Oncogene (1997) 15, 159–167

Recombinant ATM protein complements the cellular A-T phenotype

Yael Ziv[1], Anat Bar-Shira[1], Iris Pecker[1,4], Pamela Russell[2], Timothy J Jorgensen[2], Ilan Tsarfati[3] and Yosef Shiloh[1]

Departments of [1]Human Genetics and [3]Human Microbiology, Sackler School of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel and [2]Department of Radiation Medicine, Vincent T Lombardi Cancer Research Center, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington, DC 20007-2197, USA

Ataxia-telangiectasia (A-T) is an autosomal recessive disorder characterized by neurodegeneration, immunodeficiency, cancer predisposition, genome instability and radiation sensitivity. The cellular phenotype of A-T points to defects in signal transduction pathways involved in activation of cell cycle checkpoints by free radical damage, and other pathways that mediate the transmission of specific mitogenic stimuli. The product of the responsible gene, ATM, belongs to a family of large proteins that contribute to maintaining genome stability and cell cycle progression in various organisms. A recombinant vector that stably expresses a full-length ATM protein is a valuable tool for its functional analysis. We constructed and cloned a recombinant, full-length open reading frame of ATM using a combination of vectors and hosts that overcame an inherent instability of this sequence. Recombinant ATM was stably expressed in insect cells using a baculovirus vector, albeit at a low level, and in human A-T cells using an episomal expression vector. An amino-terminal FLAG epitope added to the protein allowed highly specific detection of the recombinant molecule by immunoblotting, immunoprecipitation and immunostaining, and its isolation using immunoaffinity. Similar to endogenous ATM, the recombinant protein is located mainly in the nucleus, with low levels in the cytoplasm. Ectopic expression of ATM in A-T cells restored normal sensitivity to ionizing radiation and the radiomimetic drug neocarzinostatin, and a normal pattern of post-irradiation DNA synthesis, which represents an S-phase checkpoint. These observations indicate that the recombinant, epitope-tagged protein is functional. Introduction into this molecule of a known A-T missense mutation, Glu2904Gly, resulted in apparent instability of the protein and inability to complement the A-T phenotype. These findings indicate that the physiological defects characteristic of A-T cells result from the absence of the ATM protein, and that this deficiency can be corrected by ectopic expression of this protein.

Keywords: ataxia-telangiectasia; ATM; FLAG epitope; radiosensitivity; cell cycle checkpoints; mutations

Introduction

Ataxia-telangiectasia (A-T) is a prime example of a pleiotropic genetic disorder that flags a central physiological function. This autosomal recessive disease involves progressive cerebellar degeneration, a broad spectrum of immune system defects, acute predisposition to malignancies, mitotic and meiotic chromosomal instability, and hypersensitivity to ionizing radiation and chemical agents that cause oxidative stress (see Lavin and Shiloh, 1997; Shiloh and Rotman, 1996 for reviews). The cellular phenotype of A-T points to defects in signal transduction pathways that mediate the activation of cell cycle checkpoints by radical producing agents, and others that transduce mitogenic stimuli (Artuso *et al*., 1995; Beamish *et al*., 1996; Canman *et al*., 1994; Khanna *et al*., 1997; Rotman and Shiloh, 1997; Taylor *et al*., 1994).

The responsible gene, *ATM*, was recently identified using a positional cloning approach (Savitsky *et al*., 1995a,b). It encodes a 350 kDa protein with a carboxy-terminal domain resembling the catalytic subunit of phosphatidylinositol 3-kinase (PI 3-kinase). The ATM protein is a member of an expanding family of large proteins sharing the PI 3-kinase-like domain which were identified in yeast, Drosophila and mammals. These proteins are involved in maintaining genome stability and cell cycle control, and in cellular responses to damaging agents (Savitsky *et al*., 1995b; Zakian, 1995; Jackson, 1995; Lavin and Shiloh, 1997). Two members of this family, DNA-dependent protein kinase (Hartley *et al*., 1995) and FRAP (Brown *et al*., 1995), were shown to be protein kinases, and it is likely that most or all of these proteins share this activity (Hunter, 1995). Protein kinase activity was recently reported in immune complexes obtained using anti-ATM antibodies (Keegan *et al*., 1996; Jung *et al*., 1997). ATM was found to be located predominantly in the nucleus (Brown *et al*., 1997; Chen and Lee, 1996; Lakin *et al*., 1996; Watters *et al*., 1997).

Stable, ectopic expression of recombinant ATM would be valuable for biochemical and functional analysis of this protein, allowing *in vitro* manipulation and introduction of wild-type or modified ATM into various cellular systems. A prerequisite of this system is functionality and normal cellular distribution of the recombinant protein. The unusual size of ATM and its coding sequence make the production of the necessary clones technically challenging. We report here the construction of recombinant vectors that produce epitope-tagged, functional ATM. Expression of this protein in A-T cells leads to full correction of hallmarks of the cellular A-T phenotype, indicating that this phenotype results from the absence of ATM.

Correspondence: Y Ziv
[4]Present address: InSight Strategy and Marketing Ltd., P.O.B. 2128, Rehovot 76121, Israel
Received 10 April 1997; accepted 2 June 1997

Results

Construction and stable cloning of full-length ATM open reading frame

Molecular cloning of a complete ATM ORF presented some technical difficulties due to inherent instability of this sequence in commonly used plasmid vectors amplified in standard bacterial strains. As a starting point we used cDNA clone pCEV/7-9, which spans the 3′ 5.1 kb of ATM ORF and part of the adjacent 3′ UTR (Savitsky *et al*., 1995a,b). While this clone appeared to be stable in a pBluescript vector under standard conditions, the addition of the 5′ half of the ORF to the sequence resulted in immediate rearrangements of the extended insert. Stable clones of the 9.2 kb ORF were finally obtained using a combination of the bacterial plasmid pFastBac1 (Life Technologies, Paisley, UK) and the STBL2 host strain (Life Technologies), which was designed for the cloning of unstable sequences. To avoid stress conditions that might enhance recombination processes, bacterial cultures were grown at 30°C and usually did not reach saturation.

A 4.6 kb SpeI–XhoI fragment of the pCEV/7-9 insert was subcloned in pFastBac1 (Clone pFB/7-9; Figure 1). The rest of ATM ORF, together with a portion of the 5′ UTR, was amplified from normal cell RNA by RT–PCR using a high fidelity extended PCR system (Boehringer Mannheim, Mannheim, Germany). A 4.95 kb *Sal*I–*Spe*I fragment derived from this PCR product was introduced into clone pFB/7-9 to create a full-length ATM ORF (Clone pFB-YZ3; Figure 1). Complete sequencing of the PCR-amplified portion of this clone showed no sequence alterations leading to changes at the protein level.

Subsequent derivatives of clone pFB-YZ3 were generated by introducing each of the following epitope combinations in-frame with the amino terminus of ATM ORF: a FLAG epitope (DYKDDDDK; Hopp *et al*., 1988) preceded by a methionine residue, and a FLAG epitope followed by a string of six histidine residues (Figure 1). The latter amino acid sequence binds efficiently to metal ions and can be used to isolate the protein using metal chelate chromatography (Hochuli *et al*, 1988).

Expression of recombinant ATM in insect cells

The pFB-YZ3 insert was subcloned in a derivative of the pFastBac1 vector, pFastBacHTa, which allows the fusion of a 6xHis sequence to the amino terminus of the cloned protein. The pFastBac plasmids serve as donor vectors in the Bac-to-Bac™ Baculovirus Expression System. Inserts cloned in these plasmids are subsequently transferred into a baculovirus shuttle vector (bacmid) by site-specific transposition carried out in a specially designed bacterial host. The recombinant bacmid is then transfected into insect cells to create a viable virus (Luckow, 1993). The ATM insert was thus transposed into the bacmid bMON14272 (Life Technologies), and the resultant recombinant bacmid was transfected into Sf9 cells (Smith and Cherry, 1983) and High Five cells (Invitrogen, Leek, Netherlands). Viral stocks harvested from these cultures were used to infect fresh cultures.

The presence of ATM in cellular extracts prepared by several different protocols (see Materials and methods) was monitored by Western blot analysis (Figure 2). The recombinant protein appeared to be expressed at low levels compared to those obtained with other proteins in this system. We also found that the majority of the protein was associated with cellular membranes, leaving a minor fraction in the supernatant. The soluble portion of the protein was, however, amenable to isolation by metal chelate chromatography (Figure 2).

Expression of recombinant ATM in human A-T cells

The inserts of clones pFB-YZ3, pFB-YZ5 and pFB-YZ6 (Figure 1) were subcloned in the mammalian expression vector pEBS7 (Peterson and Legerski, 1991). This vector contains the origin of replication of Epstein–Barr virus (oriP), and a functional EBNA-1 gene of the same virus. The EBNA-1 protein binds to the oriP sequence and enables episomal replication of the vector in mammalian cells (Yates *et al*., 1985). A

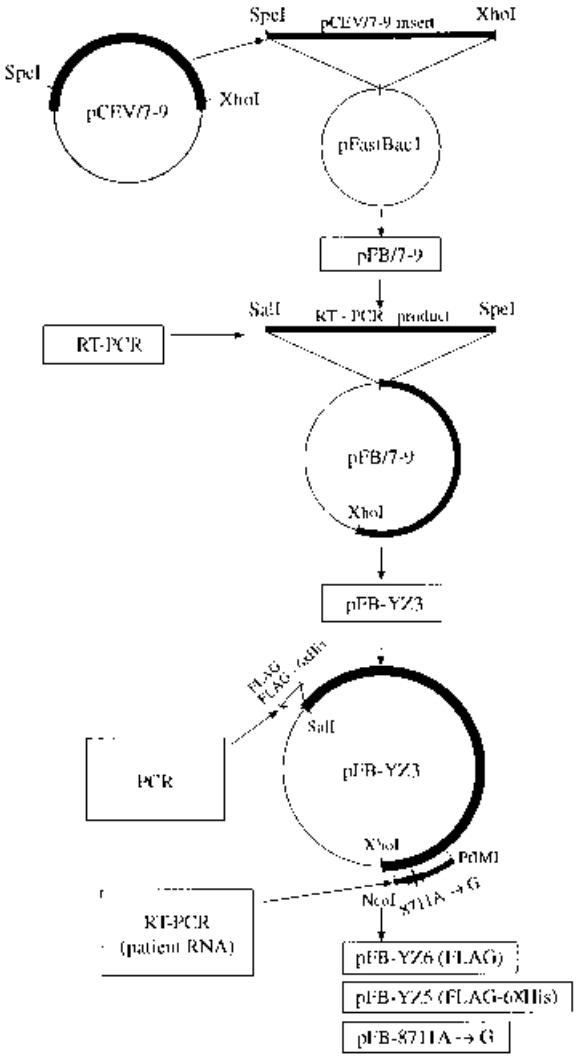

Figure 1 Stepwise construction of a full-length ATM ORF in the vector pFastBac1 and introduction of various modifications (see text). Inserts are marked by bold lines

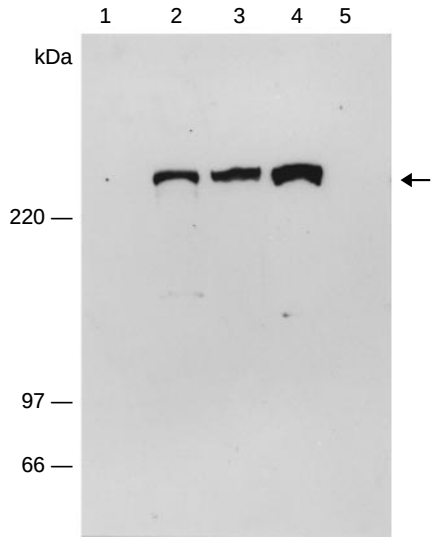

Figure 2 Expression of recombinant 6xHis-tagged ATM in insect cells using the Baculovirus vector pFastBacHTa. A Western blot of various cellular extracts was analysed with the X2361 antibody. The ATM immunoreactive band is marked with an arrow. 1: Uninfected High Five cells. 2: Cells infected with a recombinant virus. 3: Eluate from a Talon metal affinity column that had been loaded with extract of infected cells. 4: Extract of a normal lymphoblastoid cell line, L-39. 5: Extract of an A-T lymphoblastoid line, AT24RM, devoid of ATM

hygromycin resistance marker allows continuous selection of cells harboring the vector.

These constructs were transfected into an immortalized fibroblast line, AT22IJE-T, previously derived in our laboratory from primary A-T fibroblasts (Ziv *et al*., 1989). The parental cell line was recently found to harbor a homozygous frameshift mutation at codon 762 of the ATM gene (Gilad *et al*., 1996a). Since truncated versions of the ATM protein are unstable (Lakin *et al*., 1996 and unpublished results), no protein product of the ATM gene is observed in these cells.

Western blot analysis using an anti-ATM antibody showed variable rates of expression of the three ATM constructs (Figure 3). The epitope-tagged proteins were also detected by the anti-FLAG M5 monoclonal antibody (Figure 4a), but not by another monoclonal antibody, M2, directed against this epitope. However, both antibodies immunoprecipitate the recombinant proteins, as demonstrated for antibody M2 in Figure 4b. This observation indicated that the FLAG epitope remained accessible to the antibodies in the native molecule, holding promise for isolation of the protein by immunoaffinity chromatography. Cellular extracts of the transfected cells were bound to immunoaffinity gel based on the M2 antibody (Eastman Kodak), and the FLAG-tagged ATM was subsequently eluted from the gel using an excess of the FLAG peptide (Figure 4c).

Localization of recombinant ATM by immunostaining

Endogenous ATM is predominantly nuclear (Brown *et al*., 1997; Chen and Lee, 1996; Lakin *et al*., 1996; Watters *et al*., 1997). Immunostaining using anti-ATM and anti-FLAG antibodies was used to localize the endogenous protein in immortalized normal fibroblasts, and the recombinant protein produced in AT22IJE-T cells. The results (Figure 5) indicated that

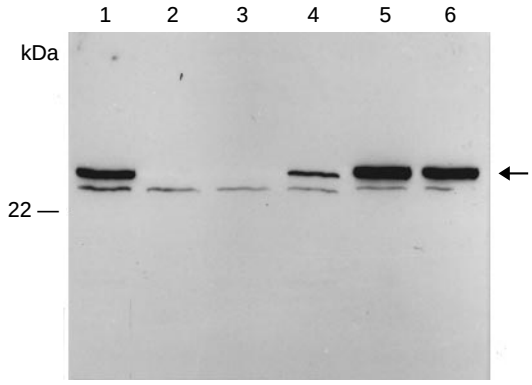

Figure 3 Expression of recombinant ATM in the A-T cell line AT22IJE-T, using the episomal vector pEBS7. Western blot analysis was performed with the ATM(Ab-3) antibody. The arrow points to the ATM band. A faint cross-reacting band detected by this antibody in human fibroblasts just below the ATM band serves as an internal control for protein amounts. 1: An immortalized human fibroblast line, GM637. 2: AT22IJE-T cells. 3: AT22IJE-T cells transfected with an empty pEBS7 vector. 4–6: AT22IJE-T cells transfected with the constructs pEBS7-YZ3, pEBS7-YZ5 and pEBS7-YZ6, respectively. These constructs contain the pFB-YZ3, pFB-YZ5 and pFB-YZ6 inserts (Figure 1) in the vector pEBS7

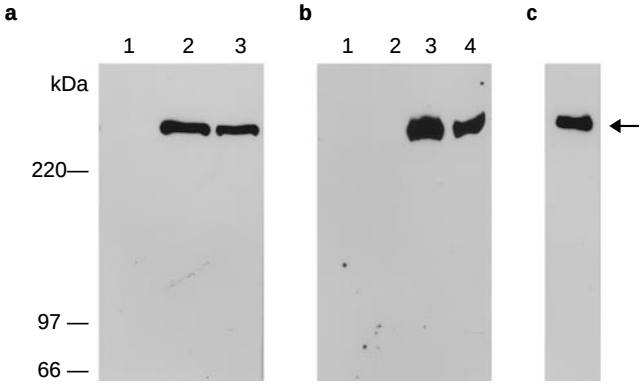

Figure 4 Detection and immunoaffinity separation of recombinant ATM tagged with an amino-terminal FLAG epitope. (**a**) The anti-FLAG M5 antibody was reacted with a Western blot containing cellular extracts of AT22IJE-T cells (1), and the same cells transfected with the pEBS7-YZ5 (2) and pEBS7-YZ6 (3) constructs (see legend of Figure 3). (**b**) Immunoprecipitation of FLAG-tagged ATM using the anti-FLAG M2 antibody. 1: AT22IJE-T cells. 2: AT22IJE-T cells transfected with empty vector. 3: AT22IJE-T cells transfected with pEBS7-YZ5. 4: AT22IJE-T cells transfected with pEBS7-YZ6. (**c**) Eluate obtained using excess FLAG peptide from an M2 anti-FLAG affinity gel that had been reacted with an extract of a pEBS7-YZ5 transfectant

the ectopically expressed molecule was localized like endogenous ATM in normal cells: in both cases, most of the protein was found in the nucleus, with a faint staining over the cytoplasm. Co-localization analysis of the ATM and FLAG staining (Figure 5a, f) underscored the predominant nuclear fraction of the protein.

Complementation of A-T phenotypic characteristics

Sensitivity to ionizing radiation and certain radiomimetic chemicals, and radioresistant DNA synthesis (RDS) are hallmarks of the A-T cellular phenotype. We examined the effect of ATM expression on the sensitivity of AT22IJE-T cells to the cytotoxic effect of

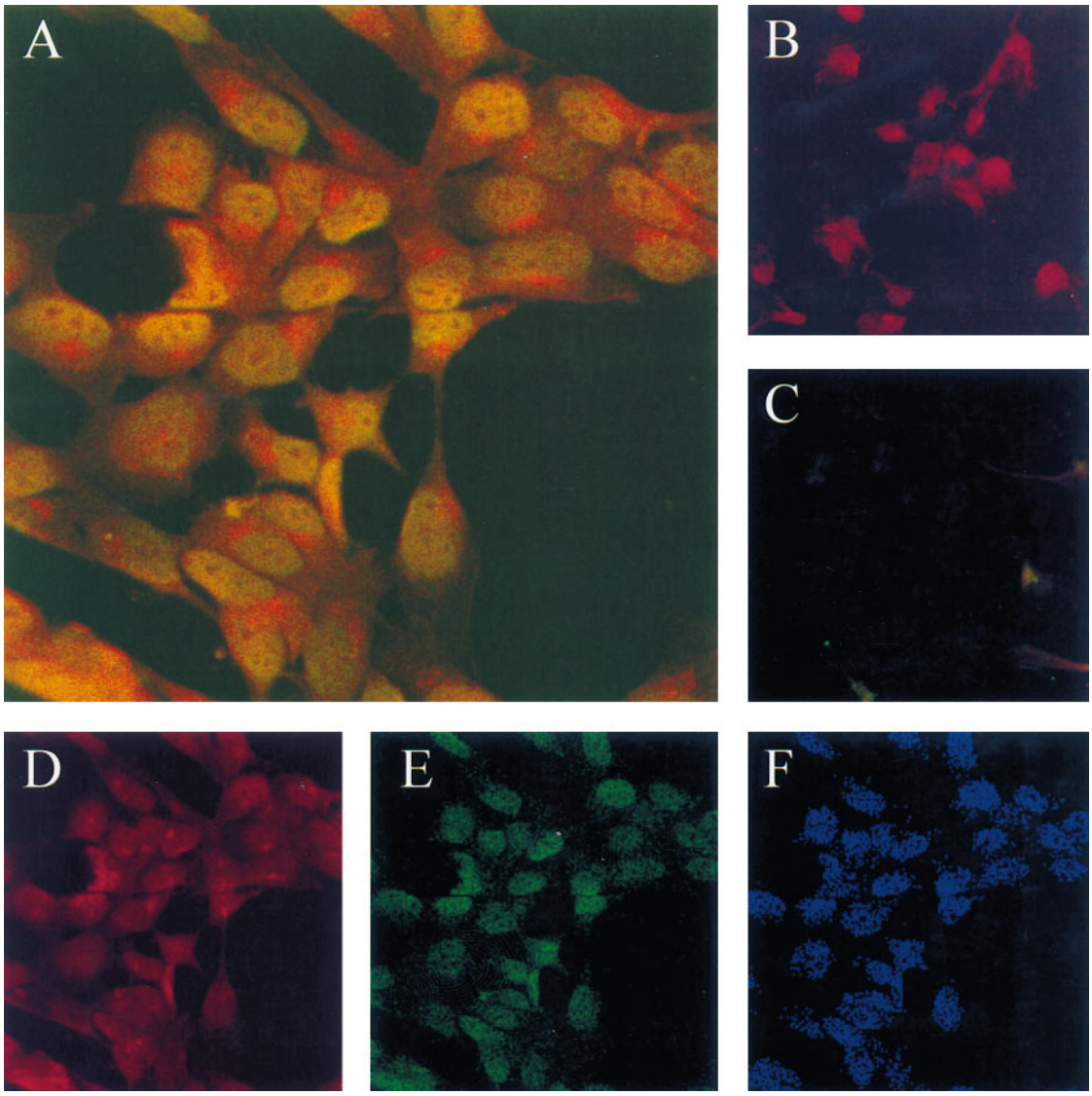

Figure 5 Cellular localization of endogenous and recombinant FLAG-tagged ATM by immunostaining. Normal immortalized fibroblasts (GM637) and AT22IJE-T cells containing clone pEBS7-YZ5 were subjected to double immunofluorescence staining and the results were analysed using confocal laser scan microscopy. Staining with anti-ATM antibody (Ab-3) was detected by Texas Red (red), and M5 anti-FLAG was detected by fluorescein isothiocyanate (green). (**A**) Superposition of the red and green immunostaining in AT22IJE-T transfectants. Yellow staining indicates co-localization of the two antibodies. (**B**) GM637 cells stained with anti-ATM. (**C**) AT22IJE-T cells stained with both antibodies. (**D**) AT22IJE-T transfectants stained with anti-ATM. (**E**) The same cells stained with anti-FLAG. (**F**) Result of co-localization analysis performed on the transfectants using the Zeiss procedure (see Materials and methods)

ionizing radiation and the radiomimetic drug neocarzinostatin (Shiloh *et al*., 1982). Complete restoration of normal sensitivity to these agents was observed in cells expressing recombinant ATM (Figure 6). Likewise, full complementation of the defect in post-irradiation DNA synthesis was obtained in the transfectants (Figure 7). This observation suggests that the recombinant protein is functionally active and can replace the endogenous one in maintaining ATM functions.

Effect of an A-T mutation

Manipulations of the ATM vectors, such as *in vitro* mutagenesis, should allow functional dissection of the protein molecule and identification of domains critical for its activity and stability. Most A-T mutations are clearly null alleles leading to protein truncations or large protein deletions (Baumer *et al*., 1996; Byrd *et al*., 1996; Gilad *et al*., 1996a,b; 1997; Savitsky *et al*., 1995a; Telatar *et al*., 1996; Wright *et al*., 1996). Only a few missense mutations were identified in patients. One of them, 8711A→G, leads to substitution of a highly conserved glutamic acid residue by glycine at position 2904. This position is within the carboxy-terminal PI 3-kinase-like domain which probably contains the catalytic site of the protein (Brown *et al*., 1995). While this mutation was expected to affect primarily the protein's activity, the patient's cells also showed a significantly low level of the mutant protein (Figure 8a). We introduced the 8711A→G mutation into the cloned ATM ORF. Although the mutant construct produced the expected mRNA in AT22IJE-T cells (Figure 8b), it did not produce any protein detectable by immunoblotting (Figure 8a). It appears, therefore, that the mutation leads to instability of the protein molecule. Accordingly, expression of the mutant clone

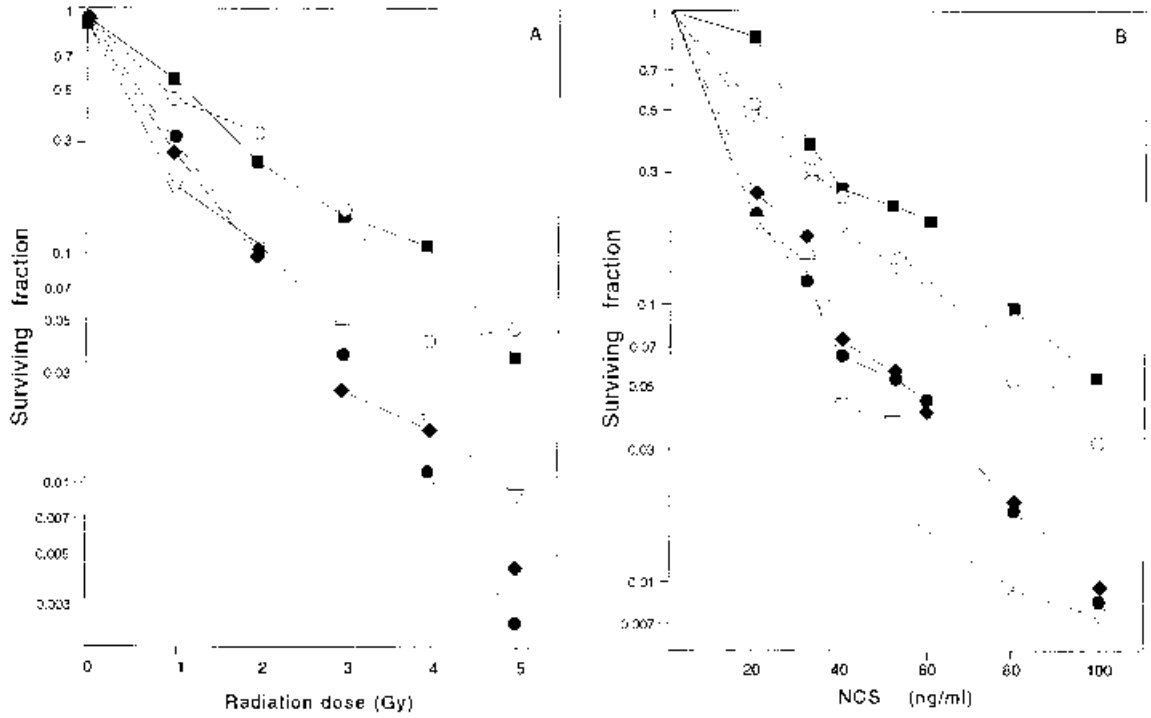

Figure 6 Survival curves of various human cell lines following treatment with increasing doses of gamma-rays (**A**) and the radiomimetic drug neocarzinostatin (**B**). ○: normal fibroblast line, GM637. ●: AT22IJE-T cells. Symbols for AT22IJE-T transfectants: ▽ – empty pEBS7; ◇ – pEBS7-YZ3; ■ – pEBS7-YZ5; ◆ – pEBS78711A→G (Glu2904Gly mutation)

did not complement the cellular A-T phenotype (Figures 6 and 7), underscoring the necessity of adequate level of functional ATM for phenotypic complementation.

Discussion

Molecular cloning of a large, unstable sequence requires a combination of vectors, hosts and growth conditions. We found that stable amplification of ATM ORF in bacterial cells can be obtained by using the pFastBac vectors when grown in STBL2 bacterial strain at 30°C.

The baculovirus expression system is based on the powerful polyhedrin promoter, which usually leads to vigorous expression of the cloned fragment. However, the capacity of insect cells to house increasing amounts of this large protein could not be predicted. Human ATM apparently cannot exceed certain levels in these cells, in addition to which it becomes associated with various cellular structures, leaving only a minor portion in the soluble fraction. Although the catalytic activity of ATM produced in this way remains to be determined, the results suggest this system may not be an ideal source of high amounts of recombinant ATM.

Long-term expression of the ATM protein in an immortalized A-T fibroblast line was obtained using an episomal vector. We preferred this vector over an integrating one because we were in doubt that the large insert would safely integrate into the unstable genome of A-T cells. Our previous experience with this system did indicate that episomal constructs are stably expressed in these cells over extended periods (Ziv *et al*., 1995).

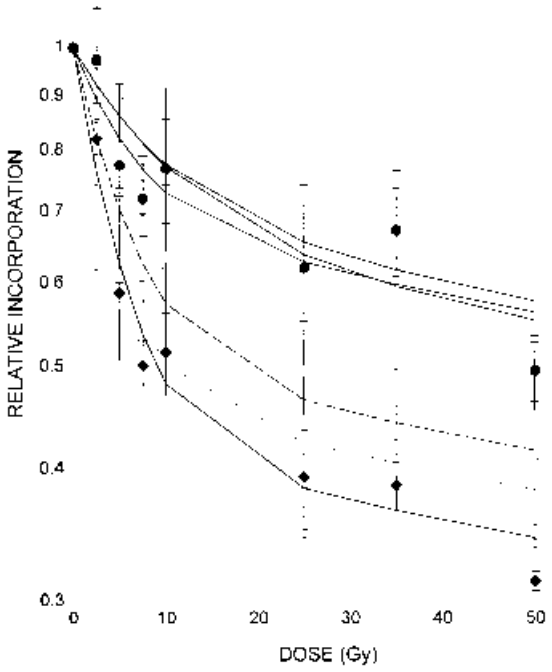

Figure 7 Effect of gamma-ray treatment on DNA synthesis in various cell lines. Relative nucleotide incorporation was measured following increasing radiation doses. □ and ◇: normal human fibroblast lines GM637 and MRC-5VI, respectively. △: AT22IJE-T cells. Symbols for AT22IJE-T transfectants: ▽ – empty pEBS7; ◆ – pEBS7-YZ5; ● – pEBS7-8711A→G (mutant)

Placing an artificial epitope at the amino-terminal end of the molecule allowed us to use highly specific antibodies to trace the protein. Anti-FLAG antibodies were previously shown to detect and efficiently

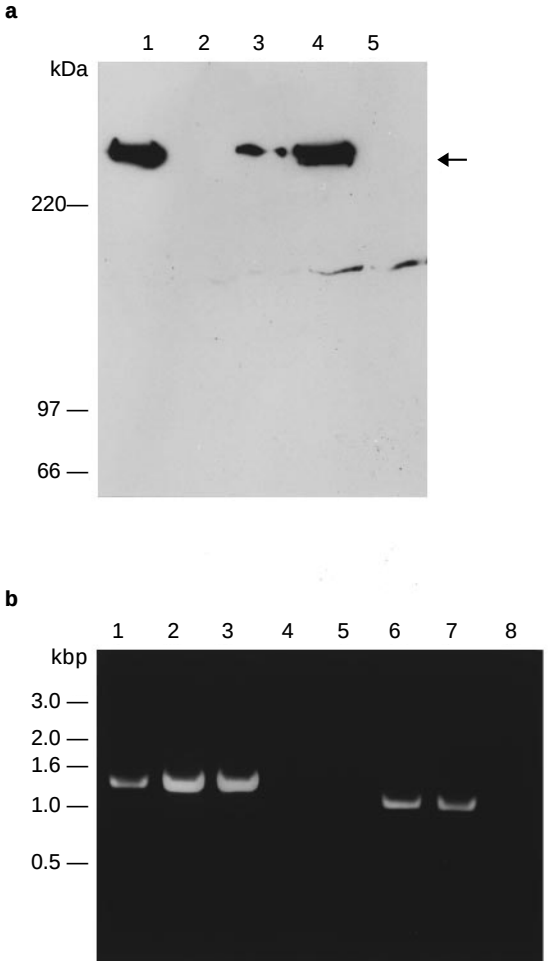

Figure 8 Effect of the 8711A→G mutation. (**a**) Western blot analysis of cellular extracts. 1: Normal human lymphoblastoid cell line, L-39. 2: An A-T lymphoblastoid cell line, AT24RM. 3: An A-T lymphoblastoid cell line, AT41RM, which is homozygous for the 8711A→G mutation. 4: AT22IJE-T cells transfected with the construct pEBS7-YZ5. 5: AT22IJE-T cells containing the construct pEBS7-8711A→G. Equal amounts of protein were loaded in the different lanes. (**b**) RT–PCR products obtained using RNA extracted from various cell lines. In lanes 1–4 the PCR product was obtained using two primers within the ATM ORF: 7K forward primer and 7L reverse primer corresponding to nucleotide positions 7979–8002 and 9184–9206 of the ATM ORF, respectively. 1: AT22IJE-T cells containing an empty pEBS7 vector. This product represents the transcript of the endogenous ATM gene. Lanes 2 and 3: AT22IJE-T cells containing the pEBS7-YZ5 and pEBS7-8711A→G constructs, respectively. These bands reflect the sum of PCR products obtained from the endogenous and recombinant transcripts. Lane 4: control reaction containing no template. In order to distinguish between the endogenous and recombinant products, the experiment was repeated with a forward primer corresponding to the pEBS7 vector (83–104 nucleotides upstream of the XhoI cloning site), and a reverse primer spanning positions 868–891 of the ATM ORF. These primers are expected to amplify only the recombinant transcript. Lanes 5–8 show the products obtained with these primers. 5: cells containing empty pEBS7. 6: pEBS7-YZ5 transfectants. 7: pEBS7-8711A→G transfectants. 8: A control reaction containing no template

immunoprecipitate a variety of recombinant proteins tagged with this epitope (see Brizzard *et al*., 1994; Dent *et al*., 1995; Derijard *et al*., 1994; Chiang and Roeder, 1993; Liu *et al*., 1995; Molloy *et al*., 1994 for examples). We placed the epitope at the amino-terminus of the protein, because the carboxy terminal PI 3-kinase-like domain probably harbors the molecule's catalytic site where conformational alterations could compromise the protein's activity. Furthermore, we previously identified an A-T mutation that abolished the legitimate termination codon of ATM, thereby extending the protein by an additional 29 amino acids (Gilad *et al*., 1996a). It turned out this patient's cells were devoid of ATM, probably due to instability of the modified molecule (R Khosravi *et al*., unpublished data). Still, we could not be sure whether the amino-terminal epitope would not be masked by tertiary structures at this region of the protein. It appeared, however, that the two monoclonal antibodies designed to detect an amino-terminal FLAG recognized the epitope and immunoprecipitated the recombinant protein. This observation led us to use immunoaffinity gel to adsorb the protein and subsequently elute it using excess FLAG peptide. This method holds promise for gentle isolation of the protein that would leave it enzymatically active and allow the identification of proteins interacting with it.

One of the prerequisites for functionality of ectopically expressed protein is proper localization in the cell. Immunostaining using anti-ATM and anti-FLAG antibodies localized the recombinant ATM predominantly in the nucleus, and a minor fraction remaining in the cytoplasm. This localization is similar to that of endogenous ATM in various cell types, as recently reported by several laboratories (Brown *et al*., 1997; Chen and Lee, 1996; Lakin *et al*., 1996; Watters *et al*., 1997). The cytoplasmic fraction of ATM appears to be microsomal (Brown *et al*., 1997; Watters *et al*., 1997), and immunoelectronmicroscopy recently performed by Watters *et al*. (1997) showed association of the protein with certain vesicular structures.

We examined the functionality of the recombinant protein by testing its effect on two hallmarks of the cellular A-T phenotype: sensitivity to ionizing radiation and radiomimetic chemicals, and radioresistant DNA synthesis (RDS), which represents a defective radiation-induced S-phase checkpoint. Immortalization of A-T primary fibroblasts by SV40 sequences was recently reported to alter their response to ionizing radiation and bring it closer to that of normal cells (Jorgensen *et al*., 1995). Indeed, following establishment of the AT22IJE-T cell line, we noticed that its radiomimetic sensitivity was somewhat reduced compared to that of the parent primary fibroblasts (Ziv *et al*., 1989), but still profoundly different from normal sensitivity; the typical RDS was also retained (Ziv *et al*., 1995). On the other hand, the constitutive level of the p53 protein in these cells is higher than in primary fibroblasts (Y Ziv, unpublished data). This precludes examination of the effect of ectopic ATM expression on the delayed post-irradiation accumulation of p53 which represents a defect in a G1 checkpoint (Canman *et al*., 1994). The ability of the recombinant protein to complement these two major characteristics of the cellular A-T phenotype suggests that it is functional, and that the lack of this protein is indeed responsible for the cellular phenotype of A-T cells. Interestingly, Morgan *et al*. (1997 recently showed that expression of the PI 3-kinase-like domain of ATM ORF in A-T cells results in complete rescue of their radiosensitivity and partial correction of chromosomal instability.

Introduction of the missense A-T mutation Glu2904Gly resulted in instability of the recombinant

protein. The net effect of this mutation on the recombinant protein is therefore similar to that of most A-T mutations, which are null alleles that produce unstable ATM derivatives and leave the cells without any protein product of the ATM gene. The highly conserved glutamic acid residue at position 2904 may, therefore, be critical for the protein's stability, in addition to its possible role in maintaining the molecule's kinase domain. The observed inability of the mutant clone to complement the A-T phenotype confirms that only an active ATM protein, rather than the mere expression of ATM mRNA, can exert this effect.

The results presented here indicate that the complex cellular phenotype associated with A-T results from functional absence of ATM, and can be reversed by ectopic expression of the corresponding recombinant protein. The expression constructs described here should add a valuable tool to the study of the numerous functions of this multifaceted protein.

Materials and methods

Human cell lines and transfections

The establishment of the AT22IJE-T cell line was previously described (Ziv *et al*., 1989). GM637 and MRC-5V1, immortalized human fibroblast lines, were obtained from the Coriell Institute for Medical Research (Camden, NJ) and Dr Colin E Arlett, respectively. Lymphoblastoid cell lines AT2YRM and ATY1RM were received from Dr Luciana Chessa. Fibroblast cell lines were grown in Dulbecco's Modified Eagle Medium supplemented with 15% fetal bovine serum (Beit Ha'Emek Biological Industries, Israel). Human lymphoblastoid cell lines were grown in RPMI 1640 medium with 10% fetal bovine serum. For each construct introduced into AT22IJE-T, 5×10^5 cells were transfected with 10 μg DNA using a mammalian transfection kit (Stratagene, La Jolla, CA). Selection with 200 μg/ml hygromycin B (Boehringer Mannheim) was initiated 48 h later. After 14–21 days, hygromycin-resistant colonies were trypsinized and the resultant cultures were grown in the presence of 100 μg/ml hygromycin.

RT–PCR

Total RNA was extracted from cultured cells using the Tri-Reagent system (Molecular Research Center, Inc., Cincinatti, OH). Reverse transcription was performed on 2.5 μg of total RNA as previously described (Gilad *et al*., 1996a). The products were analysed by electrophoresis in 1.0% agarose gel containing ethidium bromide.

Cloning in a baculovirus vector and isolation of the recombinant protein

The Bac-to-Bac system (Life Technologies) was used according to the manufacturer's instructions. Cloning in the pFastBac1 and pFastBacHTa plasmids was carried out in the STBL2 bacterial strain. Recombinant clones were transformed into DH1Bac cells for transposition into the bacmid bMON14272, and the resultant recombinant bacmids were isolated and transfected into Sf9 or High Five cells using the CellFECTIN Reagent (Life Technologies). Monolayer cultures were grown in Grace's medium supplemented with 10% fetal bovine serum, lactalbumin hydrolysate, yeastolate and 50 μg/ml gentamycin sulphate (Beit Ha'Emek Biological Industries, Israel). Cells growing in suspension were cultivated in Sf900-II serum-free medium (Life Technologies) supplemented with 50 μg/ml gentamycin sulphate.

Several protocols were tested for preparing cellular or nuclear extracts for immunoblotting analysis, in an attempt to identify the method that would yield the highest soluble ATM fraction:

1. Infected cells were washed twice with cold PBS, and resuspended in 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl. The suspension was left on ice for 15 min and then pushed ten times through a 25 gauge syringe needle. The resultant nuclear suspension was centrifuged for 5 min at 12 000 g, the nuclear pellet was resuspended in 20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 10 mM KCl, put on ice, and an equal volume of a 600 mM NaCl solution slowly added with constant mixing. The lysate was mixed at 4°C for 30 min, and centrifuged at 12 000 g; the supernatant was used for further analysis.
2. Whole cell extracts were lysed by rapid freezing and thawing as described in White *et al*. (1995). Cellular debris was removed by centrifugation at 12 000 g, and the supernatant was further used.
3. Infected cells were lysed for 45 min in ice-cold insect lysis buffer supplied with the Baculogold system (Pharmingen, San Diego, CA). The lysates were cleared of cellular debris by centrifugation, and the supernatant was further processed.
4. The cells were lysed in RIPA buffer (PBS, 1% NP40, 0.5% deoxycholate, 0.1% SDS). The lysates were left on ice for 30 min and sonicated several times. Cellular debris was removed by centrifugation at 12 000 g for 15 min at 4°C, and the supernatant was used for further analysis.

His-tagged ATM was isolated from the extracts by metal chelate chromatography using the Talon Metal Affinity Resin (Clontech, Palo Alto, CA), according to the manufacturer's instructions.

Epitope tagging

A 600 bp fragment at the 5′ region of the ATM ORF was amplified using a downstream primer that spanned a *Sac*I site within the ORF and an upstream primer that contained the desired epitope and a *Sal*I restriction site at the 5′ end. The amplified fragment was sequenced, digested with *Sac*I and *Sal*I, and used to replace the corresponding fragment in clone pFB-YZ3. In order to introduce the FLAG epitope, the upstream primer 5′-AAGACGGTCGACCACCATGGACTACAAGGACGATGACGACAAGATGAGTCTAGTACTTAATGAT-3′ was used with clone pFB-YZ3 (Figure 1) as template (the sequence encoding the FLAG epitope is underlined). To introduce a FLAG epitope and an adjacent 6XHIs sequence, the 5′ primer 5′-AAGACGGTCGACCACCATGGACTACAAGGACGATGACGACAAGATGCATCATCATCATCATCATATG-3′ was used with the clone containing the ATM ORF in the vector pFastBacHta as template. (In this clone, a 6xhis supplied by the vector already precedes the ATM ORF).

Introduction of the 8711A→G mutation

The use of conventional site-directed mutagenesis procedures with the large, unstable ATM cDNA constructs can be problematic. We preferred to replace a portion of the cDNA with the corresponding fragment obtained from patient material. A 1.8 kb fragment of the ATM ORF was amplified by RT–PCR using total RNA from the A-T cell line AT41RM, which is homozygous for the 8771A→G mutation (Gilad *et al*., 1996a). Primers for amplification were 5′-GTACTATCAGAATAGGAGACC-3′ (forward) and 5′-CTACAATGGTTAACAGAGTTTCC-3′ (reverse).

PflMI and *Nco*I sites present in the amplified fragment were used to generate a 0.9 kb derivative of this fragment, which replaced the corresponding PflMI-NcoI fragment in clone pFB-YZ3. The replaced region was sequenced in order to verify that the A-T mutation was the only sequence alteration introduced into the cDNA as a result of this procedure. The entire insert was then subcloned in the episomal vector pEBS7 (Figure 1).

Antibodies

A polyclonal antibody, X2361, was raised in rabbits against a portion of the ATM protein spanning amino acids 2361-2547. A cDNA fragment containing nt 7081–7640 of the ATM ORF was subcloned in the *Xba*I site of the bacterial expression vector pTrxFus (Invitrogen, San Diego, CA), and the construct was expressed in the bacterial strain GI472 (Invitrogen). Since the recombinant protein formed inclusion bodies, the protein band was excised from an SDS polyacrylamide gel and used as immunogen following addition of incomplete Freund's adjuvant. Rabbits were immunized with 200 μg of this protein and boosted periodically with 100 μg. ATM(Ab-3), a polyclonal antibody directed against a peptide spanning positions 819–844 of the ATM protein, was obtained from Oncogene Research Products, Cambridge, MA, and the anti-FLAG M2 and M5 monoclonal antibodies were obtained from Eastman Kodak Imaging Systems, New Haven, CT.

Western blot analysis

Cellular extracts were prepared as described by White *et al*. (1995), boiled for 5 min and fractionated on a 6% polyacrylamide gel containing 0.1% SDS. Transfer to a nitrocellulose membrane (Pharmacia Biotech, Uppsala, Sweden) was performed overnight in 50 mM Tris, 10% methanol, 200 mM glycine and 0.005% SDS at 250–300 mA. The membrane was blocked for 1.5 h in TTBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 3% BSA. Antibodies were added to the blocking solution for 1–4 h at room temperature. The blots were rinsed six times, 5 min each, with TTBS containing 2% low fat milk and 0.2% BSA. A horseradish peroxidase-conjugated secondary antibody was added for 1 h and the blots were washed five times, 5 min each, with TTBS. Immunoreactive bands were visualized using the Super Signal system (Pierce, Rockford, Il).

Immunoprecipitation

Whole cell extracts were prepared from 5×10^6–10^7 cells as described under immunoblotting analysis, and the extracts were diluted at a 1:3 ratio with 20 mM HEPES, 0.3% NP-40, to give a final solution of 20 mM HEPES, 150 mM NaCl, 0.2% NP-40. This mixture (usually 500 μl) was precleared with 40 μl of a 50% slurry of protein G-sepharose (Pharmacia Biotech) for 4 h and incubated overnight at 4°C with 9 μg of anti-FLAG M2 or M5 antibody. The immune complexes were then incubated with 40 μl of protein G-sepharose for 2 h at 4°C, washed three times with lysis buffer, and resuspended in loading buffer (50 mM Tris-HCl, pH 6.8, 2% 2-mercaptoethanol, 2% SDS, 0.2% bromophenol blue, 10% glycerol). The mixture was boiled for 5 min and fractionated on an SDS polyacrylamide gel and immunoblotting analysis proceeded as described above.

Separation of recombinant ATM by immunoaffinity

Five hundred μl of cellular extract in 20 mM HEPES, pH 7.6, 150 mM NaCl, 0.1% NP-40 were mixed with 20 μl of M2-affinity gel (Eastman Kodak), and the mixture was left at 4°C for 16 h with gentle shaking. The gel was washed five times, 5 min each, with the same buffer, and FLAG peptide was added to final concentration of 200 μg/ml. After an additional 30 min of gentle shaking at 4°C the mixture was centrifuged in a microfuge at maximum speed and aliquots of the supernatant were processed for Western blot analysis.

Immunofluorescence staining and confocal analysis

Eight-well Lab-Tek chamber slides (Nalge Nunc, Haperville, Il) were inoculated with 10^4 cells per well, and the cultures were grown for 48 h. Cells were fixed in PBS containing 4% paraformaldehyde and 0.1% Triton X-100 for 10 min at room temperature. Following blocking with 1% BSA, 10% normal donkey serum in PBS for 10 min at room temperature, cells were stained with the ATM(Ab-3) polyclonal antibody at dilution of 1:50 and the M5 anti-FLAG monoclonal antibody at dilution of 1:25 for 1 h at room temperature. Following three washes in PBS, the cells were stained with donkey anti-rabbit IgG antibody conjugated to fluorescein isothiocyanate (FITC) and anti mouse IgG antibody conjugated to Texas Red (Jackson Immuno Research Laboratories, Inc., West Grove, PA) diluted at 1:50 for 1 h at room temperature. The cells were washed three times in PBS and coverslipped with gel mount (Biomeda, Foster City, CA). Fluorochrome-labeled cells were analysed using a 410 Zeiss confocal laser scan microscope (CLSM) (Zeiss, Oberkochen, Germany) with the following configuration: 25 mW argon and HeNe lasers, 488, 568 and 633 maximum lines. Co-localization analysis was performed on simultaneously detected ATM(Ab-3) and M5 anti-FLAG antibodies using the Zeiss co-localization procedure. This procedure yields a graphic representation of the distribution of green (Y axis) and red (X axis) fluorescence for each pixel. A range of fluorescence intensities was selected from graphic representation of pixel distribution, and overlaid as a blue image on the red/green image. Images were stored on an optical disk drive and printed using a Codonics NP1600 printer (Codonics, Middleburg Heights, OH).

Cellular sensitivity to damaging agents

Experiments were carried out according to previously published protocols (Al-Nabulsi *et al*., 1994; Ziv *et al*., 1995). Briefly, monolayer cultures at late logarithmic stage were trypsinized and the cells replated at densities of 10^3–5×10^4 per 60 mm dish. The cultures were irradiated 24 h later with varying doses of gamma-rays using a JL Shepard Mark I cesium-137 irradiator (JL Shepard and Associates, Glendale, CA). Treatment with various concentrations of neocarzinostatin (Kayaku Antibiotics, Tokyo, Japan) was performed on logarithmic cultures for 30 min at 37°C and followed by replating. After 10–14 days the resultant colonies were fixed and stained with 2% crystal violet in 50% ethanol and counted under a dissecting microscope.

Radioresistant DNA synthesis (RDS)

RDS was measured as previously described in detail (Ziv *et al*., 1995). The cells were pre-labeled with 2-^{14}C-thymidine and irradiated with various doses of gamma-rays. The rate of DNA synthesis was estimated by measuring the incorporation of tritiated thymidine into chromosomal DNA during 1 h beginning 1 h after irradiation.

Acknowledgements
We are grateful to Kayaku Pharmaceutical Manufacturers (Tokyo, Japan) for a generous gift of neocarzinostatin, to Dr Randy Legerski for the pEBS7 vector, to Dr Luciana Chessa for the cell lines AT24RM and AT41RM, and to

Dr Colin F Arlett for the cell line MRC-5V1. The valuable contribution of Dr Yuval Reiss to the production of antibodies and the excellent technical help of Judith Horev are gratefully acknolwedged. This study was supported by research grants from the A-T Medical Research Foundation, the A-T Children's Project, The United States-Israel Binational Science Foundation, and the National Institute of Neurological Disorders and Stroke (NS31763).

References

Al-Nabulsi I, Takamiya Y, Voloshin Y, Dritschilo A, Martuza RL and Jorgensen TJ. (1994). *Cancer Res.*, **54,** 5614–5617.

Artuso M, Esteve A, Bresil H, Vuillaume M and Hall J. (1995). *Oncogene*, **11,** 1427–1435.

Baumer A, Bernthaler U, Wolz W, Hoehn H and Schindler D. (1996). *Hum. Genet.*, **98,** 246–249.

Beamish H, Williams R, Chen P and Lavin MF. (1996). *J. Biol. Chem.*, **271,** 20486–20493.

Brizzard BL, Chubet RG and Vizard DL. (1994). *BioTechniques*, **16,** 730–734.

Brown EJ, Beal PA, Keith CT, Chen J, Shin TB and Schreiber SL. (1995). *Nature*, **377,** 441–446.

Brown K, Ziv Y, Sadanandan SN, Chessa L, Collins FS, Shiloh Y and Tagle DA. (1997). *Proc. Natl. Acad. Sci. USA*, **94,** 1840–1845.

Byrd PJ, McConville CM, Cooper P, Parkhill J, Stankovic T, McGuire TM, Thick JA and Taylor AMR. (1996). *Hum. Mol. Genet.*, **5,** 145–149.

Canman CE, Wolff AC, Chen C-Y, Fornace AJ and Kastan MB. (1994). *Cancer Res.*, **54,** 5054–5058.

Chen G and Lee EYHP. (1996). *J. Biol. Chem.*, **271,** 33693–33697.

Chiang C-M and Roeder RG. (1993). *Peptide Res.*, **6,** 62–64.

Dent P, Reardon DB, Morrison DK and Sturgill TW. (1995). *Mol. Cell. Biol.*, **15,** 4125–4135.

Derijard B, Hibi M, Wu H, Barrett T, Su B, Deng T, Karin M and Davis RJ. (1994). *Cell*, **76,** 1025–1037.

Gilad S, Khosravi R, Uziel T, Ziv Y, Rotman G, Savitsky K, Smith S, Chessa L, Harnik R, Shkedi D, Frydman M, Sanal O, Portnoi S, Goldwicz Z, Jaspers NGJ, Gatti RA, Lenoir G, Lavin MF, Tatsumi K, Wegner RD, Shiloh Y and Bar-Shira A. (1996a). *Hum. Mol. Genet.*, **5,** 433–439.

Gilad S, Bar-Shira A, Harnik R, Shkedy D, Ziv Y, Khosravi R, Brown K, Vanagaite L, Xu G, Frydman M, Lavin MF, Hill D, Tagle D and Shiloh Y. (1996b). *Hum. Mol. Genet.*, **5,** 2033–2038.

Gilad S, Khosravi R, Harnik R, Ziv Y, Shkedy D, Galanty Y, Frydman M, Levi J, Sanal O, Chessa L, Smeets D, Shiloh Y and Bar-Shira A. (1997). *Hum. Mutat.* (in press).

Hartley KO, Gell D, Smith GC, Zhang H, Divecha N, Connelly MA, Admon A, Lees-Miller SP, Anderson CW and Jackson SP. (1995). *Cell*, **82,** 849–856.

Hochuli E, Bannwarth W, Dobeli H, Gentz R and Stuber D. (1988). *Bio/Technology*, **6,** 1321–1325.

Hopp TP, Prickett KS, Price VL, Libby RT, March CJ, Ceretti DP, Urdal DL and Conion PJ. (1988). *Bio/Technology*, **6,** 1204–1210.

Hunter T. (1995). *Cell*, **83,** 1–7.

Jackson SP. (1995). *Curr. Biol.*, **5,** 1210–1211.

Jorgensen TJ, Russell PS and McRae DA. (1995). *Radiat. Res.*, **143,** 219–223.

Jung M, Kondratyev A, Lee SA, Dimtchev A and Dritschilo A. (1997). *Cancer Res.*, **57,** 24–27.

Keegan KS, Holtzman DA, Plug AW, Christenson ER, Brainerd EE, Flaggs G, Bentley NJ, Taylor EM, Meyn MS, Moss SB, Carr AM, Ashley T and Hooekstra MF. (1996). *Genes and Dev.*, **10,** 2423–2437.

Khanna KK, Yan J, Watters D, Hobson K, Beamish H, Spring K, Shiloh Y, Gatti RA and Lavin MF. (1997). *J. Biol. Chem.* (in press).

Lakin ND, Weber P, Stankovic T, Rottinghaus ST, Taylor AMR and Jackson SP. (1996). *Oncogene*, **13,** 2707–2716.

Lavin MF and Shiloh Y. (1997). *Ann. Rev. Immunol.*, **15,** 177–202.

Liu F, Ventura F, Doody J and Massague J. (1995). *Mol. Cell. Biol.*, **15,** 3479–3486.

Luckow VA. (1993). *Curr. Opin. Biotechnol.*, **4,** 564–572.

Molloy SS, Thomas L, VanSlyke JK, Stenberg PE and Thomas G. (1994). *EMBO J.*, **13,** 18–33.

Morgan SE, Lovly C, Pandita TK, Shiloh Y and Kastan MB. (1997). *Mol. Cell. Biol.*, **17,** 2020–2029.

Peterson C and Legerski R. (1991). *Gene*, **107,** 279–284.

Rotman G and Shiloh Y. (1997). *Cancer Surveys*, (in press).

Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S, Ashkenazi M, Pecker I, Frydman M, Harnik R, Patanjali SR, Simmons A, Clines GA, Sartiel A, Gatti RA, Chessa L, Sanal O, Lavin MF, Jaspers NGJ, Taylor AMR, Arlett CF, Miki T, Weissman S, Lovett M, Collins FS and Shiloh Y. (1995a). *Science*, **268,** 1749–1753.

Savitsky K, Sfez S, Tagle D, Ziv Y, Sartiel A, Collins FS, Shiloh Y and Rotman G. (1995b). *Hum. Mol. Genet.*, **4,** 2025–2032.

Shiloh Y, Tabor E and Becker Y. (1982). *Cancer Res.*, **42,** 2247–2249.

Shiloh Y and Rotman G. (1996). *J. Clin. Immunol.*, **16,** 254–260.

Smith GE and Cherry CL. (1983). *Proc. Natl. Acad. Sci. USA*, **82,** 8404–8408.

Taylor AMR, Byrd PJ, McConville CM and Thacker S. (1994). *Int. J. Radiat. Biol.*, **65,** 65–70.

Telatar M, Wang Z, Udar N, Liang T, Bernatowska-Matuszkiewicz E, Lavin M, Shiloh Y, Concannon P, Good RA and Gatti RA. (1996). *Am. J. Hum. Genet.*, **59,** 40–44.

Watters D, Khanna KK, Beamish H, Birrell G, Spring K, Kedar P, Gatei M, Stenzel D, Hobson K, Kozlov S, Zhang N, Farrell A, Ramsay J, Gatti R and Lavin M. (1997). *Oncogene*, (in press).

White RJ, Gottlieb TM, Downes CS and Jackson SP. (1995). *Mol. Cell. Biol.*, **15,** 1983–1992.

Wright J, Teraoka S, Onegut S, Tolun A, Gatti RA, Ochs HD and Concannon P. (1996). *Am. J. Hum. Genet.*, **59,** 839–846.

Yates JL, Warren N and Sugden B. (1985). *Nature*, **313,** 812–815.

Zakian VA. (1995). *Cell*, **82,** 685–687.

Ziv Y, Etkin S, Danieli T, Amiel A, Ravia Y, Jaspers NGJ and Shiloh Y. (1989). *Cancer Res.*, **49,** 2495–2501.

Ziv Y, Bar-Shira A, Jorgensen TJ, Russell PS, Sartiel A, Shows TB, Eddy RL, Buchwald M, Legerski R, Schimke RT and Shiloh Y. (1995). *Somatic Cell Mol. Genet.*, **21,** 99–111.