactca aaaatgtttt gatggtctta
9541 aggaacatct ctgctttcac tctttagaaa taatggtcat tcgggctggg cgcagcggct
9601 cacgcctgta atcccagcac tttgggaggc cgaggtgagc ggatcacaag gtcaggagtt
9661 cgagaccagc ctggccaaga gaccagcctg gccagtatgg tgaaaccctg tctctactaa
9721 aaatacaaaa attagccgag catggtggcg ggcacctgta gtcccagcta ctcgagaggc
9781 tgaggcagga gaatctcttg aacctgggag gtgaaggttg ctgtgggcca aaatcatgcc
9841 attgcactcc agcctgggtg acaagagcga aactccatct caaaaa

α-Tubulin mRNA (cDNA)

The start and stop codons are highlighted in red, and the sequences chosen to design the primers and fluorescent probe for real time RT PCR are highlighted in blue.

```
LOCUS       BC000696                1643 bp    mRNA    linear   PRI 22-APR-2003
DEFINITION  Homo sapiens tubulin, alpha, ubiquitous, mRNA (cDNA clone MGC:2215
            IMAGE:3349131), complete cds.
ACCESSION   BC000696
VERSION     BC000696.1  GI:12653814

ORIGIN
        1 ggcacgaggc ttacctcgac tcttagcttg tcggggacgg taaccgggac ccggtgtctg
       61 ctcctgtcgc cttcgcctcc taatccctag ccactatgcg tgagtgcatc tccatccacg
      121 ttggccaggc tggtgtccag attggcaatg cctgctggga gctctactgc ctggaacacg
      181 gcatccagcc cgatggccag atgccaagtg acaagaccat tgggggagga gatgactcct
      241 tcaacacctt cttcagtgag acgggcgctg gcaagcacgt gccccgggct gtgtttgtag
      301 acttggaacc cacagtcatt gatgaagttc gcactggcac ctaccgccag ctcttccacc
      361 ctgagcagct catcacaggc aaggaagatg ctgccaataa ctatgcccga gggcactaca
      421 ccattggcaa ggagatcatt gaccttgtgt tggaccgaat tcgcaagctg gctgaccagt
      481 gcaccggtct tcagggcttc ttggttttcc acagctttgg tgggggaact ggttctgggt
      541 tcacctccct gctcatggaa cgtctctcag ttgattatgg caagaagtcc aagctggagt
      601 tctccattta cccagcaccc caggtttcca cagctgtagt tgagccctac aactccatcc
      661 tcaccaccca caccaccctg gagcactctg attgtgcctt catggtagac aatgaggcca
      721 tctatgacat ctgtcgtaga aacctcgata tcgagcgccc aacctacact aaccttaacc
      781 gccttattag ccagattgtg tcctccatca ctgcttccct gagatttgat ggagccctga
      841 atgttgacct gacagaattc cagaccaacc tggtgcccta cccccgcatc cacttccctc
      901 tggccacata tgcccctgtc atctctgctg agaaagccta ccatgaacag ctttctgtag
      961 cagagatcac caatgcttgc tttgagccag ccaaccagat ggtgaaatgt gaccctcgcc
     1021 atggtaaata catggcttgc tgcctgttgt accgtggtga cgtggttccc aaagatgtca
     1081 atgctgccat tgccaccatc aaaaccaagc gcagcatcca gtttgtggat tggtgcccca
     1141 ctggcttcaa ggttggcatc aactaccagc ctcccactgt ggtgcctggt ggagacctgg
     1201 ccaaggtaca gagagctgtg tgcatgctga gcaacaccac agccattgct gaggcctggg
     1261 ctcgcctgga ccacaagttt gacctgatgt atgccaagcg tgcctttgtt cactggtacg
     1321 tgggtgaggg gatggaggaa ggcgagtttt cagaggcccg tgaagatatg gctgcccttg
     1381 agaaggatta tgaggaggtt ggtgtggatt ctgttgaagg agagggtgag gaagaaggag
     1441 aggaatacta attatccatt ccttttggcc ctgcagcatg tcatgctccc agaatttcag
     1501 cttcagctta actgacagac gttaaagctt tctggttaga ttgttttcac ttggtgatca
     1561 tgtcttttcc atgtgtacct gtaatatttt tccatcatat ctcaaagtaa agtcattaac
     1621 atcaaaaaaa aaaaaaaaaa aaa
```