The EMBO Journal vol.14 no.18 pp.4442–4449, 1995

Negative feedback regulation of wild-type p53 biosynthesis

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When growth-arrested mouse fibroblasts re-entered the cell-cycle, the rise in tumour suppressor p53 mRNA level markedly preceded the rise in expression of the p53 protein. Furthermore, γ-irradiation of such cells led to a rapid increase in p53 protein biosynthesis even in the presence of the transcription inhibitor actinomycin D. Both findings strongly suggest that p53 biosynthesis in these cells is regulated at the translational level. We present evidence for an autoregulatory control of p53 expression by a negative feed-back loop: p53 mRNA has a predicted tendency to form a stable stem–loop structure that involves the 5′-untranslated region (5′-UTR) plus some 280 nucleotides of the coding sequence. p53 binds tightly to the 5′-UTR region and inhibits the translation of its own mRNA, most likely mediated by the p53-intrinsic RNA re-annealing activity. The inhibition of p53 biosynthesis requires wild-type p53, as it is not observed with MethA mutant p53. p53-catalysed translational inhibition is selective; it might be restricted to p53 mRNA and a few other mRNAs that are able to form extensive stem–loop structures. Release from negative feed-back regulation of p53 biosynthesis, e.g. after damage-induced nuclear transport of p53, might provide a means for rapidly increasing p53 protein levels when p53 is required to act as a cell-cycle checkpoint determinant after DNA damage.

Keywords: autoregulatory feedback loop/p53/RNA secondary structures/translationary control/tumour suppressor

Introduction

Expression of a functional wild-type (wt) p53 protein is pivotal for preserving the integrity of the genome (Lane, 1992a,b; Perry and Levine, 1993; Berns, 1994). After DNA damage, p53 levels increase and halt the cell-cycle by up-regulating expression of the $p21^{WAF1/CIP1}$ protein, which in turn inhibits cyclin-dependent kinases required for cell-cycle progression (El-Deiry *et al.*, 1993, 1994; Harper *et al.*, 1993) and stop PCNA-dependent DNA replication (Waga *et al.*, 1994). An important requirement for the induction of this cascade is a rise in p53 levels after DNA damage (Kuerbitz *et al.*, 1992; Nelson and Kastan, 1994), asking for a delicate regulation of p53 levels. The increase in p53 levels occurs post-transcriptionally (Fritsche *et al.*, 1993), with metabolic stabilization of the p53 protein being an important feature of this process (Maltzman and Czyzyk, 1984; Deppert, 1994). Whereas metabolic stabilization of p53 might be sufficient to account for the rapid rise in p53 levels in cycling cells, where p53 expression is largely independent from the cell-cycle (Coulier *et al.*, 1985), one has to postulate additional mechanisms in cells re-entering the cell-cycle. In such cells, p53 protein expression is very low throughout the G_1 phase; a substantial increase in p53 biosynthesis is observed only with the onset of cellular DNA synthesis (Mosner and Deppert, 1994). As one can assume that in such cells p53 also exerts its function as a guardian of the genome, we considered translatability of the p53 mRNA as an additional mechanism for regulating p53 expression under such conditions.

In this paper we suggest a model for a translational control of p53 protein expression which is based on two different lines of evidence. First, when resting cells re-enter the cell-cycle, the expression of p53 protein lags behind the accumulation of p53 mRNA by several hours. Upon DNA damage in G_1, i.e. at a time when p53 mRNA levels are high but p53 protein biosynthesis is low, p53 protein expression is upregulated within 1 h, even in the presence of the transcription inhibitor actinomycin D. Second, we show that purified wt p53 protein inhibits the translation of its own mRNA in an *in vitro* assay system. Taken together, these data strongly suggest that p53 protein biosynthesis is regulated at the translational level, most probably via a negative autoregulatory loop of feed-back inhibition.

Results

Disparity between p53 mRNA production and p53 protein expression in growth-stimulated Swiss 3T3 cells

When Swiss 3T3 cells, made quiescent by serum starvation, were stimulated to re-enter the cell-cycle, p53 protein expression closely paralleled the course of cellular DNA synthesis, i.e. p53 biosynthesis was low in the G_0 and G_1 phase of the cell-cycle, and increased with the onset of S phase (Mosner and Deppert, 1994) (Figure 1A and C). In contrast, the p53 mRNA level rose rapidly after serum stimulation and reached a maximum 12 h after stimulation, which was shortly after the onset of cellular DNA replication (Figure 1A and B). From then on the amount of p53 mRNA declined. Thus, the rapid rise of the p53 mRNA level after growth stimulation was not accompanied by a

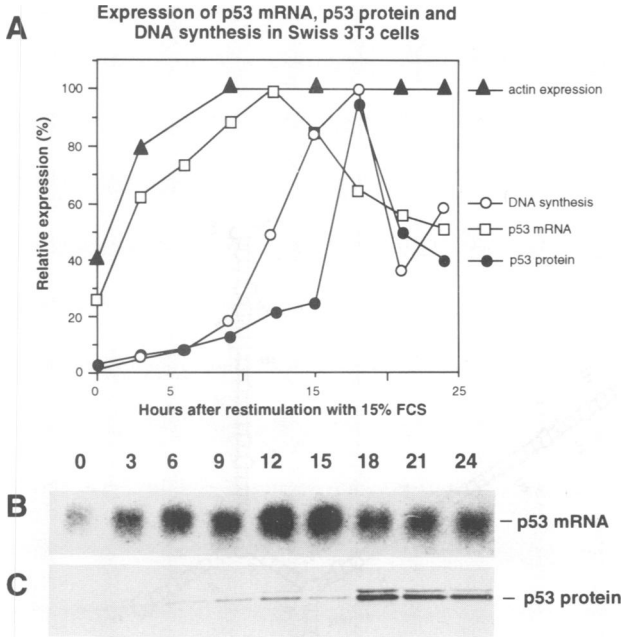

Fig. 1. Kinetics of p53 mRNA and protein expression after release of Swiss 3T3 cells from the resting state into proliferation. (**A**) Graphical evaluation of different growth parameters. The values for the rate of cellular DNA synthesis and for actin biosynthesis were taken from Mosner and Deppert (1994). The amounts of p53 mRNA and p53 protein at different time points were determined by laser-densitometric scanning of the individual RNA and protein bands of the respective autoradiographs shown below. The highest amounts were arbitrarily set as 100%. (**B**) Northern blot analysis of p53 transcripts in growth-arrested and in re-stimulated Swiss 3T3 cells. (**C**) Analysis of p53 protein expression in growth-arrested and in re-stimulated Swiss 3T3 cells. For each given time point 10^7 cells were pulse-labelled with 250 μCi [^{35}S]methionine/cysteine for 30 min. The cells were lysed and p53 was immunoprecipitated with the monoclonal antibodies PAb421 and PAb248 as described previously (Mosner and Deppert, 1994). Immunoprecipitates were analysed on an 11.5% SDS–polyacrylamide gel; radiolabelled p53 was visualized by fluorography.

corresponding rise in p53 protein synthesis. A significant increase in p53 protein synthesis was only observed at 18 h after stimulation, i.e. 6 h after maximal mRNA accumulation and at a time when the p53 mRNA level was already declining (Figure 1B). In contrast, both mRNA production and protein biosynthesis of the 'house-keeping protein' actin increased in parallel within 3 to 4 h after serum stimulation (Figure 1A). The different time course of p53 mRNA and protein expression after serum stimulation suggests a translational control of p53 biosynthesis. As p53 is cytoplasmic in serum-stimulated 3T3 cells in G_1, but translocates into the cell nucleus with the onset of cellular DNA synthesis (Shaulsky *et al.*, 1990), this opens the possibility that cytoplasmic p53 could be involved in the control of its own synthesis. As an alternate possibility to explain the apparent disparity of p53 mRNA accumulation and p53 protein expression in cells re-entering the cell-cycle as shown in Figure 1, we considered that the p53 protein in G_1 cells has a much shorter half-life than p53 in S phase cells, leading to an apparently lower p53 protein expression in G_1 cells than in S phase cells after metabolic labelling. This possibility, however, could be excluded. We determined by pulse–chase experiments the half-life of p53 in re-stimulated Swiss 3T3 cells during the course of stimulation, and found it to be 20 min at all times after stimulation (data not shown).

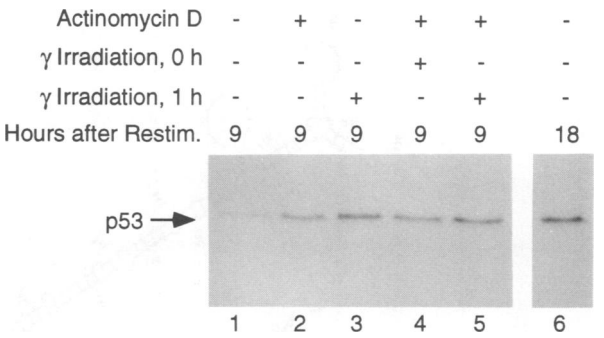

Fig. 2. Rapid increase of p53 protein biosynthesis after γ-irradiation in the presence of the transcription inhibitor actinomycin D. Swiss 3T3 cells were growth-arrested and re-stimulated as described (Mosner and Deppert, 1994). Seven hours after re-stimulation, actinomycin D (2 μg/ml) was added to the cell culture. After a further 2 h, i.e. 9 h after re-stimulation, the cells were γ-irradiated with 10 Gy of a ^{134}Cs source. Cells were pulse-labelled immediately (indicated as 0 h) or 1 h after γ-irradiation (indicated as 1 h) with 100 μCi [^{35}S]methionine/cysteine for 20 min. The cells were lysed and p53 was immunoprecipitated with the monoclonal antibodies PAb421 and PAb248. Immunoprecipitates were analysed on an 11.5% SDS–polyacrylamide gel; radiolabelled p53 was visualized by fluorography.

Rapid increase of p53 protein after DNA damage even in the presence of actinomycin D

As shown earlier by Kastan and coworkers (Kuerbitz *et al.*, 1992), p53 protein expression rapidly responds to genotoxic stress. Although p53 protein biosynthesis in Swiss 3T3 cells was barely detectable 9 h after growth stimulation (Figures 1C and 2, lane 1), γ-irradiation of such cells resulted in a very rapid (within 1 h) and marked increase in p53 biosynthesis (Figure 2, lane 3). This rapid increase in p53 protein biosynthesis was also observed in the presence of actinomycin D (Figure 2, lane 5). As actinomycin D exhibits genotoxic effects (Fritsche *et al.*, 1993), actinomycin D treatment by itself led to an increased p53 biosynthesis (Figure 2, lanes 2, 4 and 5). Nevertheless, the increase in p53 biosynthesis was considerably stronger when actinomycin D treatment was followed by γ-irradiation (Figure 2, lanes 4 and 5). Both the rapid increase in p53 biosynthesis after γ-irradiation and the finding that this increase was observed although transcription was blocked, strongly suggest a translational regulation of p53 protein expression *in vivo*. The following experiments were designed to find a mechanistic explanation for this phenomenon.

p53 mRNA can form a stable stem–loop structure at its 5′ terminus

Previous analyses predicted a capacity of the p53 mRNA to form a stable stem–loop structure between nucleotides –216 and –108 in exon I of the 5′-untranslated region (5′-UTR) with a calculated free energy of –56 kcal/mol (Bienz *et al.*, 1984; Bienz-Tadmor *et al.*, 1985). We calculated possible secondary structures for a set of successively elongated sequences of the p53 mRNA of up to almost its full length of 1435 nucleotides. This analysis revealed further stem–loop structures, including the one depicted in Figure 3, that displayed a calculated free energy of –170 kcal/mol. It is noteworthy that all calculations either showed the previously described stem–loop element spanning nucleotides –216 and –108 (Bienz *et al.*, 1984; Bienz-Tadmor *et al.*, 1985 and data not shown), or the

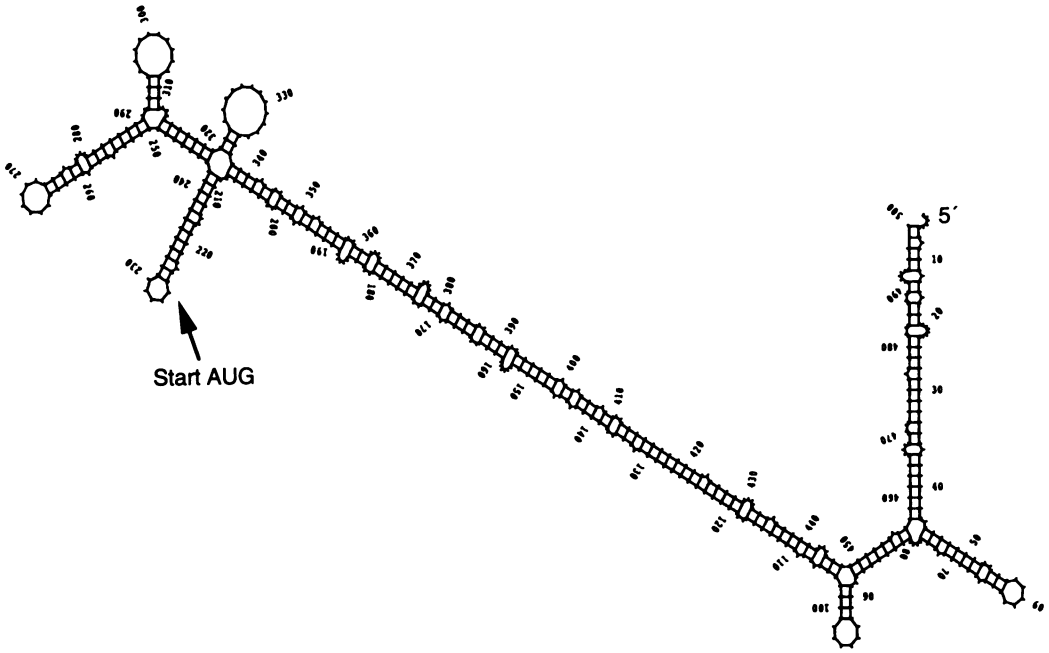

Fig. 3. Secondary structure model of the 5′ region of mouse wt p53 mRNA. The structure of the murine p53 mRNA was calculated with the program 'Heidelberg Unix Sequence Analysis Resources' (HUSAR) which makes use of a secondary structure prediction algorithm (Zuker and Stiegler, 1981). Nucleotide numeration starts with position 1 of the full-length p53 mRNA; this is identical to position –216 regarding the +1 position of the AUG start codon.

element made up by nucleotides –216 to +284 depicted in Figure 3. The opening of either of these stem–loop structures is thought to require energy-driven processes, such as the action of an RNA helicase, provided by, e.g. the eukaryotic translation initiation factor 4A (eIF-4A) (Jaramillo *et al.*, 1991). Similar 5′ stem–loop structures were described for the rat p53 mRNA (Bienz-Tadmor *et al.*, 1985). Unfortunately, the complete 5′-ends of p53 mRNAs from other species are not yet available for a comparative analysis of the phylogenetic conservation of 5′ stem–loop structures in the p53 mRNA.

5′ stem–loop structures in mRNAs are considered to be important for translational regulation of vertebrate mRNAs (Kozak, 1991). Thus their existence in p53 mRNAs from mouse and rat suggested to us that these structures play an important role in the postulated translational regulation of p53 biosynthesis. p53 is an RNA binding protein and has a well-known RNA•RNA reannealing activity that displays helicase-inhibitory effects (Oberosler *et al.*, 1993). Furthermore, it was shown that p53 can exert anti-helicase activity by physically associating with helicases (Wang *et al.*, 1994). This led us to speculate that the p53 protein counteracts its own translation by stabilizing secondary structures of its own mRNA, mediated by its RNA•RNA annealing capacity and/or its anti-helicase activity. A pre-requisite for either of these activities, however, is a capacity of p53 to bind selectively to its own mRNA.

p53 protein binds selectively to the 5′-UTR of its own mRNA

To demonstrate p53 binding to its autologous mRNA, we first produced ^{35}S-labelled p53 protein by *in vitro* translation. This reaction generated two proteins of 53 and 40 kDa, respectively (Figure 4A). A very similar pattern of translation products was obtained with either wt p53 or MethA mutant p53 (Figure 4A). Both the shorter and longer translation product could be precipitated with monoclonal antibody PAb248, an antibody that recognizes an N-terminal epitope on p53 (Yewdell *et al.*, 1986). In contrast, monoclonal antibody PAb421 with a specificity for the C-terminus of p53 (Harlow *et al.*, 1981) precipitated only the larger translation product (data not shown). Hence, the larger protein most likely represents full-length p53, whereas the shorter protein is a premature translation termination product. To study RNA binding, *in vitro* translated and labelled p53 proteins (wt and MethA mutant) were incubated with full-length p53 mRNA, with the isolated 5′-UTR region of exon I (nucleotides –216 to +1) of p53 mRNA and with the isolated coding region (nucleotides +1 to +1161). In addition, p53 proteins were incubated with mRNA encoding the large SV40 T antigen. All RNAs were transcribed *in vitro* from T7/T3 promoter-containing plasmids in the presence of biotinylated rUTP. Biotinylated RNAs together with possibly bound p53 were removed from the binding assay by using magnetic streptavidin beads. RNA-bound p53 was eluted with an SDS-containing buffer, subjected to gel electrophoresis and subsequently detected by autoradiography. Figure 4A demonstrates that significantly more ^{35}S-labelled p53 was retained by the p53 mRNA and particularly its 5′-UTR than by the control SV40 T-antigen mRNA, suggesting that p53 preferentially binds to its own mRNA at the portion of the 5′-UTR. Furthermore, both mutant p53 as well as wt p53 bound RNA to a comparable extent. Figure 4A also reveals that predominantly the full-length p53 translation product, but not the 40 kDa premature termination product of either mutant or wt p53 was able to bind to RNA. Therefore, an intact C-terminus is necessary for autologous RNA binding. Neither SV40 T antigen nor luciferase control proteins, both translated *in vitro* (Figure 4B), bound to biotinylated p53-encoding mRNA

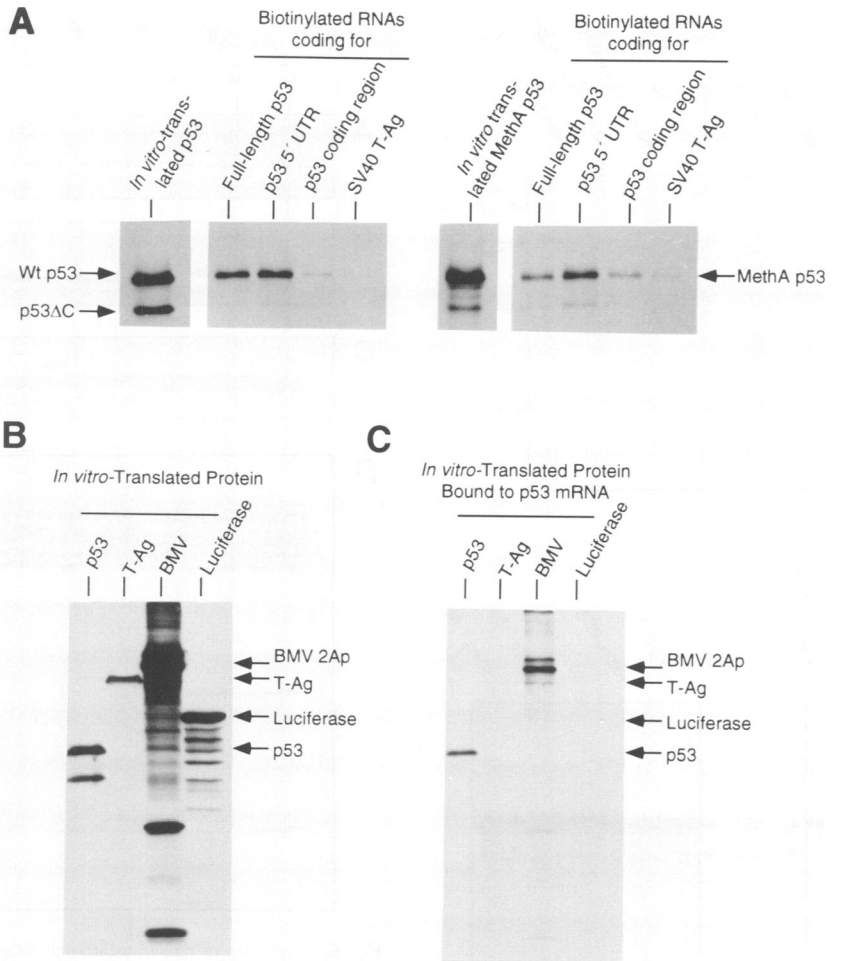

Fig. 4. Wild-type and MethA mutant p53 protein bind selectively to the 5′ UTRs of their corresponding mRNAs. (**A**) Murine wt and MethA mutant p53 were translated *in vitro* and incubated with various biotinylated RNAs. Binding of both wt and mutant p53 protein to 100 ng each of biotinylated mouse full-length p53 mRNA, the isolated 5′-UTR, the coding region of p53 mRNA and SV40 T antigen mRNA, are shown from left to right. The left panel displays binding properties of wt p53, the right panel displays binding of MethA mutant p53 to these RNAs. (**B**) *In vitro* translation of 10 ng (each) of mouse p53 mRNA, SV40 T antigen mRNA, BMV genomic RNA and luciferase mRNA. Five microlitres of each of the *in vitro* translation mixtures were analysed on an 11.5% SDS–polyacrylamide gel. (**C**) Binding of *in vitro* translated proteins (p53, T antigen, BMV proteins and luciferase) to biotinylated full-length mouse wt p53 mRNA. Portions taken from the translation reaction (5 µl) shown in (B) were incubated with 100 ng (each) of biotinylated wt p53 mRNA. Bound proteins were removed from the binding assay and were analysed by electrophoresis on an 11.5% SDS–polyacrylamide gel.

(Figure 4C). On the other hand, *in vitro* translated (Figure 4B) 2A protein of bromomosaic virus (BMV), a known RNA binding protein (Kao *et al.*, 1992) used as a positive control, bound p53 mRNA as expected (Figure 4C).

p53 protein, bound to its own mRNA, inhibits the autologous translation reaction

Human thymidylate synthase inhibits the autologous translation reaction by binding to its own mRNA (Chu *et al.*, 1991). Analogously, the selective binding of p53 to the 5′-UTR of its own mRNA, as demonstrated in Figure 4, was a pre-requisite for the suggested autoregulatory feedback control for p53 translation. Further evidence for the proposed mechanism came from studies, in which *in vitro* translation of p53 mRNA was analysed in the presence of various amounts of purified wt p53 protein (Figure 5A). While the addition of 0.5 or 2.5 ng wt p53 protein did not markedly affect the yield of the newly generated p53 and the p53-related 40 kDa proteins, addition of more than 5 ng p53 inhibited the autologous translation reaction (Figure 5A). A complete translational inhibition could be achieved by the addition of 50 ng of purified p53 protein. This inhibition could be overcome by pre-incubation of p53 protein with the monoclonal antibody PAb421, while pre-incubation of p53 with the SV40 T antigen-specific monoclonal antibody PAb108 (Gurney *et al.*, 1986) had no effect. The reversion of translation inhibition by PAb421 proved the specificity of the inhibition reaction and further stresses the importance of the C-terminus of p53 protein (and possibly also the reannealing activity) for the inhibition of its own biosynthesis. Translational autoregulation seems to be a wt p53-specific activity, as addition of up to 250 ng of purified MethA mutant p53 (Eliyahu *et al.*, 1988) had little or no effect on the translation of wt p53 mRNA (Figure 5B). This is in a striking contrast to our finding that the RNA binding affinities of MethA p53 and wt p53 were comparable (Figure 4A). Hence, binding of p53 protein to its own mRNA as such is not sufficient for inhibition of the translation reaction. Like other mutant forms of p53 protein (Stürzbecher *et al.*, 1988; Wang *et al.*, 1989; Friedman *et al.*, 1990), MethA p53 lacks RNA•RNA re-annealing activity (data not shown). There-

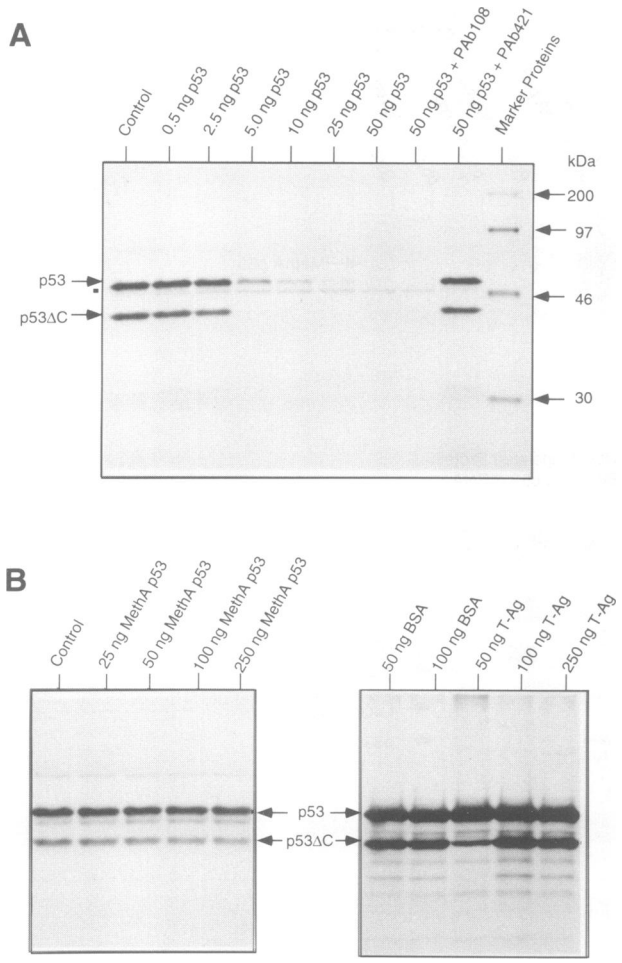

Fig. 5. Autoregulation of *in vitro* translation of p53 mRNA by p53 protein. (**A**) *In vitro* translation of full-length mouse wt p53 mRNA (10 ng) in the absence (control) and in the presence of increasing amounts of purified mouse wt p53 protein. Where indicated, 1 μg of the T antigen-specific antibody PAb108 or 1 μg of the p53-specific antibody PAb421 were added to the wt p53 protein just before starting the translation reaction. Five microlitres from the translation reaction were loaded onto the SDS gel. The 42 kDa band denoted by a square probably represents covalent binding of [^{35}S]methionine to a pre-existing acceptor protein (Clemens, 1984). (**B**) *In vitro* translation of full-length mouse wt p53 mRNA (10 ng) in the absence (control) and in the presence of the indicated amounts of purified mutant MethA p53 protein (25–250 ng), BSA (bovine serum albumin; 50 and 100 ng) and purified SV40 T antigen (100 and 250 ng).

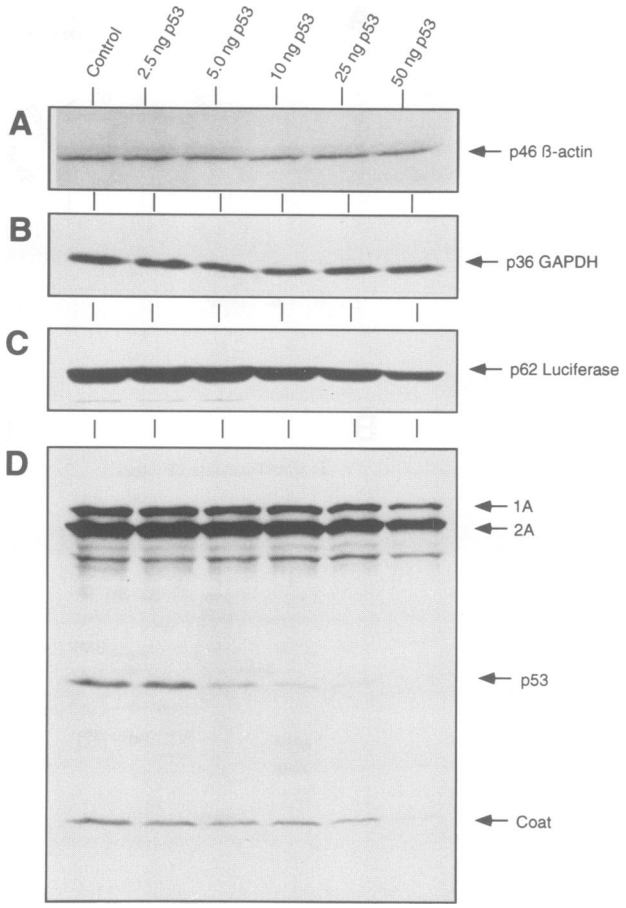

Fig. 6. Specificity of p53 translational autoregulation. Ten nanograms of each of the mRNAs coding for β-actin (**A**), glyceraldehyde dehydrogenase (**B**), luciferase (**C**) and genomic BMV RNA (**D**), were titrated with the indicated amounts of highly purified wt p53. As an internal control, BMV RNA was also co-translated with 10 ng p53 mRNA. Only p53 translation was affected by the addition of various amounts of wt p53 protein (D), in spite of the capability of BMV RNA to form extensive secondary structures (Ahlquist, 1992).

fore, we conclude that the re-annealing property of p53 rather than its RNA binding is responsible for the p53-mediated inhibition of its own translation.

Translational inhibition by wt p53 is selective. Figure 6 demonstrates that addition of up to 50 ng of purified p53 to *in vitro* translation reactions using β-actin mRNA (Figure 6A), mRNA encoding glyceraldehyde dehydrogenase (GAPDH; Figure 6B), luciferase (Figure 6C) or BMV genomic RNA (Figure 6D), did not significantly affect the translation efficiencies of these RNAs. The slight inhibition seen at the highest p53 concentration probably reflects the non-specific interaction of p53 with these RNAs (see also Figure 4). This conclusion was further supported by the experiment shown in Figure 6D, where p53 mRNA was co-translated with genomic BMV RNA in the presence of increasing amounts of p53. Whereas p53 biosynthesis was selectively inhibited by the addition of p53, co-translated genomic BMV RNA was not (Figure 6D). These data therefore suggest that translational inhibition by wt p53 is not a general phenomenon, but may be restricted to a limited number of mRNAs, possibly characterized by their ability to form extensive 5′ terminal stem–loop structures.

Discussion

The present paper provides evidence for a translational control mechanism for p53 expression in living cells. Translational control of p53 expression has already been inferred by other studies (Bienz-Tadmor *et al.*, 1985; Dony *et al.*, 1985; Steinmeyer *et al.*, 1990), but so far has not been demonstrated directly. *In vivo* evidence for a translational control of p53 protein biosynthesis comes from the following observations. First, after growth stimulation of resting Swiss 3T3 cells, the observed p53 mRNA production was not accompanied by a comparable induction of p53 protein expression. Rather, there was a time lag of 6–8 h between maximal expression of p53 mRNA and maximal expression of p53 protein. This leads to a scenario in which p53 mRNA production peaks before or at the G_1/S boundary of the cell-cycle, while p53

protein is maximally expressed at mid-S. Second, upon γ-irradiation of Swiss 3T3 cells, there was an immediate response in p53 protein production that took no longer than 1 h following irradiation, i.e. the earliest time point analysed. This time period is too short for making it likely that transcriptional processes, i.e. pre-mRNA production and maturation to mRNA, packaging into small nuclear particle proteins, mRNA transport through the nuclear membrane and subsequent unpackaging for translation, account for the observed rise in p53 protein expression. Furthermore, γ-ray-induced upregulation of p53 biosynthesis was not inhibited by actinomycin D. Hence, translational control rather than transcriptional regulation of p53 production must be responsible for the rapid increase of p53 protein seen after DNA damage *in vivo*.

In our search for a possible mechanism for this translational control we noticed that p53 mRNA has the capacity to form stem–loop structures with a high negative energy at its 5′ terminus (Bienz *et al.*, 1984; Bienz-Tadmor *et al.*, 1985). This previously published structure, spanning nucleotides –216 and –108 (Bienz *et al.*, 1984; Bienz-Tadmor *et al.*, 1985), was confirmed by us. However, our extended calculations revealed further stem–loop structures, including an extremely stable one with a calculated free energy of –170 kcal/mol that was formed by nucleotides –216 to +284 of the p53 mRNA. It is well known that 5′-situated stem-and-loop structures with a free energy of about –50 kcal/mol inhibit translation profoundly, both *in vivo* (Kozak, 1986) and *in vitro* (Kozak, 1989). Therefore, it is highly likely that the calculated stem–loop elements are involved in the translational control mechanisms of p53 protein expression, although we are aware that computer-supported predictions of secondary structures of RNA have to be viewed with caution. However, our finding that the p53 protein selectively recognized the isolated 5′-UTR, but not the isolated coding region of its own mRNA, lends strong experimental support to the view that the 5′-UTR region of the p53 mRNA assumes a configuration specifically recognized by the p53 protein. As a model, we propose that p53 either stabilizes stem–loop structures of its own mRNA or refolds its autologous mRNA from a less stable to a thermodynamically more stable form. Both structure stabilization and/or refolding might be catalysed by the p53-intrinsic RNA•RNA re-annealing activity (Oberosler *et al.*, 1993) and would lead to an attenuation of p53 mRNA translation.

Strong support for such a model was obtained from studies on the autologous inhibition of p53 protein synthesis in *in vitro* translation systems. Fifty nanograms of p53 were sufficient to block the translation reaction completely. Considering that half-maximal inhibition was achieved upon addition of 5 ng p53, i.e. at a 5:1 molar ratio of p53 monomers over input p53 mRNA, we conclude that a single p53 tetramer is able to mediate translational inhibition, most probably by catalysing the re-annealing of the 5′-UTR stem–loop structures of its mRNA. Strong support for a catalytic rather than a mere binding mechanism for p53's inhibition of its own translation reaction came from the observation that mutant p53 protein binds to its own mRNA with a similar affinity as the wild-type form but, in spite of this, was not able to inhibit the translation reaction. In all these assays, both p53 binding to its mRNA and the inhibition of translation required only 5 ng purified protein per 50 μl assay mixture. This roughly corresponds to a concentration of ~600 molecules monomeric p53 per cell (with an estimated intracellular volume of 0.5 pL). Since we do not expect that our p53 preparation is 100% active, a concentration of <600 molecules of p53 per cell was causing all the effects seen *in vitro*. These biochemical data and the quantitative considerations strongly support the view that binding and inhibition of translation do also occur *in vivo*.

Translational inhibition represents an efficient control mechanism that may be particularly suited for the fast and delicate regulation of expression of regulatory proteins (Rhoads, 1988; Sonenberg, 1988). Such a mechanism might enable a rapid rise of wt p53 levels after DNA damage under conditions where p53 biosynthesis is low, for example in cells stimulated to re-enter the cell-cycle, and even if the p53 gene has been damaged as well (provided the cell contains enough p53 mRNA). The autoregulation of p53 protein expression demonstrated here suggests a model, according to which wt p53 interacts with its own mRNA in translational complexes to slow down p53 protein production in normal cells. After DNA damage, the generation of DNA breaks and ssDNA regions might induce the translocation of p53 into the cell nucleus, where p53 binds to ss/dsDNA junctions (Oberosler *et al.*, 1993; Bakalkin *et al.*, 1994) or DNA breaks (Nelson and Kastan, 1994). The resulting decrease of cytosolic p53 should release p53 mRNA from its autocatalysed translational block and thereby up-regulate the production of p53 protein within a very short time scale, a model which is strongly supported by the data shown in Figure 2.

The ability of wt p53 to exert translational control, as demonstrated here for its own mRNA, might not be restricted to autoregulation of p53 biosynthesis, but may constitute an activity which is of more general importance for the function of p53 as a cell-cycle protein. While this manuscript was in preparation, evidence has been provided suggesting that wt p53 is involved in the control of TGF-β mediated growth arrest by inhibiting translation of the CDK4 mRNA (Ewen *et al.*, 1995). The finding that the 5′-UTR of the CDK4 mRNA is important for this translational inhibition, and that the p53-dependent repression of CDK4 translation is abrogated by mutant p53 in a dominant-negative fashion, is consistent with the biochemical model for translational regulation by wt p53 put forward in this study.

Materials and methods

Cells and viruses

Swiss 3T3 fibroblast cells were grown at 37°C in DMEM medium (Gibco) containing 5% fetal calf serum (FCS). For cell synchronization, about equal numbers of exponentially growing cells were washed with medium free of FCS and were then incubated in DMEM containing 0.1% FCS for 5 days (time point 0). Cultures were then re-stimulated with 15% FCS.

Spodoptera frugiperda insect cells (Sf9 cells) were grown at 27°C in SF900 medium (Gibco) containing 10% FCS. A total of 5×10^8 cells were infected with either wt p53-, MethA mutant p53- or SV40 T antigen-recombinant baculoviruses and harvested 48 h post-infection.

Metabolic cell labelling and immunoprecipitation

For each time point, 10^7 cells were pulse-labelled with 250 μCi [^{35}S]methionine/cysteine (Translabel, ICN) for 30 min. The cells were

lysed with detergent-containing isotonic buffer and p53 was immunoprecipitated with a mixture of the monoclonal antibodies PAb421 and PAb248 as described previously (Mosner and Deppert, 1994). Immunoprecipitates were analysed on an 11.5% SDS–polyacrylamide gel (Laemmli, 1970); radiolabelled p53 was visualized by fluorography (Skinner and Griswold, 1983).

Purification of recombinant proteins

Mouse wt p53 protein, MethA mutant p53 protein and SV40 T antigen produced in Sf9 insect cells infected with the respective recombinant baculoviruses, were purified by immunoaffinity chromatography on immobilized monoclonal antibodies PAb421 for the purification of either wt or mutant p53 protein, and PAb108 for the purification of T antigen by using an alkaline elution protocol (Nasheuer and Grosse, 1987). Protein-containing fractions were concentrated by dialysis against solid polyethylene glycol 40 000 (Merck, Darmstadt, Germany). The immunoaffinity-purified proteins had purities of >95% as estimated from SDS–polyacrylamide gels.

Isolation of RNA and Northern blotting

Total RNA from Swiss 3T3 cells was extracted by the guanidinium isothiocyanate-phenol method (Chomczynski and Sacchi, 1987) at the indicated time points after re-stimulation. Following quantitation by measuring absorbence at 260 nm, 5 μg of total RNA per lane were electrophoresed on a denaturing 1.2% agarose gel containing 3% formaldehyde and transferred onto a nylon membrane (Hybond-N, Amersham). Sample loading and RNA integrity were controlled by ethidium bromide staining of the gels. The filter was hybridized with a full-length p53 cDNA probe, labelled with [^{32}P]dCTP by random primer DNA synthesis (Feinberg and Vogelstein, 1983). Hybridization of the filter and washing procedures followed standard protocols. Autoradiographs were exposed overnight at –80°C using an intensifying screen.

In vitro transcription assays

A full-length mouse wt p53 (Bienz *et al.*, 1984) and a MethA mutant p53 (Eliyahu *et al.*, 1988) cDNA clone were both transcribed *in vitro* by T7/T3 RNA polymerases using the Riboprobe Gemini Transcription System (Promega) as described by the manufacturer. p53 mRNA was 1435 nucleotides long. The *in vitro* transcribed product contained 40 additional nucleotides spanning the start site of the T7 promotor to the first nucleotide of p53 mRNA, and missed ~250 nucleotides of the 3′-UTR. The 5′-UTR mRNA contained the untranslated exon I sequence of the murine p53 gene transcribed from the T7 promotor as described above. All mRNAs used for *in vitro* translation were synthesized in the presence of 5 mM of the cap analogue m^{7}G(5′)ppp(5′)G (Boehringer Mannheim). Biotinylated RNAs were synthesized in the presence of biotinylated uridine triphosphate (Boehringer Mannheim) as described (Ashley *et al.*, 1993) at a molar ratio of biotinylated UTP to UTP of 1:10.

In vitro translation assays

In vitro translation of 10 ng each of *in vitro* transcribed mouse wt p53 mRNA, SV40 T antigen mRNA, BMV genomic RNA and luciferase mRNA (BMV and luciferase mRNA were purchased from Promega) was performed in the presence of 30 μCi of [^{35}S]-labelled methionine, using a rabbit reticulocyte lysate system according to the manufacturer's protocol (Promega). Newly synthesized proteins (5 μl portions from a 50 μl translation reaction) were analysed by SDS–PAGE (Laemmli, 1970). Labelled proteins were detected by fluorography (Skinner and Griswold, 1983).

p53 binding assays

[^{35}S]methionine-labelled proteins were derived from *in vitro* translation reactions. Precipitation of these proteins, after binding to biotinylated p53 RNA, was performed as described elsewhere (Boelens *et al.*, 1993). The binding reaction was performed with 1 μl of biotinylated p53 RNA (100 ng/μl), 10 μg yeast tRNA as competitor and 5 μl of [^{35}S]-labelled protein in 10 μl KHN buffer (150 mM KCl, 20 mM HEPES–KOH, pH 7.9, 0.05% NP40, 0.2 mM DTT, 0.5 mM PMSF) for 1 h at 25°C. The mixture was diluted with 500 μl KHN buffer, transferred to a tube containing 15 μl of packed, prewashed streptavidin-magnetic beads (Dynabeads M-280, Dynal) and rotated end over end for 1 h at 20°C. The beads were collected by magnetic attraction, washed five times with 1 ml KHN buffer (each) and two times with KHN buffer without detergent, resuspended in 15 μl Laemmli loading buffer (Laemmli, 1970) and boiled for 5 min. After centrifugation the supernatant was loaded onto an 11.5% SDS–polyacrylamide gel.

Acknowledgements

This study was supported by a grant from the Deutsche Krebshilfe (Dr Mildred Scheel Stiftung) and the Fonds der Chemischen Industrie. The Heinrich-Pette-Institute is financially supported by Freie und Hansestadt Hamburg and Bundesministerium für Gesundheit. This work is part of the Ph.D. thesis of J.M.

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Received on April 24, 1995; revised on June 19, 1995