Ataxia-telangiectasia (AT) is an autosomal recessive disease. The relevant gene has been cloned and designated ATM. We studied the expression of both ATM mRNA and the ATM protein in unirradiated and X-irradiated EBV (Epstein-Barr virus)-transformed lymphoblastoid cell lines (LCLs) derived from donors who were normal (ATM+/+), AT heterozygotes (ATM+/−), or AT homozygotes (ATM−−−−), respectively. In ATM+/+ LCLs, the levels of ATM mRNA were found to have increased by approximately 1.5-fold within 1 h of exposure to 10 Gy of X-rays, while the ATM protein levels had increased by 1.5−2.0-fold within 2 to 3 h of irradiation. The wild-type mRNA and protein levels both returned to their basal values fairly quickly after this time. The results obtained with the ATM+/− LCLs were quite different, however: neither the mRNA nor protein levels were found to have increased as a consequence of X-irradiation in any ATM+/− LCL. Twelve of the mutations in the ATM−−−− LCLs we used were truncating mutations, and we suspected that the corresponding truncated ATM proteins would be too labile to be detected by western blot analysis. However, five of the ATM−−−− LCLs produced mutant ATM proteins that were identical in molecular weight to the wild-type ATM protein. When cells from three of these five clones were exposed to X-rays, transcription of the mutant ATM genes appeared to reduce somewhat, as were the levels of protein being produced. These results suggest that the normal ATM gene responds to ionizing radiation by up-regulating its activity, whereas none of the mutant ATM genes we studied were able to respond in this way.

Key words: Ataxia-telangiectasia (AT) — X-Irradiation — ATM expression — Up-regulation — Lymphoblastoid cell lines (LCLs)
cultured cell lines in media which support their proliferation, and have therefore decided to examine the effects of radiation on ATM mRNA and protein levels in serum-starved cell lines, on the assumption that experiments on cultured cells treated in this way may provide us with a much more realistic idea of what can be expected to occur under physiological conditions in vivo.

Our experimental approach involved using cells that had been serum-starved for 48 h as controls or as suspensions for X-irradiation prior to measuring (1) ATM mRNA levels by RNase protection assays, and (2) ATM protein levels by polyclonal antibody dependent western blot analysis. We found that both the ATM mRNA and ATM protein levels did in fact increase after exposure to ionizing radiation in ATM+/− lymphoblastoid cell lines (LCLS), but that they did not appear to increase at all in the class of ATM−/− LCLS in which an ATM-sized (360 kDa) protein can still be detected by western blotting.

MATERIALS AND METHODS

Cell lines and their characteristics The ATM−−/− LCLS GM3350 and two ATM+/− LCLSs, GM3187 and GM3188, were purchased from the National Institute of General Medical Sciences, Camden, NJ. The ATM−−/− LCL AT10ABR was kindly provided by Dr. M. F. Lavin, Queensland Institute of Medical Research, Brisbane, Australia. Three ATM−−/− LCLSs (ATM01, ATF04, and ATF05), two ATM+/− LCLSs (HATM1OS and HATF05), and six ATM+/+ LCLSs (T.H., F.H., N.T., H.O., M.I., and N.N.) were established in our laboratory by superinfecting peripheral blood mononuclear cells with Epstein-Barr virus (EBV) obtained from the supernatant of marmoset (M. nemestrina) lymphoblastoid cell lines (LCLS), but that they did not appear to increase at all in the class of ATM−−/− LCLSs in which an ATM-sized (360 kDa) protein can still be detected by western blotting.

Table I. Cell Lines and ATM Gene Mutations

| Cell lines | ATM gene |
|------------|----------|
| ATM01      | no data  |
| AT10ABR    | no data  |
| GM3350     | no data  |
| GM3187     | 8152 del 117 |
| GM3188     | 8152 del 117 |
| HATF05     | no data  |
| HATM1OS    | 4612 del 165 |

gel (Pharmacia Biotech, Uppsala, Sweden). Crude sera were then bound in batch mode to this column. The flow-through was collected and the column washed in at least ten column volumes of buffer. Pure antibody was eluted in 1.0 ml of 0.1 M glycine-HCl (pH 2.8) and collected on ice into 800 ml of 1 M Tris-HCl (pH 8.8) to ensure that the final pH of the solution would be between 7.0 and 7.5. The resulting antibody preparation was then fractionated on a GPC3000SW column (TOSOH, Tokyo) and the immunoglobulin (Ig) fractions collected (anti-ATM3 Ab).

Protein extraction and western blot Cells were washed twice in phosphate-buffered saline (PBS), lysed in SDS (sodium dodecyl sulfate) solubilizing solution (10 mM Tris-HCl (pH 7.4), 2% SDS, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 20 μM Na-O-vanadate, 100 mM NaF, 0.1% NaN3) and sonicated. Protein concentrations were determined using the DC Protein assay (Bio-Rad Lab., Hercules, CA). Cellular extracts corresponding to 50 μg of protein were fractionated by 2–15% SDS-PAGE (polyacrylamide gel electrophoresis) and transferred to Immoblot PVDF membranes (Bio-Rad Lab.). Membranes were blocked for 1 h in PBS containing 7.5% nonfat dry milk, 1% BSA, 0.1% NaN3, and 0.1% Tween 20 and incubated overnight at room temperature with 1.5 μg/ml anti-ATM3 Ab or for 1 h with anti-β-actin Ab (mAb, Sigma) at a dilution of 1:1000. Membranes prepared in this way were then washed with PBS containing 7.5% nonfat dry milk, 1% BSA, 0.1% NaN3, and 0.1% Tween 20 and incubated at 37°C in a humidified 5% CO2 incubator unless otherwise specified. We have no knowledge of the germline mutations of the ATM gene in the ATM−−/− LCLSs. These LCLSs had one wild-type allele and one mutated allele (Table I). GM3187 and GM3188 carried the ATM mutation 8152 del 117 while HATM1OS carried the 4612 del 165 mutation. Nothing appears to be known about HATF05.

Antibody preparation and affinity purification The peptide NH2-GAHKRYPNDFSAF-COOH corresponding to amino acid residues 2786–2799 of the predicted ATM gene primary structure was synthesized and coupled to bovine serum albumin (BSA, Sigma, St. Louis, MI) prior to use as an antigen by injection into adult rabbits. Peptide synthesis and coupling to BSA were performed by the Peptide Institute Inc. (Osaka). The ATM protein could be detected in immune serum following the fourth boost. To prepare an affinity purification column, the peptide used as an antigen was attached to CNBr-activated Sepharose 4B.
were expressing as little ATM mRNA and/or protein as possible at the start of the experiment. Thus, rather than making use of exponentially growing cells as all previous workers appear to have done, we began by incubating each LCL in a relatively low concentration (0.05%) of FCS at 37°C for 48 h with the intention of accumulating as many of the lymphoblastoid cell population as possible at G0/G1. In practice, some 70–80% of the cells in the resulting populations turned out to be at G0/G1. (By contrast, an asynchronous culture could be expected to have 30–60% of its cells at G0/G1.) These partially-starved cell populations were then exposed to 5 or 10 Gy of X-rays at a dose rate of 1.21 Gy/min or to 50 or 100 Gy at a dose rate of 2.72 Gy/min at room temperature in an X-ray irradiator (WSI-250S, Shimadzu, Kyoto) equipped with 0.5-mm Al and 0.3-mm Cu filters at 220 kVp and 8 mA prior to being suspended in culture medium supplemented with 10% FCS at 37°C for the indicated times.

**Preparation of radio-labeled antisense RNA probe**

Total RNA was isolated using TRIZol reagent (GIBCO-BRL, Rockville, MD). ATM and β-actin cDNA fragments were prepared by reverse transcription PCR (RT-PCR) using total RNA as substrates. These cDNA fragments were then cloned into pGEM-T Easy Vector (Promega, Madison, WI). A 687-base long cDNA molecule corresponding to a segment covering bases 8520 to 9206 of the ATM gene (pATM), and a 379-base long cDNA molecule corresponding to bases 1352 to 1730 of the human β-actin gene (pAc) were identified.

Radio-labeled anti-sense RNA probes were synthesized in vitro using a transcription buffer (Promega) consisting of 10 mM dithiothreitol, 20 units of RNase inhibitor, 1 µg pATM or pAc linearized by digestion with a restriction enzyme BamH1, 1.85 MBq [α-32P]UTP with 12 µM UTP, 0.5 mM ATP, GTP and CTP and 20 units of T7 RNA polymerase in a total volume of 10 µl for 60 min at 37°C. Subsequently, five units of RNase-free DNase were added for 15-min incubation at 37°C. Labeled RNAs were precipitated after extraction with phenol/chloroform/isoamyl alcohol (25:24:1) solution.

**Ribonuclease protection assay**

Approximately 100 000 cpm and 10 000 cpm of the radio-labeled anti-sense RNA probes for ATM and β-actin, respectively, were added to 20 µg of total RNA to be tested in the hybridization buffer composed of 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl, and 80% formamide in a total volume of 10 µl for 60 min at 37°C. Subsequently, five units of RNase-free DNase were added for 15-min incubation at 37°C. Labeled RNAs were precipitated after extraction with phenol/chloroform/isoamyl alcohol (25:24:1) solution.

**Fig. 1. Western blot analysis of ATM gene product in representative ATM+/+, ATM+-/−, and ATM−/− LCLs.** Cell lysates were separated by 2–15% SDS-PAGE, transferred to membrane, and immunoblotted with anti-ATM3Ab. Cell lines: lane 1, T.H.; lane 2, N.T.; lane 3, GM3187; lane 4, GM3188; lane 5, ATF04; lane 6, ATF05; lane 7, AT10ABR; lane 8, GM3350; lane 9, ATM01.

**Fig. 2. ATM expression level after stimulation with 10% FCS.** LCLs were cultured in RPMI 1640 supplemented with 0.05% FCS at 37°C for 48 h and thereafter cultured in RPMI 1640 supplemented with 10% FCS for the indicated time. The same blot was reacted with antibody against ATM and β-actin. The band levels were quantified using densitometry. The amount of ATM protein was normalized to that of β-actin. The ratio of intensity was normalized to that of 0 h. Error bars are standard deviation of the mean from three cell lines. ATM+/+ LCLs (H.O., F.H., M.I.), ATM+/− LCLs (GM3187, GM 3188), ATM−/− LCLs (GM3350). ▲ ATM+/+ (n=3), ○ ATM+/− (n=2), ▲ ATM−/− (n=1).

**Fig. 3. Dose-response curve for an ATM+/+ LCL (H.O.) with X-irradiation.** Cultures were treated with different doses of X-radiation and harvested after 2 h. The ratio of intensity was normalized to that of 0 Gy. Error bars are standard deviation of the mean from three to four separate experiments.
RESULTS

Detection of ATM protein in LCLs  Fig. 1 shows representative results of western blot analysis of the ATM protein in wild-type and ATM mutation carrying LCLs. Using rabbit anti-ATM3 polyclonal Ab, we were able to detect a single protein with a molecular weight of approximately 350 kDa in ATM+/+ LCLs. Most of the ATM−/− LCL preparations we examined did not have this band, but three of them (ATM01, AT10ABR and GM3350) appeared to produce proteins that were all but identical in molecular weight to the normal ATM protein. No protein fragments were detected in any of these LCLs. Previous authors have also been unable to demonstrate the presence of truncated proteins in ATM−/− cells.16–18

Dose response and time course of ATM expression in ATM+/+ LCLs following X-irradiation  Lymphoblastoid cell lines that had been incubated with 0.05% FCS to induce partial serum starvation were released back into the cell cycle by refeeding them with complete media containing 10% FCS. Some 2 h after the FCS concentration had been elevated, the ATM protein levels had increased by 1.5- to 2-fold in each of the ATM+/+, ATM+/- and 350 kDa molecular weight protein-producing ATM−/− LCLs (Fig. 2). Following X-irradiation, the various individual ATM protein levels were found to have been elevated still further.

A typical dose response for ATM protein levels in ATM+/+ cells 2 h after exposure to X-rays is shown in Fig. 3. The protein levels continued to increase up to a dose of 100 Gy. Time-course experiments indicated that ATM levels would reach a peak some 2 to 3 h after exposure to 10 Gy of X-rays, and would return to basal levels by about 5 h (Fig. 4). In all subsequent experiments, we recorded the changes in ATM protein levels resulting from 10 Gy of X-rays dose in a post-irradiation incubation time of 2 h, since this appeared to maximize discrimination.

Effects of X-rays on ATM protein level  As summarized in Table II, by 2 h after exposure to 10 Gy of X-rays the ATM protein levels had increased substantially in ATM+/+ LCLs, remained the same in ATM+/- LCLs, and decreased in ATM−/− LCLs, all as compared with the levels found in their unirradiated but 10% FCS-stimu-

| Cell lines | Relative abundance of ATM protein levels |
|------------|-----------------------------------------|
| Normal     |                                         |
| N.T.       | 0.80±0.14                               |
| H.O.       | 0.90±0.08                               |
| M.I.       | 1.10±0.1                                |
| N.N.       | 0.94±0.07                               |
| mean±SD    | 1.00±0.06                              |

AT heterozygotes

| GM3188     | 0.51±0.01                               |
| HATM1OS    | 0.53±0.03                               |
| GM3187     | 0.52±0.02                               |
| HATF05     | 0.42±0.09                               |
| mean±SD    | 0.49±0.03                              |

AT homozygotes

| ATM01      | 1.24±0.24                               |
| AT10ABR    | 1.09±0.09                               |
| GM3350     | 1.23±0.23                               |
| mean±SD    | 0.50±0.14                              |

Two to four separate assays were performed on each LCL.

a) Unexposed LCLs.
b) LCLs exposed to 10 Gy ionizing radiation.
c) The ratio of protein levels in exposed to unexposed LCLs for each LCL.

* By the t test, the ratio of normal LCLs was statistically significant versus AT heterozygotes (P<0.01).

Fig. 4. Time-response curve for an ATM+/+ LCL (H.O.) after X-irradiation. Cultures were treated with 10 Gy X-irradiation and re-incubated for different time periods before harvest. The ratio of intensity was normalized to that of 0 h. Error bars are standard deviation of the mean from four separate experiments.
lated counterpart LCLs (Fig. 5). β-Actin expression was not altered to any significant extent by any of these treatments.

**Effects of X-rays on ATM mRNA levels** ATM mRNA levels were examined using an RNase protection assay involving a probe that contains the PI-3 kinase domain of the ATM gene (Fig. 6); the results are summarized in Fig. 7. The ATM mRNA levels were found to have increased slightly (1.4-fold) at 1 h in the ATM+/+ LCLs that had been exposed to 10 Gy of X-rays, but showed virtually no change in the similarly X-irradiated ATM+/− LCLs and had actually decreased slightly within 1 h of the exposure.
of their ATM−/− counterparts to 10 Gy of X-rays. Fig. 8 shows the time course of both ATM protein and mRNA levels in three different ATM+/+ LCLs. There is clear evidence of the post-irradiation increase in ATM mRNA levels occurring prior to there being any obvious increase in the levels of ATM protein. To our knowledge, this is the first suggestion that transient increases in both ATM mRNA levels and ATM protein levels occur in human cells as a result of their exposure to ionizing radiation.

DISCUSSION

The results reported here show that we can detect increased levels of both ATM mRNA and its protein product in normal human cells soon after they have been exposed to 10 Gy of X-rays. Previous workers reported that they detected no such increases. At least two factors may be important in explaining this discrepancy. The first is that subtle changes in ATM mRNA levels are always going to be difficult to detect by the northern blot technique, if only because the wild-type ATM transcript is extremely long (ca. 13 kb; see 7, 25) and its efficient transfer to filters is virtually impossible to accomplish on a routine basis. We adopted a different strategy in which we replaced the northern blotting step with an RNA protection assay in which the critical RNA/RNA hybridization reactions took place in solution rather than on solid surfaces. This method turned out to be much more sensitive than the northern blot method in the detection of its target ATM mRNA species, and should be extremely useful whenever it is necessary to quantify tiny amounts of any particular mRNA.

A second factor concerns our finding that ATM protein levels vary dramatically with cell-cycle stage. We could detect little or no ATM protein in peripheral blood mononuclear cells, and yet there appeared to be substantial amounts in phytohemagglutinin (PHA)-stimulated blast mononuclear cells. We were nonetheless able to detect significant quantities of ATM-specific mRNA in peripheral blood mononuclear cells whether or not they had previously been exposed to PHA (data not shown). Similar results have recently been reported by Fukao et al. Thus, ATM mRNA appears to be present in significant quantities throughout the cell cycle, but its protein product appears to be absent at G0 and reaches measurable levels only in G1 to S; taken together, these observations strongly suggest that synthesis of the ATM protein is regulated at the post-transcriptional level. Since our results reveal that ATM protein levels are not very high either before or after X-irradiation, we now suspect that it may only be possible to detect radiation-associated increases in ATM protein levels of the order of 2–3-fold if the experimental protocol involves the use of cells which are producing minimal quantities of the ATM protein at the start of the experiment. When we were attempting to use exponentially growing cultures of the ATM+/+ LCLs, ATM protein levels did not appear to change in any discernable way in response to X-irradiation. Thus cells that are currently producing minimal quantities of the ATM protein, such as those stalled at G0/G1 by incubating them in 0.05% FCS, may prove to be very well suited to the detection of radiation-associated changes in ATM protein levels. This may mean that exponentially growing cell populations are not suitable for use in attempts to detect radiation-induced changes in ATM protein synthesis; it may also explain why previous authors appear to have had so much difficulty in detecting radiation-related enhancements of ATM gene expression in their experiments.

Other features of the ATM transcriptional regulation may also be important. Thus, for example, the ATM sequence is organized in head-to-head configuration with the sequence of a second gene, NPAT/E14/CAND3, with which it shares a bi-directional promoter that contains CCAAT boxes and 4 consensus sites for the Sp1 transcription factor and whose transcription start site is only 550 base pairs away from the ATM start site. Sp1 is a candidate factor for common regulation of the activities of these two genes. Additional evidence that may be of some relevance includes a recent report by Gueven et al. which indicates that epidermal growth factor (EGF) increases radiosensitivity in both human fibroblasts and lymphoblasts and that this is associated with a controlled down-regulation of ATM. This down-regulation appears to occur at the level of transcription, and may be accompanied by a decrease in DNA-binding activity of the Sp1 transcription factor. Following pretreatment of these cells with EGF, Sp1 binding activity was found to decrease, but a subsequent exposure to radiation appeared to restore Sp1 binding activity to normal levels. Thus incubation of cells with EGF appeared to lead to down-regulation of ATM expression, while a subsequent radiation exposure appeared to restore intracellular ATM protein to constitutive levels. Although we did not measure Sp1 binding activity in our experiments, it may be worth investigating the possibility that down-regulation of ATM levels in ATM+/+ LCLs treated with serum starvation and their subsequent up-regulation in response to X-irradiation are simply manifestations of changes in Sp1 binding activity and hence in ATM transcription levels.

A key part of our study involved examining pre- and post-irradiation ATM mRNA and protein levels in ATM+/− and ATM−/− LCLs. The ATM+/− LCLs we examined showed 10–30% as much of the ATM protein as did the normal LCLs, and their radiosensitivities were intermediate between those of the normal and ATM−/− LCLs. The ATM−/− LCLs were clearly radiosensitive. The changes in expression level observed following X-irradiation of these heterozygous and homozygous mutant LCLs
differed from the changes detected in their wild-type equivalents, since neither the mRNA nor protein levels appeared to change in ATM+/− LCLs while both the mRNA and protein levels decreased significantly in ATM−/− LCLs in response to X-irradiation. This appears to be the first time comparative studies of all three genotypes have been carried out. We suspect that the differences in ATM protein and mRNA expression levels that were observed following X-irradiation of these various LCLs are a reflection of their differing radiosensitivities. Gueven et al. reported that EGF treatment of ATM−/− cells did not appear to increase their radiosensitivity. This may mean that transcriptional regulation by Sp1 is deficient in ATM−/− cells.

Our results suggest that regulation of ATM involves not only the activation of pre-existing protein molecules, but also alterations in the amounts of ATM protein that are being produced in response to a range of different stimuli. We have provided the first evidence that alterations in the amount of ATM protein produced can vary in response to serum-starvation and radiation exposure. Our findings indicate that the control of ATM gene expression and function after X-irradiation is likely to involve a degree of complexity that is only just beginning to be understood.

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