Participation of the human p53 3'UTR in translational repression and activation following γ -irradiation

Loning Fu and Samuel Benchimol¹

Ontario Cancer Institute/Princess Margaret Hospital, Department of Medical Biophysics, University of Toronto, 610 University Avenue, Toronto, Ontario, Canada M5G 2M9

¹Corresponding author

p53 protein levels have been shown to increase in a number of cells after treatment with genotoxic agents through a post-transcriptional mechanism. In γ -irradiated human cells, the accumulation of p53 protein is accompanied by an increase in the association of p53 mRNA with large polysomes without any change in the level of p53 mRNA. This redistribution of p53 mRNA on polysomes in response to irradiation is consistent with enhanced translational activity of p53 mRNA. We demonstrate that a region of the p53 3'-untranslated region (3'UTR) inhibits translation of a chimeric reporter mRNA *in vivo*. Induced elevation of reporter activity after γ -irradiation was seen in cells expressing chimeric reporter–p53 3'UTR transcripts. These data taken together demonstrate translational control of p53 gene expression after γ -irradiation and denote a previously unsuspected and novel role for the p53 3'UTR in controlling translation.

Keywords: γ -irradiation/p53/translational control/3'UTR

Introduction

The intracellular concentration of the p53 tumor suppressor protein increases in cells treated with a wide variety of DNA-damaging agent (Kastan *et al.*, 1991; Fritsche *et al.*, 1993; Lu and Lane, 1993). p53 protein appears to play a central role in the response to DNA damage by promoting cell cycle arrest in G1 or apoptosis (Kuerbitz *et al.*, 1992; Clarke *et al.*, 1993; Lowe *et al.*, 1993a,b). The ability of p53 to mediate these cellular processes is considered to be important for its tumor suppressor activity. Indeed, disruption of the p53 response pathway is associated with oncogenesis. While elevated levels of p53 protein are believed to be important to initiate the events that lead to G1 arrest or apoptosis after DNA damage, there is compelling evidence that post-translational modification of p53 protein is also required to activate the latent sequence-specific DNA-binding function of p53 (Hupp *et al.*, 1992, 1995; Jayaraman and Prives, 1995). The accumulation of p53 protein can be induced by the double-strand breaks in chromosomal DNA resulting from the introduction of a restriction endonuclease into cells (Lu and Lane, 1993; Nelson and Kastan, 1994). Hence, DNA strand breaks appear to be necessary and sufficient to initiate the events leading to the increased expression of p53 protein.

We have investigated the mechanism underlying the accumulation of p53 protein in γ -irradiated human cells

and have considered the possibility that the increased expression of p53 protein is regulated in part at the translational level. Our rationale is based on previous studies showing that p53 mRNA levels do not change after irradiation (Kastan *et al.*, 1991) and on the identification of a region in the 3'-untranslated region (3'UTR) of p53 mRNA that acts to repress translation of p53 mRNA and of a reporter RNA *in vitro* (Fu *et al.*, 1996). We present evidence showing that the increase in p53 protein that occurs in response to ionizing irradiation is achieved, at least in part, by enhanced translation of p53 mRNA. Furthermore, we demonstrate that a negative regulatory element, capable of inhibiting translation *in vivo*, resides on a 330 nucleotide region at the distal end of the human p53 3'UTR. The translational efficiency of chimeric reporter transcripts bearing the p53 3'UTR is enhanced by γ -irradiation. We propose that interaction between RNA-binding factors and the p53 3'UTR contributes to the regulation of human p53 mRNA translation.

Results

Repression of translation by the p53 3' UTR

We have shown previously that translation of synthetic human p53 transcripts in cell-free extracts is inhibited by a region at the distal end of the p53 3'UTR that contains an Alu-like repetitive element (Fu *et al.*, 1996). Deletion of the distal end of the p53 3'UTR increased the efficiency of translation without affecting RNA stability *in vitro*. Moreover, the addition of the distal end of the p53 3'UTR onto a reporter transcript inhibited its translation *in vitro* (Fu *et al.*, 1996). To investigate whether or not a negative regulatory domain resides within the p53 3'UTR that represses translation *in vivo*, we generated a series of chimeric luciferase reporter constructs that included: (i) the entire p53 3'UTR (pRLucRU); (ii) the proximal half of the 3'UTR that is missing the Alu-like element (pRLucAB); (iii) the distal half of the p53 3'UTR containing the Alu-like element (pRLucAlu); and (iv) the p53 3'UTR with an internal 348 nucleotide deletion that removes the Alu-like sequence (pRLucRU Δ) (Figure 1). Each of these plasmids contains the gene conferring resistance to G418 to allow selection of stable transfected clones. MCF-7 cells were electroporated with linearized plasmid DNA, and stable transfectants were selected for growth in G418. Northern blot analysis with a luciferase probe allowed a determination of the relative levels of expression obtained in clones stably transfected with each type of chimeric luciferase plasmid (Figure 2). Differences in luciferase RNA abundance were evident among clones, presumably due to copy number and site of integration of the plasmid. Fifteen independent clones, selected only on the basis of having detectable luciferase RNA, were studied further and are described in Table I. The mean

Fig. 1. Plasmids expressing chimeric luciferase-p53 3'UTR transcripts. All constructs contain the firefly luciferase-coding region as a reporter gene and either the entire human p53 3'UTR (pRLucRU), the proximal segment of the p53 3'UTR (pRLucAB), the distal segment of the p53 3'UTR (pRLucAlu) or an internal deletion of part of the p53 3'UTR (pRLucRUΔA). Together, the proximal AB segment and the distal Alu segment encompass the entire length of the p53 3'UTR (RU segment). Transcription of the luciferase-coding region is under the control of the RSV long terminal repeat and the polyadenylation signal (pA) is derived from the bovine growth hormone (BGH) gene.

relative abundance of luciferase RNA in each of the clones was determined by repeated Northern blotting after normalization for the amount of RNA present in each sample (Table I). Next, we compared luciferase activity in each of the 15 clones. Luciferase activity was measured in triplicate in cell extracts obtained in at least three experiments (Table I). Measurements of luciferase RNA abundance and luciferase activity allowed an assessment of the translational efficiency of each chimeric luciferase transcript. The ratio of luciferase activity/luciferase mRNA for each of the clones is shown in Table I. These ratios were lower in all clones where the chimeric luciferase RNA contained the Alu-like sequence (A clones and RU clones) compared with clones that expressed chimeric luciferase transcripts without the Alu-like sequence (AB clones and RUΔA clones). The distribution of the clones into two distinct groups on the basis of luciferase activity after correction for luciferase mRNA abundance is depicted in Figure 3. Comparison of mean values of luciferase activity in the two groups revealed a 4.6-fold difference in luciferase activity. A highly significant correlation was found between low luciferase activity and the presence of the Alu-like element on the chimeric luciferase transcript ($P < 0.001$, t -test).

The steady-state level of luciferase protein was measured in three clones to ensure that the differences in luciferase activity reflected luciferase protein levels. The Western immunoblot shown in Figure 4 was performed with equal amounts of protein extract and was probed sequentially with antibodies against luciferase and human β 1 tubulin to ensure equivalent loading of protein in each lane. The blot shows a higher level of luciferase protein

Fig. 2. The plasmids described in Figure 1 were used to transfect MCF-7 cells. The clones obtained and the corresponding plasmids used for transfection are listed in Table I. Clone RD4 was obtained after transfection of MCF-7 cells with the parental, empty vector pRDNA3. RNA was isolated from untreated cells (-IR) or 1 h after treatment with 6 Gy γ -irradiation (+IR) on three separate occasions and subjected to Northern blot analysis. The blots were hybridized sequentially with 32 P-labeled luciferase and L35 or GAPDH cDNA probes. One representative Northern blot is shown. Luciferase RNA levels were quantitated using a PhosphorImager and ImageQuant Software (Molecular Dynamics, Incorporated) and normalized to the L35 or GAPDH signal. The relative levels of luciferase mRNA present in each of the clones is entered in Table I.

in clone RUΔA14 compared with clones RU11 and A10. When differences in the abundance of luciferase RNA in these cells were considered (Figure 4), we estimated that the translational efficiency of the chimeric luciferase transcript expressed in clone RUΔA14 was between 5- and 6-fold higher than that of the luciferase transcripts expressed in clones RU11 and A10 (Figure 4). These findings are consistent with the previous set of results based on measurement of luciferase activity. Together, these data indicate that chimeric luciferase mRNAs containing sequences derived from the distal end of the human p53 3'UTR appear to be translated less efficiently than luciferase transcripts missing these sequences. We conclude that a negative regulatory element, capable of inhibiting translation, resides on a 330 nucleotide region at the distal end of the human p53 3'UTR.

γ -Irradiation increases the level of p53 protein through a post-transcriptional mechanism in OCI/AML-3 and OCI/AML-4 cell lines

The accumulation of p53 protein that occurs in cells exposed to γ -irradiation has been reported to occur through a post-transcriptional mechanism. Since the p53 3'UTR mediates repression of translation, we wished to know if this repression is relieved in irradiated cells. Two AML cell lines, OCI/AML-3 and OCI/AML-4, established from the primary blasts of two patients with acute myelogenous leukemia (AML) were chosen for this study (Wang *et al.*, 1989). Both cell lines contain endogenous wild-type p53 genes as determined by RT-PCR and direct DNA sequence analysis of the entire coding region and the 5' and 3'UTRs (Fu *et al.*, 1996). Following exposure of OCI/AML-3 and OCI/AML-4 cells to 6 Gy of γ -radiation, the amount of p53 protein in a fixed number of cells (2×10^6) was measured by Western immunoblot-enhanced chemiluminescence (ECL) analysis at different times after irradiation (Figure 5A). A rapid and pronounced increase in the level of p53 protein was observed in irradiated cells. The accumulation of p53 protein in OCI/AML-3 cells was transient, and the pre-irradiation basal level was re-established ~5 h after exposure to radiation. In OCI/AML-4 cells, the level of p53 protein continued to increase over a 5 h period. Using a quantitative Western blot

Table I. The p53 3'UTR reduces translation efficiency and γ -irradiation stimulates translation of chimeric luciferase mRNA in MCF-7 clones

Clone	Plasmid	Relative abundance of luciferase RNA		Luciferase activity		Luciferase activity/Luciferase RNA		
		-IR	+IR	-IR	+IR	-IR	+IR	Fold increase
RU9	pRLucRU	1.0	0.7	7716	10 230	7716	14 614	1.9
RU11	pRLucRU	3.8	1.9	24 479	29 812	6441	15 690	2.4
RU12	pRLucRU	0.9	0.8	8080	11 124	8977	13 905	1.6
A10	pRLucAlu	6.1	3.2	45 873	43 887	7520	13 715	1.8
A16	pRLucAlu	4.7	ND	40 954	ND	8713	ND	
A17	pRLucAlu	3.8	2.5	32 221	40 474	8479	16 189	1.9
A20	pRLucAlu	2.8	ND	21 325	ND	7616	ND	
AB11	pRLucAB	6.3	7.0	215 392	209 632	34 173	29 947	0.9
AB29	pRLucAB	3.6	3.7	176 757	180 254	49 099	48 717	1.0
RUAA4	pRLucRUAA	0.5	ND	14 971	ND	29 942	ND	
RUAA13	pRLucRUAA	6.1	ND	270 827	ND	44 398	ND	
RUAA14	pRLucRUAA	6.8	7.7	261 017	265 486	38 385	34 479	0.9
RUAA41	pRLucRUAA	1.5	ND	46 797	ND	31 198	ND	
RUAA46	pRLucRUAA	4.8	ND	164 474	ND	34 265	ND	
RUAA50	pRLucRUAA	6.3	ND	200 188	155 798	31 776	ND	

RNA quantitation is the result of data obtained from a Molecular Dynamics PhosphorImager and is the mean derived from three Northern blots for the RUAA14 clone and for the RU, A and AB series of clones. The luciferase RNA level in the remaining RUAA clones was measured once. All values were normalized to L35 rRNA to ensure equivalent loading of RNA on the blots. Reprobing the filters with a GAPDH cDNA probe gave similar results. The ratio of the luciferase RNA signal to the L35 signal in RU9 cells was arbitrarily set to 1.0. Luciferase activity (relative light units per μ g protein) represents the mean level derived from at least three independent experiments. The ratio of luciferase activity/luciferase mRNA provides a measure of translational efficiency. Cells were either left untreated (-IR) or irradiated with a dose of 6 Gy (+IR). After irradiation, cells were incubated at 37°C for 1 h prior to measurement of luciferase RNA and luciferase activity.

procedure in which bound antibody was detected directly with [125 I]protein A, the increase in the steady-state level of p53 protein in irradiated OCI/AML-3 cells was ~26-fold after 2 h (data not shown). The accumulation of p53 protein was not accompanied by any change in the level of p53 mRNA (Figure 5B). Increased levels of WAF1/CIP1 mRNA became evident 1–2 h after irradiation (Figure 5B).

Increases in the level of p53 protein following γ -irradiation were also seen in the wild-type p53-expressing human breast carcinoma cell line MCF-7 (Takahashi *et al.*, 1993) and in the mutant p53-expressing human erythroleukemia cell line OCI-M2 (Papayannopoulou *et al.*, 1988; Slingerland *et al.*, 1991) (Figure 5C). The dissimilar kinetics of p53 protein accumulation in response to irradiation displayed by the different cell lines probably indicates an influence of the cellular environment on p53 protein induction. As in OCI/AML-3 and OCI/AML-4 cells, no change in p53 mRNA was observed in irradiated MCF-7 and OCI-M2 cells by Northern blotting (data not shown).

γ -Irradiation increases the association of p53 mRNA with polysomes

To determine if γ -irradiation stimulates translation of p53 mRNA, we examined the association of p53 mRNA with polysomes in OCI/AML-3 and OCI/AML-4 cells. If p53 mRNA is more translationally active after irradiation, one should expect p53 mRNA from irradiated OCI/AML-3 and OCI/AML-4 cells to be associated with polysomes to a greater extent than p53 mRNA from non-irradiated cells. Cells, either untreated or irradiated with 6 Gy, were incubated at 37°C for 45 min, collected and lysed in the presence of cycloheximide and $MgCl_2$, which stabilize the association of ribosomes with mRNA. The lysates were sedimented through a linear sucrose gradient and fractions were collected. RNA was recovered from each fraction and subjected to dot-blot hybridization analysis with a

Fig. 3. Luciferase activity in MCF-7-derived clones expressing the luciferase-coding region attached to different segments of the p53 3'UTR. All values for luciferase activity were corrected for differences in chimeric luciferase mRNA abundance using quantitative Northern blotting as indicated in Table I. A clones (A10, A16, A17, A20), RU clones (RU9, RU11, RU12), AB clones (AB11, AB29), RUAA clones (RUAA4, RUAA13, RUAA14, RUAA41, RUAA46, RUAA50). The horizontal lines represent the group mean values.

32 P-labeled p53 cDNA probe to determine the amount of p53 mRNA in each of the fractions. The filters subsequently were stripped and reprobed with a 32 P-labeled

Fig. 4. The p53 3' UTR contains a negative regulatory element that reduces the translational efficiency of chimeric luciferase mRNA. Results from analyses of MCF-7-derived clones transfected with empty backbone vector pRDNA₃ (RD4), pRLucAlu (A10), pRLucRU (RU11) or pRLucRUΔA (RUΔA14). The amount of luciferase protein was determined by SDS-PAGE and Western immunoblotting analyses using an antibody directed against luciferase. One hundred μg of total protein was loaded on each of the lanes of the gel. Filters were reprobbed with β-tubulin antibodies to ensure equivalent protein loading on the gel. Chimeric luciferase mRNA was visualized by Northern blotting and quantitated by phosphorimage analyses. The Northern blot was reprobbed with L35 cDNA to correct for differences in RNA loading. The relative translational efficiency of each chimeric luciferase mRNA transcript (luciferase protein/luciferase RNA) is presented at the bottom of the figure normalized to the level of luciferase protein measured in the A10 clone. This figure was generated using samples derived from a single representative determination analyzed for both luciferase mRNA and luciferase protein simultaneously. The weak luciferase signal detected in RD4 cells by Western blotting probably represents an endogenous protein that is cross-reactive with the luciferase antibody. This background signal was subtracted from all the lanes.

cDNA probe that recognizes ribosomal protein L35 mRNA. The results from three independent experiments for each of OCI/AML-3 and OCI/AML-4 are summarized in Figure 6. Representative dot-blots are shown in the inserts of Figure 6. A shift in the distribution of p53 mRNA from monosomes to polysomes was seen in the irradiated cells. In non-irradiated OCI/AML-3 cells, 38% of the p53 mRNA was found in fractions 5–10 containing polysomes; in irradiated OCI/AML-3 cells, 48% of the p53 mRNA was found in fractions containing polysomes (Figure 6A). In non-irradiated OCI/AML-4 cells, 45% of p53 mRNA was found in the polysome fractions while in the irradiated sample, 86% of the p53 mRNA was found associated with polysomes (Figure 6B). For each of the cell lines, the total amount of p53 RNA recovered from the gradients of the irradiated and non-irradiated samples was similar; only the distribution changed. As an internal control for RNA recovery and for confirmation that different gradients could be compared, the distribution of L35 RNA was determined in irradiated and non-irradiated cells (Figure 6, insert). In both OCI/AML-3 and OCI/AML-4 cells, 70% of the L35 mRNA was present in fractions 5–10 containing polysomes both before and after irradiation. These data indicate that p53 mRNA is associated with larger polysomes in irradiated cells compared with non-irradiated cells and, hence, is likely to be more translationally active. The larger shift in the distribution of p53 RNA in irradiated OCI/AML-4 compared with irradiated OCI/AML-3 could reflect the lower abundance of p53 RNA in OCI/AML-4 compared with OCI/AML-3 (Figure 5), even though both cell lines

Fig. 5. Post-transcriptional regulation of p53 protein expression in γ-irradiated cells. p53 protein accumulation in OCI/AML-3 and OCI/AML-4 cells (A) and in MCF-7 and OCI-M2 cells (C) exposed to γ-radiation. Cell extracts prepared at different time points after γ-irradiation with a dose of 6 Gy were fractionated by SDS-PAGE and immunoblotted separately with monoclonal antibodies that recognize human p53 (PAb1801) and β1 tubulin. Immune complexes were detected by enhanced chemiluminescence. (B) Expression of p53 and WAF1 RNA in γ-irradiated AML cells. Samples of total RNA (20 μg) taken at different times after exposure to 6 Gy of γ-irradiation were fractionated on agarose-formaldehyde gels, transferred to nitrocellulose and hybridized sequentially with ³²P-labeled cDNA probes for human p53, WAF1 and human ribosomal protein L35 as indicated. The lanes labeled control contain protein or RNA prepared from non-irradiated cells.

have similar levels of p53 protein prior to irradiation. Recruitment of a relatively large proportion of the p53 RNA into polysomes may be required to increase the level of p53 protein in OCI/AML-4 cells. Alternatively, differences in the abundance of translational activators in the two cells may restrict the extent of ribosome loading on mRNA templates.

Changes in the distribution of RNA on polysomes, while providing compelling evidence in support of translation regulation, cannot be equated readily with changes in actual rates of translation. As a result, we cannot be certain that the increased association of p53 mRNA with ribosomes is sufficient to account for the entire increase in p53 protein. Hence, these data do not exclude the possibility that post-translational changes affecting p53 protein stability might also occur in irradiated cells and contribute to the increase in p53 protein. Rates of protein synthesis and rates of protein degradation are determined commonly by metabolic labeling and pulse-chase procedures, respectively. However, neither of these isotopic procedures is suitable for p53. Recent reports indicate that metabolic labeling of cells with [³H]thymidine or [³⁵S]methionine alters the level of p53 protein expression (Dover *et al.*, 1994; YeARGIN and Haas, 1995) and, hence, cannot be used to measure p53 protein synthetic rates.

Fig. 6. Increased association of p53 mRNA with ribosomes in γ -irradiated cells. The distribution of p53 mRNA and human ribosomal protein L35 mRNA on polysomes was determined in OCI/AML-3 (A) and in OCI/AML-4 cells (B) prior to and 45 min after γ -irradiation (6 Gy). Cell extracts were prepared from untreated and irradiated OCI/AML-3 and OCI/AML-4 cells in the presence of cycloheximide and MgCl_2 . Polysomes were fractionated by sedimentation on 15–50% linear sucrose gradients as described in Materials and methods. RNA was extracted from each of the fractions and dot-blot hybridization analysis was used to determine the amount of p53 and L35 ribosomal protein transcripts in each fraction of the gradient. Representative dot-blots are shown in the inserts. RNA levels were quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics, Incorporated). The mean distribution of p53 mRNA from three separate experiments is summarized on each bar chart; error bars represent the standard error of the mean. The gradients were calibrated with polysomes prepared from OCI/AML-3 cells in exactly the same way except for an additional purification step involving the precipitation of the polysomes with 100 mM MgCl_2 for 1 h on ice prior to sucrose gradient sedimentation. The distribution of the polysomes on the gradient was monitored by absorbance at 254 nm. The positions of free ribosomes, monosomes and polysomes are indicated.

γ -Irradiation partially relieves the inhibition of translation mediated by the p53 3'UTR in chimeric luciferase mRNA

To determine if the translational repression mediated by the p53 3'UTR could be relieved by γ -irradiation, the MCF-7 clones expressing chimeric luciferase transcripts were irradiated with a dose of 6 Gy of γ -rays. One hour after irradiation, luciferase RNA and luciferase activity were measured. The luciferase activity was normalized

Fig. 7. Accumulation of luciferase protein in response to γ -irradiation in clones expressing chimeric luciferase mRNA transcripts containing the distal end of the p53 3'UTR. Cells were irradiated (6 Gy) and extracts were prepared for Western immunoblotting at different times after irradiation. A representative Western immunoblot in which 100 μg of protein were loaded in each lane of the polyacrylamide gel is shown at the top. Mean values obtained from four experiments for RD4 and RUΔA14 cells, and from three experiments for RU11 and A10 cells are summarized in the histogram below. In these analyses, luciferase protein levels were not adjusted for differences in luciferase mRNA abundance. Error bars represent the standard error of the mean.

with respect to luciferase RNA abundance. The data are summarized in Table I and are presented alongside the untreated samples which were processed in parallel. Increased luciferase activity following γ -irradiation was observed only in the A and RU series of clones expressing the luciferase–distal p53 3'UTR transcript or the luciferase–p53 3'UTR transcript, respectively. In contrast, the two AB clones expressing luciferase–proximal p53 3'UTR RNA showed no increase in luciferase activity after γ -irradiation.

To confirm this finding, we measured the level of luciferase protein in clone A10 (expressing luciferase RNA with the distal end of the p53 3'UTR), clone RU11 (expressing luciferase RNA fused to the entire length of the p53 3'UTR) and clone RUΔA14 (expressing luciferase RNA with a deletion of the Alu-like element present in the p53 3'UTR). As shown earlier, clones A10 and RUΔA14 express similar levels of chimeric luciferase RNA (Table I) but different levels of luciferase protein (Figure 4). These clones were irradiated with a dose of 6 Gy and the level of luciferase protein was determined by Western blot analysis using cell extracts that were prepared at different times following irradiation. The data are summarized in Figure 7 and demonstrate that irradiation stimulates accumulation of luciferase protein ~2-fold in clones A10 and RU11 but not in clone RUΔA14. This is entirely consistent with the stimulation of luciferase

activity that was seen in these cells after irradiation. We believe that the increase in luciferase protein in the irradiated A10 and RU11 cells is attributable to enhanced translation rather than enhanced mRNA stability or enhanced luciferase protein stability for the following reasons: (i) the luciferase plasmids used in this study produce mRNAs that contain the identical coding region of luciferase; (ii) the clones being compared were derived from a common parental cell line, MCF-7, and may be considered isogenic; and (iii) the abundance of luciferase RNA did not increase in irradiated A10 and RU11 cells, rather a decrease in luciferase RNA was observed (Table I; see below). These results demonstrate that γ -irradiation enhances translation of human p53 transcripts and of heterologous transcripts bearing the distal end of the p53 3'UTR.

Discussion

Our results lead to three main conclusions about the regulation of human p53 gene expression. First, a 330 nucleotide region at the distal end of the p53 3'UTR contains a regulatory element that represses translation *in vivo*. This region of the p53 3'UTR contains an Alu-like element and previously was shown to repress p53 RNA translation *in vitro* (Fu *et al.*, 1996). Second, the increase in p53 protein that occurs in response to ionizing irradiation is achieved, at least in part, by translational control of p53 mRNA. Third, the p53 3'UTR is involved in modulating translational activity after exposure to ionizing radiation.

How does the p53 3'UTR inhibit translation? While there is good evidence that sequences within the 3'UTR of numerous transcripts determine mRNA stability, poly(A) tail length (Huarte *et al.*, 1992; Wickens, 1993; Sheets *et al.*, 1994) and mRNA localization (Gavis and Lehmann, 1994), and hence influence translation indirectly, recent studies indicate a more direct involvement of the 3'UTR in translational control (Jackson, 1993; Sonenberg, 1994; Standart and Jackson, 1994). In some cases, specific protein-RNA interactions at the 3' end of mRNA molecules have been shown to inhibit the initiation of translation at the 5' end (Kwon and Hecht, 1993; Kwon *et al.*, 1993; Minich *et al.*, 1993; Ostareck-Lederer *et al.*, 1994; Wolffe, 1994; Robbie *et al.*, 1995).

The Alu-like sequence present in human p53 mRNA is in the reverse transcriptional orientation with respect to the p53 gene (Matlashewski *et al.*, 1984). When the p53 3'UTR is analyzed by the FOLD program of the GCG Sequence Analysis Software Package, the Alu-like element is predicted to form an independent and stable secondary structure. In the presence of a poly(A) tail, the secondary structure formed by the Alu-like element is predicted to remain essentially intact except that a 50 nucleotide U-rich sequence at the 5' boundary of the Alu-like sequence will interact with the poly(A) tail. The extended base pairing between U and A residues will further stabilize the secondary structure formed by the Alu-like element. Our working hypothesis for the translational regulation of p53 RNA by its 3'UTR is centered around this structural motif. This structural motif, either alone or in complex with an RNA-binding (repressor) protein prevents the initiation of translation. Translation may be activated upon

interaction of an RNA-binding protein (activator) with the regulatory element present in the 3'UTR or by removal of the repressor protein.

In general, the luciferase-containing clones that contain the distal end of the p53 3'UTR fused to luciferase-coding sequences (A clones, RU clones) show only a 2-fold increase in the level of luciferase activity or luciferase protein after γ -irradiation. Upon irradiation, these clones do not attain the level of luciferase that is seen in the non-irradiated AB and RU Δ A series of clones where the luciferase RNA lacks the distal end of the p53 3'UTR. Hence, the inhibition of translation that is mediated by the p53 3'UTR in chimeric luciferase RNA is not completely relieved by γ -irradiation. While the p53 3'UTR alone appears to be sufficient to inhibit translation, activation of translation may involve additional sequences in the 5'UTR or coding region of p53 mRNA. It is also possible that the p53 3'UTR sequences present on the chimeric luciferase transcripts compete with endogenous p53 transcripts for RNA-binding factors that are required for the stimulation of p53 mRNA translation in irradiated cells. In preliminary experiments to investigate this possibility, we see far less accumulation of p53 protein in irradiated RU11 cells than in control RD4 that lack chimeric luciferase transcripts.

The results presented in Table I indicate that the abundance of the hybrid luciferase RNA decreased following irradiation in clones expressing the entire p53 3'UTR or the distal end of the p53 3'UTR fused to luciferase. In contrast, the clones expressing chimeric luciferase RNA containing the proximal end of the p53 3'UTR or the p53 3'UTR missing the Alu sequence showed either no change (clone AB29) or a slight increase in the level of luciferase RNA (clone AB11, RU Δ A14). It is unlikely that the Rous sarcoma virus (RSV) promoter is subject to regulation after irradiation since the RSV promoter is controlling expression of the chimeric luciferase gene in clones AB11, AB29 and RU Δ A14, and these clones show no decrease in the level of luciferase RNA after irradiation. One possibility is that the actively translated chimeric transcripts are destabilized and degraded. A number of studies have suggested an inverse relationship between mRNA translation and mRNA stability (Peltz and Jacobson, 1992; Ross, 1995). Actively translated mRNAs, through their association with ribosomes, may become susceptible to a ribosome-associated degradation process and exhibit increased decay in comparison with non-translated mRNAs (Adamou and Bag, 1992; Kypides *et al.*, 1995). Alternatively, the process of mRNA unfolding during translation might disrupt structural features that are required for mRNA stability and accelerate mRNA turnover (Aharon and Schneider, 1993; Weiss and Liebhauer, 1995). In this regard, we note that the level of p53 mRNA does not change in irradiated cells even though we present evidence consistent with translational activation. The possibility that the p53 promoter is activated by genotoxic agents including irradiation (Sun *et al.*, 1995) and that an increased rate of transcription is balanced by increased degradation needs to be explored further.

Purified mouse p53 protein has been shown to inhibit translation of murine p53 RNA in reticulocyte lysates through its ability to bind tightly to a predicted stable stem-loop structure in the 5'UTR of its own mRNA

(Mosner *et al.*, 1995). Translocation of p53 protein into the nucleus after DNA damage has been proposed to relieve the inhibition of p53 protein synthesis and to provide a means for rapidly increasing p53 protein levels (Mosner *et al.*, 1995). This model, while appropriate for murine p53, cannot explain the increase in p53 protein after DNA damage in human cells; the 5'UTR of human p53 mRNA is much smaller than that of mouse p53 RNA and is missing sequences capable of forming a stable secondary structure (Tuck and Crawford, 1989; Fu *et al.*, 1996).

In contrast to our findings with γ -irradiation, UV irradiation of mouse cells was shown, by pulse-chase analysis, to increase the level of p53 protein through a post-translational mechanism involving increased protein stability (Maltzman and Czyzyk, 1984). In addition, the kinetics of p53 protein induction by UV and γ -rays are distinct (Lu and Lane, 1993). It will be important to determine if the mechanism underlying p53 protein accumulation in response to DNA-damaging agents is dependent on the agent used and the type of DNA damage inflicted, and whether or not human and rodent cells utilize different mechanisms to regulate p53 expression in response to DNA damage.

Materials and methods

Cells and culture conditions

The OCI/AML-3 and OCI/AML-4 cell lines were established from the primary leukemic blasts obtained from two patients with AML. OCI/AML-3 cells (Wang *et al.*, 1989), OCI-M2 cells (Papayannopoulou *et al.*, 1988) and MCF-7 cells (ATCC) cells were grown in α -minimal essential medium (α -MEM) containing 10% fetal calf serum (FCS); OCI/AML-4 cells were maintained in the same medium supplemented with 10% conditional medium obtained from the 5637 bladder carcinoma cell line (Wang *et al.*, 1989).

Plasmids

The cytomegalovirus (CMV) promoter in plasmid pCDNA₃ (Invitrogen) was replaced with the RSV long terminal repeat (LTR) promoter from plasmid pREP₄ (Invitrogen) to generate plasmid pRDNA₃. The pRDNA₃ expression plasmid carries a neomycin resistance gene driven by the SV40 early promoter. The pRLuc plasmid was constructed by inserting the *HindIII*–*BamHI* DNA fragment containing the firefly luciferase cDNA sequence from plasmid pJD206 (de Wet *et al.*, 1987) into the multiple cloning site immediately following the RSV LTR in plasmid pRDNA₃. Four plasmids were constructed in which different portions of the human p53 3'UTR from plasmid p2516 (Fu *et al.*, 1996), which contains nearly full-length human p53 cDNA, were inserted immediately downstream of the luciferase cDNA sequence in the multiple cloning site present in plasmid pRLuc. Plasmid pRLucRU contains the entire p53 3'UTR (nucleotides 1270–2516 from plasmid p2516) fused to luciferase; plasmid pRLucAB contains the proximal segment of the p53 3'UTR (nucleotides 1270–2034 from plasmid p2516) fused to luciferase; plasmid pRLucAlu contains the distal segment of the p53 3'UTR (nucleotides 2034–2516 from plasmid p2516) fused to luciferase; and plasmid pRLucRUAA contains the full-length p53 3'UTR with an internal deletion (between nucleotides 2038 and 2386 from plasmid p2516) that removes the Alu-like sequence. The fusion transcripts expressed from these plasmids will have identical 5' ends, identical luciferase-coding sequences and distinct 3' ends derived from different regions of the human p53 3'UTR. The polyadenylation site present on these plasmids was derived from the bovine growth hormone gene.

DNA transfection

Subconfluent cultures of MCF-7 cells (5×10^6 cells) were trypsinized and washed three times with α -MEM without FCS and resuspended in ice-cold α -MEM containing 10 μ g of linearized plasmid DNA. Plasmids pRDNA₃, pRLucAB and pRLucRUAA were linearized with *BglII*; pRLucRU and pRLucAlu were linearized with *ScaI*. The cells were kept

on ice for 10 min prior to electroporation using a Bio-Rad electroporator at 250 V and 960 μ F. After electroporation, the cells were kept on ice for a further 10–15 min, resuspended in α -MEM containing 10% FCS and incubated at 37°C. Forty eight hours after electroporation, the culture medium was replaced with fresh medium containing geneticin/G418 (Gibco) at a final concentration of 0.4 mg/ml. After 4 weeks of selection, the G418-resistant clones were transferred to 6-well plates; the cells were dispersed and grown in α -MEM containing 10% FCS and 0.2 mg/ml of G418.

γ -Irradiation

The leukemia cell lines were irradiated in their flasks at a density of 5×10^5 cells/ml at room temperature with a Co⁶⁰- γ -irradiator for an appropriate length of time to deliver a dose of 6 Gy. The MCF-7 cells and all of the MCF-7-derived transfected clones were irradiated in their culture dishes under subconfluent growth conditions. Immediately after irradiation, the cells were cultured at 37°C and collected at various time points for further analysis.

Western blot analysis

OCI/AML-3 and OCI/AML-4 cells (2×10^6) and OCI-M2 cells (0.2×10^6) were pelleted and lysed directly in an equal volume of 2 $\times$ SDS sample buffer (4% SDS, 20% glycerol, 50 mM Tris pH 6.8, 0.2% bromophenol blue, 0.2 M dithiothreitol). The MCF-7 cells (2×10^6) were lysed directly in their culture dishes using 2 $\times$ SDS sample buffer. Samples were passed through a 20 gauge syringe needle to shear genomic DNA and heated for 10 min at 100°C prior to electrophoresis through a polyacrylamide gel containing 10% SDS. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane and the abundance of p53 protein was estimated by immunoblotting with the PAb1801 mouse monoclonal antibody against human p53 (Banks *et al.*, 1986). Immune complexes were visualized by ECL. Membranes were stripped and reprobed with antibodies against human β 1 tubulin isotype I and II (Sigma). For the analysis of luciferase protein, cells were lysed directly in 2 $\times$ SDS sample buffer and 100 μ g of protein was applied to each well of the polyacrylamide gel. After transfer, filters were probed with a monoclonal antibody against firefly luciferase (Promega) and reprobed with the β 1 tubulin antibody.

Northern blot analysis

Total RNA was isolated from untreated and irradiated cells at different times after exposure to 6 Gy of γ -radiation. The RNA was fractionated on a 1% agarose gel containing 6% formaldehyde, transferred to nitrocellulose and hybridized with ³²P-labeled cDNA probes for human p53 (Harlow *et al.*, 1985) or firefly luciferase (de Wet *et al.*, 1987). The filters were stripped and reprobed with ³²P-labeled cDNA probes for human ribosomal protein L35 (Herzog *et al.*, 1990), human WAF1 (El-Deiry *et al.*, 1993) or mouse GAPDH (Fort *et al.*, 1985). The filters were exposed to DuPont reflection film. RNA levels were quantitated using a PhosphorImager and ImageQuant Software (Molecular Dynamics, Incorporated).

Polysome analysis

Polysomes were isolated as described (Fu *et al.*, 1996). Briefly, cell extracts were prepared from untreated and irradiated OCI/AML-3 and OCI/AML-4 cells in the presence of cycloheximide and MgCl₂. For the irradiated samples, extracts were prepared 45 min after exposure to 6 Gy of γ -irradiation. Polysomes were fractionated by centrifugation over a 15–50% linear sucrose gradient. Ten fractions of equal volume were collected and the RNA was extracted from each of the fractions, precipitated with ethanol and resuspended in 200 μ l of diethylpyrocarbonate-treated water. The amount of p53 mRNA in each fraction (100 μ l of the RNA sample) was determined by dot-blot hybridization analysis using a ³²P-labeled human p53 cDNA probe (Harlow *et al.*, 1985). The blot was stripped and reprobed with a human ribosomal protein L35 cDNA probe (Herzog *et al.*, 1990). The gradients were calibrated with polysomes prepared from OCI/AML-3 cells in exactly the same way except for an additional purification step involving the precipitation of the polysomes with 100 mM MgCl₂ for 1 h on ice prior to sucrose gradient sedimentation. The distribution of the polysomes on the gradient was monitored by absorbance at 254 nm.

Luciferase assay

Luciferase activity was measured in untreated or irradiated cells, 1 h after exposure to 6 Gy of γ -radiation by the detection method recommended by the supplier of the Luciferase Assay System (Promega). Cells in 55 mm culture dishes were washed twice in phosphate-buffered saline and lysed

with 250 µl of Cell Culture Lysis Reagent (Promega). Cells were scraped from the culture dishes, transferred to centrifuge tubes and spun briefly to remove debris. Total protein concentration in the cell extracts was determined as described by Bradford (1976) using the dye reagent purchased from Bio-Rad Laboratories and bovine serum albumin as the standard. Luciferase activity was measured on a LB9507 luminometer (Fisher Scientific Limited) using 5 or 25 µg of cell extract. The intensity of light emission was assayed by integration over a 5 s interval after the injection of 5.3 µM ATP and 4.7 µM luciferin.

Acknowledgements

We thank S.Minkin for his advice regarding the statistical analysis of data, S.Chung and W.Ma for technical assistance, and our colleagues in the laboratory for many useful and spirited discussions. This work was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada.

References

- Adamou,J. and Bag,J. (1992) Alteration of translation and stability of mRNA for the poly(A)-binding protein during myogenesis. *J. Biol. Chem.*, **269**, 803–812.
- Aharon,T. and Schneider,R.J. (1993) Selective destabilization of short-lived mRNAs with the granulocyte-macrophage colony-stimulating factor AU-rich 3' noncoding region is mediated by a cotranslational mechanism. *Mol. Cell. Biol.*, **13**, 1971–1980.
- Banks,L., Matlashewski,G. and Crawford,L. (1986) Isolation of human p53-specific monoclonal antibodies and their use in the studies of human p53 expression. *Eur. J. Biochem.*, **159**, 529–534.
- Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–258.
- Clarke,A.R., Purdie,C.A., Harrison,D.J., Morris,R.G., Bird,C.C., Hooper,M.L. and Wyllie,R.G. (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, **362**, 849–852.
- de Wet,J.R., Wood,K.V., Deluca,M., Helinski,D.R. and Subramani,S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.*, **7**, 725–737.
- Dover,R., Jayaram,Y., Patel,K. and Chinery,R. (1994) p53 expression in cultured cells following radioisotope labelling. *J. Cell. Sci.*, **107**, 1181–1184.
- El-Deiry,W.S., Tokino,T., Velculescu,V.E., Levy,D.B., Parsons,R., Trent,J.M., Lin,D., Mercer,W.E., Kinzler,K.W. and Vogelstein,B. (1993) WAF-1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.
- Fort,P., Marty,L., Piechaczyk,M., Sabrouby,S.E., Dani,C., Jeanteur,P. and Blanchard,J.M. (1985) Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res.*, **13**, 1431–1442.
- Fritsche,M., Haessler,C. and Brandner,G. (1993) Induction of nuclear accumulation of the tumor-suppressing protein p53 by DNA-damaging agents. *Oncogene*, **8**, 307–318.
- Fu,L., Minden,M.D. and Benchimol,S. (1996) Translational regulation of human p53 gene expression. *EMBO J.*, **15**, 4392–4401.
- Gavis,E. and Lehmann,R. (1994) Translational regulation of *nanos* by RNA localization. *Nature*, **369**, 315–318.
- Harlow,E., Williamson,N.M., Ralston,R., Helfman,D.M. and Adams,T.E. (1985) Molecular cloning and *in vitro* expression of a DNA clone for human cellular tumor antigen p53. *Mol. Cell. Biol.*, **5**, 1601–1610.
- Herzog,H., Hofferer,L., Schneider,R. and Schweiger,M. (1990) cDNA encoding the human homologue of rat ribosomal protein L35a. *Nucleic Acids Res.*, **18**, 4600.
- Huarte,J., Stutz,A., O'Connell,M.L., Gubler,P., Belin,D., Darrow,A.L., Strickland,S. and Vassalli,J.D. (1992) Transient translational silencing by reversible mRNA deadenylation. *Cell*, **69**, 1021–1030.
- Hupp,T.R., Meek,D.W., Midgley,C.A. and Lane,D.P. (1992) Regulation of the specific DNA binding function of p53. *Cell*, **75**, 875–886.
- Hupp,T.R., Sparks,A. and Lane,D.P. (1995) Small peptides activated the latent sequence-specific DNA binding function of p53. *Cell*, **83**, 237–245.
- Jackson,R.J. (1993) Cytoplasmic regulation of mRNA function: the importance of the 3' untranslated region. *Cell*, **74**, 9–14.
- Jayaraman,T. and Prives,C. (1995) Activation of p53 sequence-specific DNA-binding by short single strands of DNA requires the p53 C-terminus. *Cell*, **81**, 1021–1029.
- 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 Res.*, **51**, 6304–6311.
- Kuerbitz,S.J., Plunkett,B.S., Walsh,W.V. and Kastan,M.B. (1992) Wild-type p53 is a cell-cycle checkpoint determinant following irradiation. *Proc. Natl Acad. Sci. USA*, **89**, 7491–7495.
- Kwon,Y.K. and Hecht,N.B. (1993) Binding of a phosphoprotein to the 3' untranslated region of the mouse protamine 2 mRNA temporally represses its translation. *Mol. Cell. Biol.*, **13**, 6547–6557.
- Kwon,Y.K., Murray,M.T. and Hecht,N.B. (1993) Proteins homologous to the *Xenopus* germ cell-specific RNA-binding proteins p54/p56 are temporally expressed in mouse male germ cells. *Dev. Biol.*, **158**, 90–100.
- Kyrpides,N., Tavernarakis,N., Papamatheakis,J. and Thireos,G. (1995) A transient GCN4 mRNA destabilization follows GCN4 translational derepression. *J. Biol. Chem.*, **270**, 17317–17321.
- Lowe,S.W., Ruley,H.E., Jacks,T. and Housman,D.E. (1993a) p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell*, **74**, 957–967.
- Lowe,S.W., Schmitt,E.M., Smith,S.W., Osborne,B.A. and Jacks,T. (1993b) p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, **362**, 847–849.
- Lu,X. and Lane,D.P. (1993) Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? *Cell*, **75**, 765–778.
- Maltzman,W. and Czyzyk,L. (1984) UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. *Mol. Cell. Biol.*, **4**, 1689–1694.
- Matlashewski,G., Lamb,P., Pim,D., Peacock,J., Crawford,L. and Benchimol,S. (1984) Isolation and characterization of a human p53 cDNA clone: expression of the human p53 gene. *EMBO J.*, **13**, 3257–3262.
- Minich,W.B., Maidebura,I.P. and Ovchinnikov,L.P. (1993) Purification and characterization of the major 50-kDa repressor protein from cytoplasmic mRNP of rabbit reticulocytes. *Eur. J. Biochem.*, **212**, 633–638.
- Mosner,J., Mummenbrauer,T., Bauer,C., Sczakiel,G., Grosse,F. and Deppert,W. (1995) Negative feedback regulation of wild-type p53 biosynthesis. *EMBO J.*, **14**, 4442–4449.
- Nelson,W.G. and Kastan,M.B. (1994) DNA strand break: the DNA template alterations that trigger p53-dependent DNA damage response pathway. *Mol. Cell. Biol.*, **14**, 1815–1823.
- Ostareck-Lederer,A., Ostareck,D.H., Standart,N. and Thiele,B.J. (1994) Translation of 15-lipoxygenase mRNA is inhibited by a protein that binds to a repeated sequence in the 3' untranslated region. *EMBO J.*, **13**, 1476–1481.
- Papayannopoulou,T., Nakomoto,B., Kurachi,S., Tweeddale,M. and Messner,H. (1988) Surface antigenic profile and globin phenotype of two new human erythroleukemia lines: characterization and interpretations. *Blood*, **72**, 1029–1038.
- Peltz,S.W. and Jacobson,A. (1992) mRNA stability: intransit. *Curr. Opin. Cell Biol.*, **4**, 979–983.
- Robbie,E.R., Peterson,M., Amaya,E. and Musci,T.J. (1995) Temporal regulation of *Xenopus* FGF receptor in development: a translation inhibitory element in the 3' untranslated region. *Development*, **121**, 1775–1785.
- Ross,J. (1995) mRNA stability in mammalian cells. *Microbiol. Rev.*, **59**, 423–450.
- Sheets,M.D., Fox,C.A., Hunt,T., Vande Woude,G. and Wickens,M. (1994) The 3'-untranslated regions of *c-mos* and cyclin mRNA stimulate translation by regulating cytoplasmic polyadenylation. *Genes Dev.*, **8**, 926–938.
- Slingerland,J.M., Minden,M.D. and Benchimol,S. (1991) Mutations of p53 gene in human acute myelogenous leukemia. *Blood*, **7**, 1500–1507.
- Sonenberg,N. (1994) mRNA translation: influence of the 5' and 3' untranslated regions. *Curr. Opin. Genet. Dev.*, **4**, 310–315.
- Standart,N. and Jackson,R.J. (1994) Regulation of translation by specific protein/mRNA interactions. *Biochimie*, **76**, 867–879.
- Sun,X., Shimizu,H. and Yamamoto,K. (1995) Identification of a novel p53 promoter element involved in genotoxic stress-inducible p53 gene expression. *Mol. Cell. Biol.*, **15**, 4489–4496.
- Takahashi,K., Sumimoto,H., Suzuki,K. and Ono,T. (1993) Protein synthesis-dependent cytoplasmic translocation of p53 protein after serum stimulation of growth-arrested MCF-7 cells. *Mol. Carcinogen.*, **8**, 58–66.
- Tuck,S.P. and Crawford,L. (1989) Characterization of human p53 gene promoter. *Mol. Cell. Biol.*, **9**, 2163–2172.

- Wang,C., Curtis,J.E., Minden,M.D. and McCulloch,E.A. (1989) Expression of retinoic acid receptor gene in myeloid leukemia cells. *Leukemia*, **3**, 264–269.
- Weiss,I.M. and Liebhaber,S.A. (1995) Erythroid cell-specific mRNA stability elements in the alpha 2-globin 3' untranslated region. *Mol. Cell. Biol.*, **15**, 2457–2465.
- Wickens,M. (1993) Messenger RNA. Springtime in the desert. *Nature*, **363**, 305–306.
- Wolffe,A.P. (1994) Structural and functional properties of the evolutionarily ancient Y-box family of nucleic acid binding proteins. *Bioassays*, **16**, 245–251.
- YeARGIN,T. and Haas,M. (1995) Elevated levels of wild-type p53 induced by radiolabelling of cells leads to apoptosis or sustained growth arrest. *Curr. Biol.*, **5**, 423–431.

Received on February 17, 1997; revised on March 19, 1997