BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **245,** 144–148 (1998)
ARTICLE NO. RC988137

Cloning and Expression of the Ataxia–Telangiectasia Gene in Baculovirus

Shaun P. Scott,* Ning Zhang,* Kum Kum Khanna,* Alexander Khromykh,† Karen Hobson,* Dianne Watters,* and Martin F. Lavin*,‡

**The Queensland Cancer Fund Research Laboratories, Queensland Institute of Medical Research, PO Royal Brisbane Hospital, Herston, Brisbane, Queensland 4029, Australia; †Sir Albert Sakzewski Virus Research Centre, The Royal Children's Hospital, Herston, Brisbane, Queensland 4029, Australia; and ‡Department of Surgery, The University of Queensland, PO Royal Brisbane Hospital, Herston, Brisbane, Queensland 4029, Australia*

Received December 26, 1997

The gene mutated in the human genetic disorder ataxia–telangiectasia, ATM, is implicated in the response to radiation-induced DNA damage and to a more widespread signalling defect. The ATM protein is predominantly a nuclear protein where it interacts with p53 and c-Abl as part of a radiation signal transduction pathway(s). We describe here the cloning of full-length ATM cDNA in a baculovirus vector to produce recombinant protein. Expression of ATM, as a soluble protein, was observed by 36 h post-infection using immunoblotting with anti-ATM antibody. The presence of a hexahistidine tag on ATM was used as the basis for purification of the protein by affinity chromatography. The protein yield was only 20 ng/100 ml of infected cells, presumably because of the size of the protein and adverse effects on cell growth when overexpressed. ATM was found to have autophosphorylation activity in immunoprecipitates with antibodies directed against the hexahistidine tag sequence. These results demonstrate that ATM can be expressed inefficiently in baculovirus infected insect cells and the data suggest that it phosphorylates itself. © 1998 Academic Press

Ataxia–telangiectasia (A-T) is a human autosomal recessive disorder, defined by a complex variety of clinical manifestations. This multisystem disease is characterized by immunodeficiency, neurological abnormalities, extreme radiosensitivity, cell cycle abnormalities and predisposition to lymphoid and other malignancies (1,2).

The gene mutated in A-T and designated the ataxia–telangiectasia mutated (ATM) gene was recently cloned and shown to be mutated in cell lines from A-T patients (3). The gene contains 66 exons, encompasses approximately 150 kb of genomic DNA and codes for a 13 kb mRNA with an open reading frame of 9168 base pairs (4). Mutation analysis has revealed that the large majority of mutations are predicted to give rise to a truncated form of the ATM protein (5-7). The protein product of the ATM gene is predicted to be 350.6 kDa in size based on the ORF size (4), which agrees well with the ATM protein size detected by immunoblotting (8-12). This protein is localized to the nucleus but has also been detected in vesicular structures in the cytoplasm (12). Use of immunoblotting failed to detect truncated protein in A-T cells suggesting that it is unstable (10,12).

The exact role of the ATM protein is not yet known but based on the pleiotropic phenotype of A-T and its relationship to a family of phosphatidylinositol 3-kinases (13-15), it is likely to be involved in signal transduction, cell cycle control and as a sensor of DNA damage due to ionizing radiation exposure. Binding of ATM to p53 further substantiates the role of this protein in radiation signal transduction (12). ATM has also been shown to bind to and activate c-Abl as part of an oxidative stress pathway (16,17).

The expression of the ATM gene and purification of the protein represent important steps in an attempt to define the role of the protein and to unravel the complexities of this multifaceted disorder. The baculovirus expression system provides an ideal system for overproducing recombinant proteins and their subsequent purification (18-20). As a eukaryotic system it uses many of the protein modification, processing and transport systems present in higher eukaryotic cells. In addition, since the viral genome of the baculovirus is large, it can accommodate large segments of foreign DNA, such as the ATM cDNA which is 9.168 kb is size. We describe here the cloning of full-length ATM cDNA in baculovirus, expression of the protein and determination of protein kinase activity.

EXPERIMENTAL PROCEDURES

Insect cell culture. The lepeidopteran insect *Spodoptera frugiperda* cell line, Sf9, was routinely cultured in TC-100 (GIBCO-BRL)

0006-291X/98 $25.00

medium supplemented with 10% foetal bovine serum at 27°C. The expression of the recombinant ATM protein within insect cells was achieved using both TC-100 and a serum free medium, SF-900 II (GIBCO-BRL). Essentially, all manipulations of insect cells and baculovirus were as described by O'Reilly *et al.* (21).

Plasmid construction and purification of recombinant virus. The ATM recombinant transfer vector was constructed by sub-cloning the full-length ATM cDNA into the baculovirus transfer vector, pBlueBacHis (Invitrogen). The ATM fragment was isolated from pEAT22 (22) using Xho I (blunt ended) and Sca I, and cloned into the Bgl II (blunt ended) site of pBlueBacHisB (Clontech), resulting in the pBAT plasmid. The cloning junctions were sequenced to ensure the integrity of the sequence. The production of recombinant ATM baculovirus (rATM) was achieved by cotransfecting insect cells with linear wildtype viral DNA together with pBAT. Repetitive rounds of plaquing were undertaken to obtain a pure stock of recombinant virus, which were selected by their ability to express β-galactosidase. For the plaque assays, 60 mm tissue culture plates were seeded with 2×10^6 Sf9 cells and infected with various dilutions (10^{-2} to 10^{-6}) of the transfection supernatant. Following the first round of recombinant plaque identification, viral DNA was isolated from 5 putative recombinants (23) and subjected to PCR. To confirm the presence of the ATM gene, a 563 bp fragment, situated at the 5′ end (nucleotides 318-880), was PCR amplified (30 cycles of 95°C × 40 sec; 55°C × 80 sec; 72°C × 60 sec). The PCR products were analysed by agarose-gel electrophoresis.

Expression and purification of recombinant ATM protein. The expression of recombinant ATM protein in the baculovirus system was achieved using both monolayer and suspension cultures of Sf9. Cells were infected with recombinant baculovirus (rATM) at a multiplicity of infection (MOI) of 5 and allowed to incubate for approximately 48 h at 27°C. Cells were then collected by centrifugation, washed with PBS, lysed in 1 ml of RIPA buffer (50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 1% NP40; 0.5% sodium deoxycholate; 0.1% SDS) supplemented with protease inhibitors and frozen at −70°C overnight. The recombinant 6 × His-tagged ATM protein was purified from the soluble supernatant fraction by absorption to Ni-NTA resin (QIAGEN), essentially according to the manufacturers instructions. Briefly, 50 μl of Ni-NTA resin previously equilibrated with RIPA buffer, was added to the fraction and rotated for 1-2 h at 4°C. Beads were then pelleted by centrifugation at 1000 × g, washed three times with RIPA buffer to remove unbound proteins and the recombinant protein was then eluted from the resin using 100 mM imidazole.

Immunoblotting. Immunoblotting was employed for the detection of purified recombinant proteins. Proteins were separated on 5% SDS-PAGE, transferred to nitrocellulose, blocked and incubated with an ATM antibody, ATM-3BA (12). After incubation with secondary antibody (anti-rabbit Ig HRP labelled), the ATM protein was visualized using ECL.

Autophosphorylation of recombinant ATM. Sf9 insect cells, infected with wild type or recombinant virus, were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 25 mM sodium flouride, 25 mM β-glycerophosphate, 0.1 mM sodium ortho-vanadate, 0.1 mM PMSF, 5 μg/ml leupeptin, 1 μg/ml aprotonin, 0.2% Triton X-100, 0.3% NP40). After incubation on ice for 30 min, the debris was removed by centrifugation at 14,000 × g for 15 min, yielding the total cell extract. Anti-His immunoprecipitates were performed by adding antibody against the hexahistidine tag (6 × His mAb, Clontech) and a mixture of protein A and G-Sepharose for 2 h at 4°C. Immune complex kinase assays were performed by incubating the resulting protein complexes in kinase buffer (25 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM DTT), with 5-10 μCi [γ^{-32}P]ATP for 30 min at 28°C and analysed by 5% SDS-PAGE and autoradiography.

RESULTS

A 4.7 kb fragment extending from immediately downstream of the ATG initiation codon of ATM and overlapping with the 5.9 kb partial ATM cDNA, described by Savitsky *et al.* (3), was prepared by PCR as reported recently (22). The 4.7 kb and 5.9 kb fragments were employed to construct a full-length ATM cDNA clone (pEAT22) in an Epstein-Barr Virus based vector (Fig. 1A). The ATM cDNA was excised with Xho I (blunt ended) and Sca I as a 9.6 kb fragment and cloned into the baculovirus vector pBlueBacHisB to produce the construct pBAT (Fig 1A). This construct also contains a sequence coding for hexahistidine which allows for isolation of the ATM protein by immunoprecipitation or nickel chelate chromatography.

Sf9 insect cells were infected with wild type baculovirus and the pBAT construct, and following the first round of recombinant plaque isolation, PCR was carried out to confirm the presence of the ATM gene. A 563 bp fragment (nucleotides 318-880) was amplified since this region has been shown to be unstable during ATM cDNA cloning (22). Four of five plaques isolated were positive for the ATM sequence (Fig 1B). Two additional rounds of plaque purification were carried out.

To confirm the expression of ATM, insect cell monolayers were infected (MOI of 5) with either wild-type baculovirus or with ATM recombinant baculovirus. Extracts were immunoprecipitated with anti-hexahistidine antibody and ATM protein was detected by immunoblotting. The results in Figure 2A demonstrate that ATM protein was present only in Sf9 cells infected with recombinant virus. This protein was of comparable size to ATM detected in extracts from human cells (Fig 2A).

Time course experiments were undertaken in monolayer and suspension cultures to determine the optimal time for expression of the recombinant ATM protein in insect cells (infected with an MOI of 5) and samples taken at 36, 48 and 60 h post-infection. Immunoblotting with ATM-3BA antibody revealed similar amounts of ATM at all the time points and expression was approximately the same after infection of monolayer or suspension cultures (Fig 2B). ATM protein was not detected in the uninfected insect cells.

The recombinant ATM protein was successfully purified using the hexahistidine tag at the N-terminus of the protein. Western blot analysis depicts the various stages of the purification (Fig 2C). Total cell lysates of Sf9 insect cells (lane 1) and Sf9 infected with wild-type baculovirus (lane 2) contained no bands corresponding to the eluted protein band. The absence of protein in the membrane pellet (lane 3) indicated the production of a soluble protein, which is also consistent with previous data showing that ATM is predominantly a nuclear protein (8,10,12). The recovery levels of ATM were found to be extremely low approximately 20 ng/100 ml of infected cells. The relatedness of ATM, through a PI3-kinase domain, to proteins such as DNA-dependent protein kinase (DNA-PK) suggest that it may have protein kinase activity. In order to check this we assayed for autophosphorylation of baculovirus produced

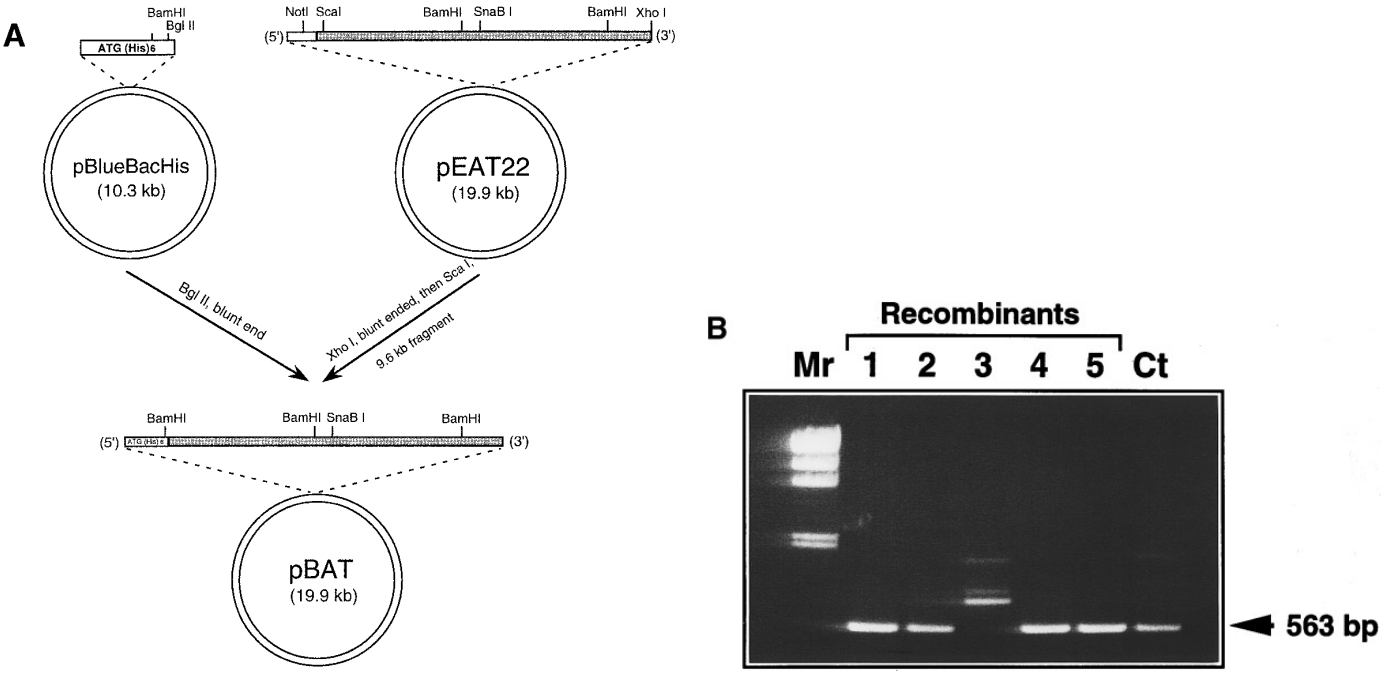

FIG. 1. Construction and identification of ATM recombinant baculovirus. (A) Schematic of the construction of the ATM recombinant baculovirus transfer vector. The strategy involved the isolation of the full-length ATM cDNA and cloning into the baculovirus transfer vector (pBlue Bac His) containing a hexahistadine tag sequence, to generate the pBAT construct. (B) Identification of recombinant baculovirus by PCR. Amplification with recombinant viral DNA (lanes 1 to 5) and control cDNA (Ct) prepared from a human cell line, with ATM specific primers designed to the 5′ region of the ATM cDNA (see Experimental Procedures). Agarose gel (1%) electrophoresis revealed a 563 bp DNA fragment, which correlates with the predicted size of the ATM cDNA. The marker (Mr) is λ DNA digested with HindIII. This approach established that the 5′ unstable region of ATM was intact.

ATM protein. As is evident in Figure 3 (rATM) phosphorylation of a protein approximately 350 kDa in size was observed only in lysates from cells expressing ATM.

DISCUSSION

We have reported here the expression of ATM protein in insect cells using a baculovirus construct. Although the full-length cDNA for ATM is not excessively large, 9.168 kb, it has proved difficult to isolate a complete cDNA clone. Savitsky *et al.* (3) initially reported the isolation of a partial ATM cDNA, 5.9 kb in size, and subsequently the complete ORF for the ATM gene based on cDNA contigs spanning the complete gene. Instability at the 5′ end of the cDNA appears to be the major stumbling block. This instability was manifested as deletions, rearrangements and the introduction of stop codons (22,24). We have recently expressed the full-length ATM cDNA in human cells and corrected aspects of the A-T radiosensitive phenotype with this cDNA (22). In this report we have cloned this cDNA in a baculovirus vector and successfully expressed the gene in insect cells. The stability of the clone was confirmed by PCR analysis of the 5′ end of the cDNA isolated from recombinant viral plaques. Immunoblotting detected a protein of approximately 350 kDa in size which corresponds well to the previously estimated size of the ATM protein in human cell extracts (10-12). The presence of the hexa-His tag at the N-terminus of the recombinant protein facilitated purification using a Ni-NTA resin.

The baculovirus system has proved to be an efficient approach for the overproduction of recombinant proteins capable of being modified and processed in a manner similar to that in higher eukaryotic cells (18,19). Recombinant proteins may comprise 1-5% of total cell protein and levels of expression between 100 μg and 50 mg/100 ml of insect cells have been reported (20). The amount of ATM protein produced falls far short of this range with only 20 ng of protein being produced per 100 ml of infected insect cells. Attempts to improve this yield by modifying MOI and growth conditions had little effect (results not shown). It is not clear why the yield is so low for ATM but may relate to the size of the protein (350 kDa) or the multi-functional nature of the protein. Similar observations have been made by Ziv *et al.* (24) who showed that the majority of recombinant ATM was associated with cellular membranes. A-T cells, in which this protein is mutated or absent due to the instability of a truncated form, are characterized by a pleiotropic phenotype (1,2). This mutation leads

to neuronal degeneration, immune dysfunction, developmental abnormalities, radiosensitivity, cell cycle anomalies, signalling defects and predisposition to cancer (1,2). Overexpression of ATM which would be expected in baculovirus vectors may have uneven effects on different pathways, leading to an antiproliferative effect overall. It should be pointed out that the presumed activation of ATM by ionizing radiation leads to the stabilization of p53 and G1/S checkpoint activation to slow the progress of cells from G1 to S phase (25,26). Since it is not yet known how radiation recruits ATM to the radiation signal transduction pathway, it is possible that overexpression of the protein might have the same effects and thus account for the poor yield in the baculovirus system.

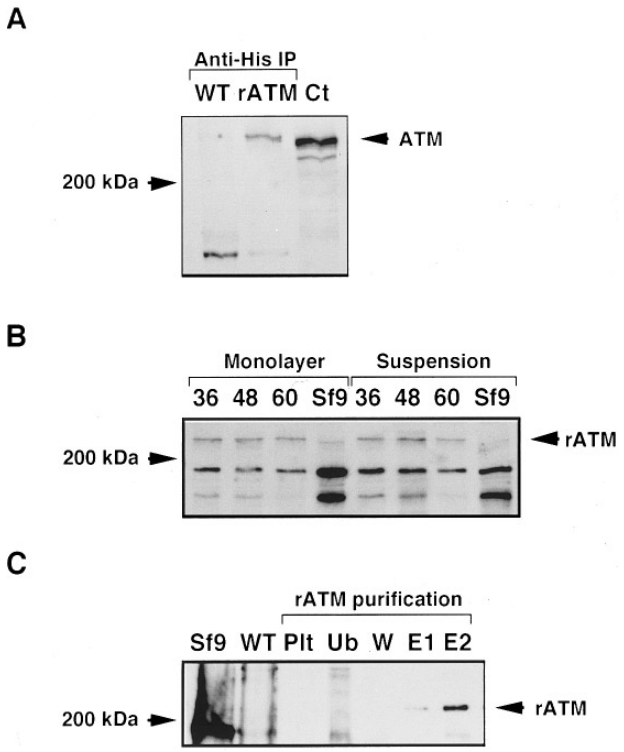

FIG. 2. Expression and purification of recombinant ATM baculovirus. (A) Expression of recombinant ATM baculovirus. Extracts were prepared from insect cells infected with wild-type baculovirus (WT); insect cells infected with recombinant ATM (rATM) and from a human lymphoblastoid cell line (Ct). Immunoprecipitation was carried out with monoclonal antibody to the hexahistidine for WT and rATM followed by immunoblotting with ATM-3BA antibody (12). (B) Time course of expression of ATM in monolayer and suspension cultures of Sf9 cells infected with rATM. Extracts were prepared at 36, 48 and 60 h post-infection. Sf9 represents extracts from uninfected cells. Immunoblotting was carried out with ATM-3BA antibody. (C) Purification of rATM using Ni-NTA resin. Sf9 insect cells; WT, insect cells infected with wild-type baculovirus; Plt, pellet after cell lysis from insect cells infected with rATM; Ub, flow through from Ni-NTA resin; W, initial wash of resin with RIPA buffer; E1, first elution with RIPA containing 25 mM imidazole and E2, second elution with RIPA containing 100 mM imidazole. Protein was separated on 5% SDS-PAGE, transfered and immunoblotted with ATM-3BA antibody.

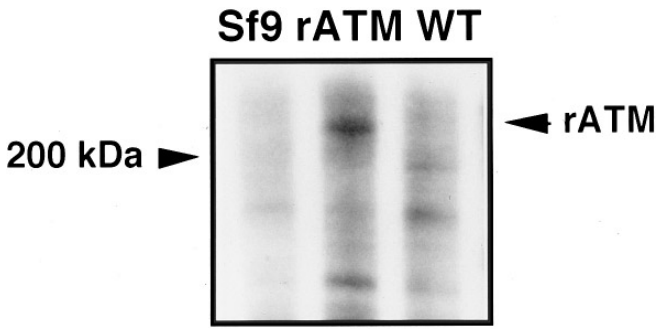

FIG. 3. Autophosphorylation of rATM *in vitro.* Extracts were prepared from insect cells and from cells infected with either recombinant (rATM) or wild-type (WT) baculovirus and immunoprecipitated with anti-hexahistidine antibody. Autophosphorylation was carried out in the presence of [γ^{32}P]ATP followed by electrophoresis on 5% SDS-PAGE and autoradiography.

The demonstration that ATM protein is capable of autophosphorylation supports the notion that it is most likely to behave as a protein kinase. In this respect it is similar to other members of the PI3-kinase family (27,28). Two recent reports demonstrate that ATM is a phosphoprotein. Metabolic labelling with ^{32}P-orthophosphate followed by immunoprecipitation demonstrated that mouse ATM is phosphorylated (8). Autophosphorylation of ATM was shown in anti-ATM immunoprecipitates in an *in vitro* protein kinase assay (9). Future emphasis will be to identify critical targets for the ATM kinase activity.

ACKNOWLEDGMENTS

We wish to thank the National Health and Medical Research Council of Australia and the A-T Childrens Foundation (Boca Raton, USA) for support. We would also like to thank members of the Westaway laboratory for their assistance with the baculovirus system. Thanks to Ann Knight for typing the manuscript.

REFERENCES

1. Sedgwick, R. P., and Boder, E. (1991) *in* Hereditary Neuropathies and Spinocerebellar Atrophies (Vianney De Jong, J. M. B., Ed.), pp. 347–423, Elsevier Science Publishers, Amsterdam.
2. Lavin, M. F., and Shiloh, Y. (1997) *Ann. Rev. Immunol.* **15,** 177–202.
3. Savitsky, K., Bar-Shira, 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., Taylor, A. M. R., Arlett, C. F., Miki, T., Weissman, S. M., Lovett, M., Collins, F. S., and Shiloh, Y. (1995) *Science* **268,** 1749–1753.
4. Savitsky, K., Sfez, S., Tagle, D., Ziv, Y., Sartiel, A., Collins, F. S., Shiloh, Y., and Rotman, G. (1995) *Hum. Mol. Genet.* **4,** 2025–2032.
5. Byrd, P. J., McConville, C. M., Cooper, P., Parkhill, J., Stankovic, T., McGuire, G. M., Thick, J. A., and Taylor, A. M. R. (1996) *Hum. Mol. Genet.* **5,** 145–149.

6. Gilad, S., Khosravi, R., Shkedy, D., Uziel, T., Ziv, Y., Savitsky, K., Rotman, G., Smith, S., Chessa, L., Jorgensen, T. J., Harnik, R., Frydman, M., Sanal, O., Portnoi, S., Goldwicz, Z., Jaspers, N. G. J., Gatti, R. A., Lenoir, G., Lavin, M. F., Tatsuni, K., Wegner, R. D., Shiloh, Y., and Bar-Shira, A. (1996) *Hum. Mol. Genet.* **5,** 433–440.
7. Telatar, M., Wang, Z., Udar, W., Liang, T., Concannon, P., Bernatowska-Matuscklewicz, E., Lavin, M. F., Shiloh, Y., Good, R. A., and Gatti, R. A. (1996) *Am. J. Hum. Genet.* **59,** 40–44.
8. Chen, G., and Lee, E. Y-H. P. (1996) *J. Biol. Chem.* **271,** 33693–33697.
9. Keegan, K. S., Holtzman, D. A., Plug, A. W., Christenson, E. R., Brainerd, E. E., Flaggs, G., Bentley, N. J., Taylor, E. M., Meyn, M. S., Moss, S. B., Carr, A. M., Ashley, T., and Hoekstra, M. F. (1996) *Genes & Development* **10,** 2423–2437.
10. Lakin, N. D., Weber, P., Stankovic, T., Rottinghaus, S. T., Malcolm, A., Taylor, R., and Jackson, S. P. (1996) *Oncogene* **13,** 2707–2716.
11. Brown, K. D., Ziv, Y., Sadanandan, S. N., Chessa, L., Collins, F. S., Shiloh, Y., and Tagle, D. A. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1840–1845.
12. Watters, D., Khanna, K. K., Beamish, H., Birrell, G., Spring, K., Kedar, P., Gatei, M., Stenzel, D., Hobson, K., Kozlov, S., Farrell, A., Ramsay, J., Gatti, R., and Lavin, M. F. (1997) *Oncogene* **14,** 1911–1921.
13. Zakian, V. A. (1995) *Cell* **82,** 685–687.
14. Jackson, S. P. (1995) *Current Biology* **5,** 1210–1212.
15. Lavin, M. F., Khanna, K. K., Beamish, H., Williams, R., Spring, K., Watters, D., and Shiloh, Y. (1995) *Trends Biochem. Sci.* **20,** 382–383.
16. Khanna, K. K., Shafman, T., Kedar, P., Yen, T., Spring, K., Gatei, M., Zhang, N., Watters, D., Egerton, M., Shiloh, Y., and Lavin, M. F. (1997) *Nature* **387,** 520–524.
17. Baskaran, R., Wood, L. D., Whitaker, L. L., Canman, C. E., Morgan, S. E., Zu, Y., Barlow, C., Baltimore, D., Wynshaw-Boris, A., Kastan, M. B., and Wang, J. Y. J. (1997) *Nature* **387,** 516–520.
18. Luckow, V. A., and Summers, M. D. (1988) *BioTechnology* **6,** 47–55.
19. Miller, L. K. (1988) *Annu. Rev. Microbiol.* **42,** 177–199.
20. Piwnica-Worms, H. (1995) *in* Current Protocols in Molecular Biology. (Ausebel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds.), pp. 16.8.1–16.11.7, John Wiley & Sons, Inc. USA.
21. O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992) Baculovirus Expression Vectors: A Laboratory Manual. pp. 109–240, W. H. Freeman & Company, New York.
22. Zhang, N., Chen, P., Khanna, K. K., Scott, S., Gatei, M., Kozlov, S., Watters, D., Spring, K., Yen, T., and Lavin, M. F. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 8021–8026.
23. Khromykh, A. A., Meka, H., and Westaway, E. G. (1995) *BioTechniques* **19,** 356–360.
24. Ziv, Y., Bar-Shira, A., Pecker, I., Russell, P., Jorgensen, T. J., Tsarfati, I., and Shiloh, Y. (1997) *Oncogene* **15,** 159–167.
25. Kastan, M. B., Zhan, O., El-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J. (1992) *Cell* **71,** 587–597.
26. Khanna, K. K., and Lavin, M. F. (1993) *Oncogene* **8,** 3307–3312.
27. Brown, E. J., Beal, P. A., Keith, C. T., Chen, J., Shin, T. B., and Schreiber, S. L. (1995) *Nature* **377,** 441–446.
28. Chan, D. W., and Lees-Miller, S. P. (1996) *J. Biol. Chem.* **271,** 8936–8941.