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Interaction of hnRNPC1/C2 with a novel cis-regulatory element within p53 mRNA as a response to cytostatic drug treatment

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Nonstandard abbreviations in text: **Act D**, actinomycin D, generic name Dactinomycin; **Cisplatin**, generic name cisplatinum or *cis*-diamminedichloridoplatinum(II); **DRB,** 5,6-dichlorobenzimidazole riboside, $C_{12}H_{12}Cl_2N_2O_4$, .

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Abstract

We describe a novel cis-element in the 5' coding region of p53 mRNA and its interaction with hnRNPC1/C2. This element is located in a putative hairpin loop structure, within the first 101 nucleotides downstream of the start codon. The binding of hnRNPC1/C2 is strongly enhanced in response to the DNA damaging drug cisplatin and the cytostatic transcriptional inhibitor actinomycin D, both known inducers of apoptosis and p53. Strongly stimulated binding is observed in both nuclear and cytoplasmic compartments and is accompanied by a cytoplasmic increase of hnRNPC1/C2. Changes in hnRNPC1/C2 protein levels are not proportional to binding activity, thus suggesting qualitative changes in hnRNPC1/C2 upon activation. Phosphorylation studies reveal contrasting characteristics of the cytoplasmic and nuclear hnRNPC1/C2 interaction with p53 mRNA. Results from chimeric p53-luciferase reporter constructs suggest that hnRNPC1/C2 regulates p53 expression via this binding site. Our results are consistent with a mechanism where the interaction of hnRNPC1/C2 with a cis-element within the coding region of the p53 transcript regulates the expression of p53 mRNA before and during apoptosis. In addition, we report that pre-apoptotic signals induced by transcriptional inhibition trigger the appearance of a truncated, exclusively cytoplasmic 43 kDa variant of p53 prior to apoptosis.

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Introduction

The p53 protein acts as a transcription factor for various genes including those that regulate cell cycle and apoptosis. Evidence suggests that p53 protects cells during stress situations and disturbances in its function often lead to cell damage, transformation, and cancer (Ryan et al., 2001). The intracellular levels of p53 are kept under tight negative control by the HDM-2 protein, which targets p53 for degradation via the proteosome in normal cells. However, cellular stress, such as DNA damage (Kastan et al., 1991), oxidative stress (Achanta and Huang, 2004), and inhibition of transcription (Blagosklonny et al., 2002; Ljungman and Lane, 2004), suppresses HDM-2 action resulting in a dramatic rise of p53 protein levels. Concurrently, several modifications such as phosphorylations and acetylations allow p53 to form a homotetramer, translocate to the nucleus and affect the transcription of target genes (Mansur, 1997), thereby halting the cell cycle and giving the cell time to repair damage. In the event of irreparable damage, p53-dependent apoptosis is initiated (Shen and White, 2001). The balance between pro- and anti-apoptotic factors, and thus the decision for the cell to undergo apoptosis, is in part determined by p53.

DNA damaging agents used in the treatment of cancer are known to affect rates of transcription, and inhibition of RNA polymerase II-dependent transcription by non-specific DNA damage has been suggested to function as a so called “lesion dosimeter” which allows the cell to sense the amount of DNA damage, and to decide upon an appropriate response (Ljungman and Lane, 2004). Inhibition of RNA polymerase II without corresponding physical DNA damage, for example by treatment with actinomycin D (Act D) or 5,6-dichlorobenzimidazole riboside (DRB), is known to trigger

p53 induction and apoptosis (Blagosklonny et al., 2002; Ljungman et al., 1999). However, the signalling mechanisms linking reduction of global RNA synthesis and downstream apoptotic processes remain unclear. The p53 protein can induce apoptosis via either transcription-dependent (through induction of pro-apoptotic genes and down regulation of anti-apoptotic genes) or transcription-independent mechanisms (through cytoplasmic actions such as direct interaction with the mitochondrial surface) (Moll et al., 2005). The mechanisms controlling the cytoplasmic role of p53, particularly during conditions of impaired global transcription, remain a subject of continued scientific interest.

The hnRNPC protein exists in two isoforms, the more abundant hnRNPC1 and its slightly larger splicing variant, including a 13 aa insertion, known as hnRNPC2 (Burd et al., 1989). They form stable heterotetramers which bind mRNA cooperatively, although the functional difference between the two variants remains unclear (McAfee et al., 1996). The hnRNP C1/C2 protein has multiple functions within the eukaryotic cell. It is involved in splicing (Dreyfuss et al., 1993), stabilization of mRNA (Rajagopalan et al., 1998; Shetty, 2005), and in IRES-dependent translation of proteins implicated in cell division and apoptosis (Holcik et al., 2003; Kim et al., 2003).

In this paper we identify a previously unknown regulatory cis-element within the coding region of p53 mRNA, which interacts with hnRNPC1/C2, and we present evidence for its involvement in the expression of p53. We show that hnRNPC1 and C2 bind strongly and specifically, in a phosphorylation dependent manner, at this site in response to DNA damage and inhibition of transcription caused by cytostatic agents. Experiments using a p53-luciferase fusion reporter gene system suggest that expression

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of the construct via this novel cis-site requires cytoplasmic hnRNPC1 binding and is dependent upon the ratio of cytoplasmic hnRNPC1/C2 binding. Our results suggest that this regulatory element and its interaction with the hnRNPC1/C2 proteins is critical for expression of p53, and may be part of the apoptotic process in response to inhibition of global cellular transcription resulting from treatment with cytostatic drugs used in the treatment of cancer.

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Materials and Methods

Cell culture and transfection.

HepG2 human hepatocellular carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA). The HepG2 cells were grown in minimal essential medium (MEM) containing 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and non-essential amino acids. HeLa and MCF-7 cells were obtained from Dr. Dan Lindholm, Uppsala University and propagated in Dulbeccos modified Eagles medium supplemented with 10% fetal calf serum. Primary human hepatocytes (a gracious gift of Dr. Patrick Maurel) were isolated and cultured as previously described (Christian et al., 2004). In short, the cells were isolated from a 79-year-old man who underwent a right lobectomy for a metastasis of a colorectal tumor. Isolation was carried out in accordance with the French National Ethics Committee. The primary hepatocytes were kept in 25 cm^2 collagen-coated flasks. The serum-free culture medium (Pichard-Garcia et al., 2002) was changed every 48 hours. All cell lines were kept in a humidified atmosphere of 5% CO_2. In some cases, the cells were treated with cisplatin (16 μg/ml) dissolved in 0.9% NaCl solution, or 4 μM Act D in DMSO, or carrier only, for the indicated times. All cell culture products were purchased from Invitrogen (Carlsbad, CA).

Reporter plasmid transfection was carried out using Fugene 6 (Roche, Basel Switzerland) according to the manufacturer's recommendations. All transfections included a co-transfected plasmid expressing ß-galactosidase (pCMV- ßgal) for internal control of transfection. HepG2 cells were plated at a density of $4.5x10^4$ cells/cm^2 in

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either 6 or 24-well plates. Treatment was carried out 24 hours post-transfection, and the cells were harvested using reporter lysis buffer (Promega, Madison WI) and snap frozen at –80º C for lysis. Luciferase activities were obtained via luminometer TD 20/20 (Turner Designs) using luciferase assay reagent (Promega, Madison WI) according to the manufacturer's protocol. The resulting values are expressed as RLU/ ßgal activity.

Isolation of cytoplasmic and nuclear proteins.

Protein extracts were prepared as previously described (Christian et al., 2004). In short, the cultured cells were scraped into PBS and centrifuged at 2000 x g for 30 s. The cell pellet was resuspended in buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenymethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.4% Igepal) and incubated at 4° C for 1 h. The cells were homogenized and centrifuged at 12000 x g, 4° C for 10 min. The supernatant (crude cytoplasmic fraction) was stored at -80° C, and the nuclear pellet was resuspended in buffer B (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 420 mM NaCl, 0.5 mM dithiothreitol, 0.2 mM phenymethylsulfonyl fluoride, 0.4% Igepal) homogenized, and centrifuged at 12000 x g for 15 min at 4° C. The supernatant containing the nuclear proteins was stored at –80° C. Protein concentrations were measured using the Lowry method (Lowry et al., 1951) with bovine serum albumin as a standard. Separation between subcellular compartments was confirmed by western blot using antibodies against nuclear or cytoplasmic specific proteins (data not shown).

Synthesis of radiolabeled RNA probes.

The full-length cDNA coding region of human wild type p53 contained within the plasmid pcDNA-p53wt (graciously provided by Dr. M. Gloria Luciani, University of

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Dundee, UK) was amplified in a PCR reaction containing the primers 5'ATGGAGGAGCCGCAGTCAGAT3' (sense) and 5'GGAGAATGTCAGTCTGAGTCA3'(antisense), utilizing Phusion high fidelity thermostable DNA polymerase (Finnzymes, Espoo Finland) according to the manufacturer's instructions. An initial denaturation at 98° C for 30 sec was followed by 35 cycles consisting of 98° C for 10 sec, 49° C for 20 sec, and 72° C for 20 sec. The PCR product was cloned into the pGEM-T vector containing a T7 promoter (Promega, Madison WI) using standard molecular biological techniques. Cloning product and orientation was confirmed using restriction digest, and a series of exonuclease III-digested 3' truncations was created using the Erase-a-Base system (Promega, Madison WI) according to manufacturer's recommendations (Fig. 1). All truncations were sequenced in order to confirm deletion location. The plasmids containing the truncations were linearized 3' of the p53 sequence, and radiolabeled RNA probes were transcribed from the truncations using T7 RNA polymerase in the presence of α-^{32}P-UTP (800Ci/mmol; GE Healthcare, Buckinghamshire UK) according to manufacturer's specifications (Promega, Madison, WI). Following digestion of the DNA template with RQ1 DNase, unincorporated nucleotides were removed via dialysis against water using VSWP 0.025 μm pore size membranes (Millipore, Billerica, MA). A probe corresponding to the 234 nt 3' untranslated region of human *CYP2A6* mRNA was synthesized as previously described for use as a negative binding control in some experiments (Christian et al., 2004). Probes were checked for quality by electrophoresis through a denaturing polyacrylamide urea gel followed by autoradiography.

Construction of the chimeric p53-luciferase reporter gene.

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Using standard molecular biological techniques, the full-length firefly luciferase coding region cDNA obtained from the plasmid pGL3 (Invitrogen, Carlsbad CA) was cloned in frame downstream of 258 bp of the human p53 cDNA containing the entire 5'UTR and part of the p53 coding region in the plasmid TC119832 (Origene Technologies, Rockville MD). The resulting plasmid 5'p53luc (Fig. 8 D) was driven via a CMV promoter. The chimeric protein product contained 57 aa from p53 on the N-terminus, a spacer of 11 aa, and the entire luciferase gene (539 aa) in frame downstream of these amino acids. A T7 RNA polymerase promoter was located 5' of the cloned sequence for in vitro transcription reactions.

Site-directed mutagenesis of the plasmid 5'p53luc and synthesis of RNA probes.

Site directed mutagenesis was performed on several sites within the plasmid 5'p53luc using the GeneEditor site-directed mutagenesis kit according to manufacturer's directions (Promega, Madison, WI). The location of the mutations and sense oligonucleotides used to create the mutations are indicated in table 1. Nucleotide assignment is in accordance with the GeneBank sequence NM_000546. All mutations located within the coding region of p53 were designed to be silent, and were confirmed to lack rare codons.

In order to create radioactive probes of the mutated sequences, the mutated plasmids were linearized 3' of the p53 sequence, and overhanging nucleotides removed with Klenow fragment. The resulting cDNA template contained 258 nt of the p53 5'UTR and coding sequence, corresponding to nt 166 to 426 in the reference sequence NM_000546. This template corresponds exactly to the sequence in Fig. 8 A.

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1 μg of the linearized DNA template was used in an in vitro transcription reaction as described above. The unincorporated nucleotides were removed using a G-50 column (GE Healthcare, Buckinghamshire UK) according to the manufacturer's protocol. Equimolar amounts of the non-mutated and site directed-mutated RNA probes were then used in UV-crosslinking reactions using equal amounts of total cytoplasmic proteins from Act D treated (6hrs) HepG2 cells.

All bound proteins were separated on the same SDS-page gel, dried, and exposed to x-ray film for visualization. The strength of individual signals was measured quantitatively via scanning of the original film, and density readings of hnRNPC1 and C2 complex taken using the program Scion Image for Windows (Scion Corp, Worman's Mill Ct.).

RNA secondary structure prediction.

The sequence corresponding to probe E (Fig. 1), or this region including the 5'UTR (data not shown) were input into M-fold version 3.2 RNA secondary structure prediction program (Zuker, 2003), using the web-based server at Rensalaer Polytechnic Institute (http://www.bioinfo.rpi.edu/applications/mfold).

UV cross-linking.

Binding reactions using nuclear or cytoplasmic extracts and the indicated RNA probes were performed as previously described by Geneste et al. (Geneste et al., 1996). Reactions were irradiated with UV-light at 5225 μJ/cm^2 in a Spectrolinker XL-1000 UV cross-linker (Spectronics, Westbury, NY). Free RNA was digested using 2 μg of RNase A (Invitrogen, Täby, Sweden) at 37° C for 20 min. The samples were denatured under non-reducing conditions at 95° C for 10 min, separated via 12% SDS-PAGE, and

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visualized by autoradiography. In some cases, the cell extract was pretreated with proteinase K or potato acid phosphatase (nr. 108227; Roche Applied Science, Indianapolis, IN), for the indicated times before crosslinking was performed. In all cases, the amount of radioactive RNA probe was quantitated via scintillation, and master mixes of components in the UV-crosslinking reaction were used to ensure equal protein and RNA probe amounts in the reactions. All experiments comparing strength of the resulting RNA-protein complex signal or experiments comparing apparent molecular weight of complexes were separated on the same gel and exposed to the same film to ensure accuracy.

Immunoprecipitation of the RNA/protein complexes.

Immunoprecipitiation was performed essentially as described previously by Hamilton et al. (Hamilton et al., 1993). In brief, UV crosslinked samples were performed as indicated, and added to antibody binding buffer containing 1μl of 4F4 monoclonal hnRNPC1/C2 specific antibody (Sigma Aldrich AB, Tyresö, Sweden), 1μl of monoclonal hnRNP I antibody (Zymed Laboratories, San Francisco, CA), 2 μl of polyclonal anti-hnRNPA1 antiserum, 2 μl of pre-immune serum, or no antibody. Immunoprecipitation was carried out with protein A sepharose beads (GE Healthcare, Buckinghamshire UK). The samples were washed 5 times with PBS, and the immunoprecipitated complexes were denatured, separated on a 12% SDS-PAGE gel, and visualized using autoradiography.

Western blot assay.

Denatured samples were separated on a 4% stacking, 12% separating SDS- PAGE gel. The separated proteins were transferred to a nitrocellulose membrane (Hybond ECL;

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GE Healthcare, Buchinghamshire UK), and blocked with 5% milk, 0.1% Tris-buffered saline-Tween 20. The primary antibodies were monoclonal anti-p53 (DO-1), HSP-90 (F-8), poly (ADP-ribose) polymerase (PARP F-2), or polyclonal anti-hnRNPC1/C2 (H-105) (Santa Cruz Biotechnology, Santa Cruz CA). Detection was performed using ECL reagents according to manufacturers recommendations (GE Healthcare, Buckinghamshire UK).

Statistical analysis.

Statistical analysis of luciferase data was carried out with Minitab v.14 statistical software (Minitab Inc, State College PA) using a general linear model ANOVA. Statistical significance was assumed at $p<0.01$(**), while $p<0.001$ is denoted as "***".

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Results

Several cellular proteins interact with the coding region of human p53 mRNA.

To identify proteins interacting with the coding region of human p53 mRNA, UV crosslinking experiments were performed using nuclear or cytoplasmic protein extracts from untreated HepG2, HeLa cells or primary human hepatocytes and the full length coding region of human p53 as a radioactively labelled probe (probe A, Fig. 1).

The results showed several complexes of varying size and sub-cellular localization (Fig. 2 A). A strong, approximately 41 kDa, nuclear complex appeared in all cell types. Complexes with apparent sizes of 90, 78, 44 and 34 kDa were also observed. The 90 and 78 kDa complexes were mainly cytoplasmic in HepG2 and nuclear in HeLa cells. The 44 kDa complex was primarily nuclear in both HepG2 and HeLa cells, but was absent or weak in primary hepatocytes.

The intensity of the 44 and 41 kDa complexes increased with increasing protein concentration, and all complexes were destroyed by proteinase K pre-treatment of extracts (data not shown), showing that the complexes include proteins.

Formation of the 41 and 44 kDa complexes is influenced by cisplatin.

In order to determine if the RNA-protein complexes are influenced by conditions known to induce p53-dependent apoptosis, HepG2 cells were treated with 16 μg/ml cisplatin. This concentration is thought to be encountered during treatment of hepatocellular carcinoma, and is known to induce both p53 protein levels and result in apoptosis in HepG2 cells (Qin and Ng, 2001).

Treatment for 6 hours markedly increased the intensity of the 41 and 44 kDa complexes obtained with both nuclear and cytoplasmic extracts (Fig. 2 B). The increase

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appeared to be specific to these protein complexes, as the others remained unaffected by cisplatin treatment. These results indicate that a cytostatic agent known to halt the cell cycle and induce p53-dependent apoptosis stimulates the interaction of the 41 and 44 kDa complexes with p53 mRNA.

Inhibition of transcription induces formation of the 41 and 44 kDa complexes.

One of the suggested effects of cisplatin is to interfere with transcription through DNA adduct formation (Jung and Lippard, 2006). To investigate the possible role of transcriptional inhibition on the formation of the 41 and 44 kDa complexes, HepG2 cells were exposed to 4 μM of Act D, a potent transcriptional inhibitor widely used in the treatment of cancer (Green et al., 2006; Hauer et al., 2006). This concentration of Act D is known to fully prevent RNA polymerase II- dependent cellular transcription (Perry and Kelley, 1970) thereby inducing p53 levels (Ljungman et al., 1999), as well as p53-dependent apoptosis.

Within 6 hours of exposure, a massive increase of both the 41 and 44 kDa complexes took place (Fig. 3 A). The induction was much greater than that observed with cisplatin treatment, and was most marked with cytoplasmic, but also clearly evident with nuclear extracts. As with cisplatin, complexes other than the 41/44 kDa remained apparently unaffected by Act D. Maximal binding intensity in the cytoplasm was reached at 24 hours post-treatment, followed by a decrease at 48 hours. In contrast, the corresponding nuclear complex remained at maximal level at 48 hours. These results suggest that inhibition of transcription is sufficient to strongly and specifically induce the binding of the 41 and 44 kDa complexes to p53 mRNA.

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The binding of the complexes to p53 mRNA is phosphorylation dependent.

In order to test the phosphorylation dependence of the 41/44 kDa complexes, protein extracts from Act D treated cells were pre-treated with increasing concentrations of potato acid phosphatase (PAP) and subjected to UV-crosslinking.

As shown in Fig. 3 B, pre-treatment of extracts with amounts of phosphatase above 0.2 U for 20 minutes resulted in a complete loss of binding activity suggesting that the interaction is highly phosphorylation-dependent in both the cytoplasmic and nuclear compartments. Notably, the use of low phosphatase doses revealed differences between the subcellular fractions. Whereas in the cytoplasm, 41/44 kDa binding activity increased in response to lowest phosphatase dose, a substantial loss of binding activity was seen when the nuclear extract was treated with the same dose.

In conclusion, the results show that the 41/44 kDa proteins binding to p53 mRNA are strongly regulated by phosphorylation either directly, or through other cellular proteins that affect the binding.

Identification of the 41/44 kDa complexes as hnRNPC1/C2.

The apparent size, subcellular localization, and RNA binding characteristics led us to believe that the 41 kDa complex might contain hnRNPC1.

To investigate this possibility, immunoprecipitiation with the monoclonal antibody 4F4 specific to hnRNPC1/C2 was performed. The UV-crosslinked nuclear or cytoplasmic complexes were incubated with monoclonal antibodies against hnRNPC1/C2, hnRNP I (polypyrimidine tract binding protein), polyclonal anti-hnRNPA1 antiserum, pre-immune serum or no antibody. We observed that only the use

of the hnRNPC1/C2 specific antibody resulted in an immunoprecipitiation of both the 41kDa and the 44 kDa complexes (Fig. 4 A).

These results suggest that the 41 kDa protein contained within nuclear and cytoplasmic extracts of HepG2 cells is in fact hnRNPC1, and the 44 kDa protein may be the slightly larger splice variant of hnRNPC1 known as hnRNPC2.

The actinomycin D-induced binding of hnRNPC1 to p53 mRNA is not cell-specific.

In order to investigate if the Act D-inducible binding of hnRNPC1/C2 to human p53 mRNA was specific to HepG2 cells, primary human hepatocytes were treated with Act D and nuclear extracts used in a UV-crosslinking assay (Fig. 4 B).

The results revealed a very similar increase of the 41 kDa complex to that observed in HepG2 cells. A single band was observable in the primary hepatocytes, and was identified as the 41 kDa protein (data not shown). This protein was identified by immunoprecipitation as hnRNPC1 (Fig. 4 B).

These results confirm that the increased binding activity of hnRNPC1 to the coding region of p53 mRNA upon transcriptional inhibition is not specific to HepG2 cells, and is conserved in primary, non-transformed, human cells.

Inhibition of transcription leads to an apparent partial nucleo-cytoplasmic translocation of hnRNPC and enhances its affinity towards p53 mRNA.

The next series of experiments were designed in order to investigate if differences in the protein levels of hnRNPC1/C2 were responsible for the strongly increased binding to p53 mRNA in response to transcriptional inhibition. For that purpose, a western blot analysis was performed on nuclear (Fig. 5 A) and cytoplasmic (Fig. 5 B) proteins from untreated and Act D-treated HepG2 cells. Monoclonal antibodies, specific for

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hnRNPC1/C2 as well as for HSP-90 and poly-ADP-ribose polymerase (PARP) were used. HSP-90 and PARP-1 served as both internal loading and sub-cellular fractionation controls.

The results showed that while the nuclear levels of hnRNPC1/C2 were unchanged or in some experiments slightly decreased upon treatment, the cytoplasmic levels were increased. The initial increase in the cytoplasmic hnRNPC1/C2 levels began to subside by 48 hours (Fig. 5 B). This decrease correlated with the appearance of an 85 kDa fragment of PARP (Fig. 5 A) in the nucleus at 24 and 48 hours. The appearance of the PARP fragment is a sign of caspase 3 activation as part of apoptotic processes (Kaufmann et al., 1993). Therefore, the results suggest that the cytoplasmic increase of hnRNPC1/C2 precedes caspase 3 activation in Act D-treated HepG2 cells.

Notably, changes in the cytoplasmic and nuclear hnRNPC1/C2 protein level in response to Act D treatment do not account for the observed changes in RNA binding activity to p53 mRNA. Indeed, the increase in mRNA binding activity in the cytoplasm is massive compared to the modest increase in the protein levels, and the mRNA binding activity in the nucleus is increased despite a modest decrease in the levels of hnRNPC1/C2.

A 43 kDa, p53-related protein appears in the cytoplasm of Act D-treated cells.

In order to confirm the subcellular localization of p53 after Act D treatment, nuclear or cytoplasmic extracts were analyzed by Western blotting (Fig. 5 A and B) using a monoclonal antibody specific for p53 (DO-1). The DO-1 antibody is directed against amino acids 11-25 of the N-terminus of human p53 (Fig. 6).

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As expected, the treatment of HepG2 cells with Act D led to a dramatic translocation of p53 from cytoplasm to the nucleus by 6 hours. This effect was less pronounced at the 24 and 48-hour time points.

Notably, Act D treatment resulted in the transient appearance of a 43 kDa protein in the cytoplasm at 6 hrs post-treatment, recognized by the monoclonal antibody DO-1 (Fig. 5 B). The 43 kDa protein was not visible in nuclear extracts and was not visible in the cytoplasm by 24 to 48 hours of Act D treatment.

Mapping and specificity of the hnRNPC1/C2 binding site on the coding region of p53 mRNA.

In order to determine the binding site(s) of hnRNPC1/C2 on the p53 mRNA, UV-crosslinking reactions were performed with truncated probes corresponding to various fragments of the p53 mRNA coding region (Fig. 1) and extracts from HepG2 cells treated with Act D for 24 hrs.

The binding activity of hnRNPC1/C2 was retained in all truncations (data not shown for larger truncations) as shown in Fig. 7 A. These data suggest that the main binding site for hnRNPC1/C2 is located within the region of p53 mRNA covered by probe E, a 101nt sequence at the extreme 5' end of the p53 coding mRNA. Identical results were obtained using protein extracts from cisplatin-treated cells (data not shown).

In order to investigate the specificity of hnRNPC1 binding to p53 mRNA during Act D induced conditions, a UV- crosslinking reaction was performed using an unrelated 234 nt RNA probe (CYP2A6) and the resulting binding pattern was compared with that of the p53 probe A. The unrelated probe lacked the ability to bind hnRNPC1 using Act D

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induced human hepatocyte nuclear extracts, as shown by the lack of a 41 kDa RNA-protein complex (Fig. 7 B).

To further characterize the interaction of hnRNPC1/C2 with the p53 mRNA, the full-length, radioactive p53 coding region RNA (probe A) was incubated with varying concentrations of non-radioactive competitors E or F (Fig. 7 C) and UV-crosslinked with Act D-treated HepG2 cytoplasmic extracts. Probe F corresponds to nucleotides 435-536, downstream of probe E within the p53 mRNA (Fig. 1). The results presented in Fig. 7 C show that equimolar concentrations of the non-radioactive probe E is superior to probe F at competing the hnRNPC1/C2 signal, suggesting binding specificity to probe E.

The primary binding site for hnRNPC1/C2 is a U-rich sequence 55 nt downstream the start codon.

To confirm previous mapping results, and to more precisely map the location of hnRNPC1/C2 binding to p53 RNA, several potential binding sites were modified using site-directed mutagenesis. HnRNPC proteins are known to bind U-rich sequences (Dreyfuss et al., 1993). Therefore, one such sequence within the 5'UTR (site A), and two downstream of the first known start codon (sites B and C) were modified (Fig. 8 A). The mutated probes were radiolabeled and used in UV- crosslinking experiments using Act D-treated extracts.

Cytoplasmic hnRNPC1/C2

As shown in Fig. 8 B, mutation at site A resulted in a slight decrease in binding activity (lane 2) of hnRNPC1. However, mutation at site B (lane 3) consisting of nucleotide U308, almost completely abolished binding of hnRNPC1 to p53 mRNA.

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Notably, the binding of cytosolic hnRNPC2 towards p53 mRNA appeared to be less sensitive to the mutations than hnRNPC1.

Nuclear hnRNPC1/C2

In the wild type and C mutant, (WT and C, lanes 5 and 8) no visible hnRNPC2 binding was evident in the nucleus. In contrast, hnRNPC2 binding was evident in mutants A and B. HnRNPC1 binding appeared to be particularly strong in the nuclear compartment to mutant A, but was otherwise not affected by the mutations.

In summary, these data suggest that two poly U elements at the 5´ end of p53 mRNA are of importance for the cytoplasmic binding of hnRNPC1/C2. The major binding site is located within probe E (see Fig. 1) and consists of four U nucleotides downstream of the start codon (site B, Fig. 8 A). In particular, nucleotide U308 is pivotal for binding of hnRNPC1/C2. Another, minor, site contains three U nucleotides and is situated upstream of the start codon (site A, Fig. 8 A). In addition, the binding characteristics of the two proteins towards the mutated sequences appear to differ in the cytoplasmic and nuclear compartments.

Prediction of the secondary structure of probe E.

The predicted secondary structure for probe E shows an ordered secondary conformation. Within this conformation the principal hnRNPC1/C2 binding site resides on a hairpin loop (Fig. 8 C). The predicted structure is preserved regardless of the presence of extraneous sequences, such as 5' UTR and extra coding region (data not shown), supporting the idea that the predicted secondary structure hairpin may be locally stable.

Mutation of the hnRNPC1/C2 binding site downregulates gene expression.

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The sequence denoted in Fig. 8 A, corresponding to 258 nt of the intact p53 5'UTR and coding region, and point-mutated sequences A, B, and C described above were cloned in front of a luciferase reporter gene (Fig. 9 A). The constructs were transfected into HepG2 cells and 24 hours post transfection Act D was added to the cells for 6 hours. The point mutations were designed to be silent, therefore the amino acid sequence of the resulting chimeric protein was unchanged. In addition, codon usage tables were consulted to ensure the lack of rare codons in the silent mutations.

Fig. 9 B shows the effects of the mutations on the luciferase activity in Act D-treated cells. A point mutation changing a single U nucleotide (U308), critical for hnRNPC binding, (mutation B) significantly reduced the gene expression by approximately 50%, while a mutation upstream (mutation A) upstream of this critical site resulted in a less pronounced but still significant ($P<0.01$) decrease in luciferase expression when compared with the wild type reporter. Mutation C, located downstream of these sites, did not cause a significant change of the reporter gene expression.

These results suggest that the hnRNPC binding sites located at site A in the 5'UTR, and in particular, site B in the coding region are regulatory elements playing a role in p53 expression.

The ratio of cytoplasmic hnRNPC1 to hnRNPC2 binding towards p53 mRNA correlates with luciferase expression.

The two hnRNPC proteins are known to interact, and bind cooperatively (McAfee et al., 1996). Therefore, in order to better understand the relationship between the binding activity of hnRNPC1/C2 to the p53 RNA sequence and luciferase expression of the chimeric reporter gene, the signal density of hnRNPC1 or hnRNPC2 (Fig. 8 B) in both

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the cytoplasm and nucleus of Act D treated cells was quantitated using the program Scion Image. A correlation between the ratio of cytoplasmic hnRNPC1 to hnRNPC2 binding and the expression of the mutants was observed (Fig. 9 C). In contrast, no such correlation was found in the nuclear compartment (data not shown).

These results suggest that not only the absolute binding activity of cytoplasmic hnRNPC1, but also the relative binding activity of hnRNPC1 and hnRNPC2, is important for the expression of the p53 reporter gene.

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Discussion

This paper describes identification and mapping of a previously unknown regulatory element of the p53 gene: a cis-site located in a putative hairpin loop structure in the coding region of human p53 mRNA. This site binds hnRNPC1/C2 with high affinity in a phosphorylation-dependent manner. Binding is up regulated by the DNA-damaging drug cisplatin and by the transcriptional inhibitor Act D, both strong inducers of the p53 protein and apoptosis. Mutations that reduce the binding affinity of hnRNPC1/C2 towards the mRNA decrease the expression of a luciferase reporter gene.

Previous studies have shown that p53 is regulated via post-transcriptional mechanisms, including translational control of p53 mRNA via proteins binding in its 3' and 5' UTR (for a review, see (Ryan et al., 2001). Increased translation of p53 mRNA in response to ionizing (Fu and Benchimol, 1997) and UV radiation (Mazan-Mamczarz et al., 2003) has also been described. During apoptosis, there is a reduction of normal cap-dependent translation due to the cleavage of several initiation factors via caspases (Spriggs et al., 2005). However, many mRNAs necessary for the apoptotic process continue to be translated via internal ribosome entry site (IRES)-dependent mechanisms. Recently, one such IRES segment in the 5'UTR of p53 mRNA was shown to increase translation of p53 mRNA in response to the DNA-damaging agent etoposide (Yang et al., 2006). In addition, the RNA- binding protein HuR was found to stabilize p53 mRNA by binding the 3'UTR in response to polyamine depletion (Zou et al., 2006). Thus, it is clear that mRNA binding proteins play an important role in p53 expression during cellular stress by both modifying translation and mRNA stability.

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Our present findings that the interaction of hnRNPC1/C2 with a regulatory site within the coding region of p53 mRNA is strongly activated by both transcriptional inhibition and DNA damage reveal that this site may be part of the machinery that controls p53 expression in pre-apoptotic and apoptotic conditions where transcription is disturbed.

The doses of cisplatin used in this study are known to create inter- and intra-strand crosslinks and adducts on DNA, thus interfering with transcription (Jung and Lippard, 2006). On the other hand, Act D was used in concentrations known to shut down global cellular transcription, yet not induce discernable DNA damage (Ljungman et al., 1999). Both induce a very rapid change of cytoplasmic hnRNPC1/C2 binding to p53 mRNA. The speed of this activation suggests that it may be part of the cell's acute response to disturbed transcription.

Accordingly, it has previously been shown that DNA-dependent protein kinase (DNA-PK) phosphorylation of hnRNPC is dependent upon intact cellular RNA in the protein extracts (Zhang et al., 2004). Therefore, it is plausible that the phosphorylation state of hnRNPC1/C2, and consequently its interaction with p53 mRNA are affected by changes in global RNA synthesis.

We observed an apparent partial nucleo-cytoplasmic translocation of hnRNPC1/C2 in response to Act D treatment, concurrent with a massively increased affinity towards p53 mRNA. HnRNP C1/C2 has been until recently thought to be strictly nuclear in non-damaged interphase cells (Nakielny and Dreyfuss, 1996). However, it has been found as an AU-rich sequence binding factor in the cytoplasm of mouse lymphocytes (Hamilton et al., 1993), and to affect the translation of XIAP and c-myc

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mRNA (Holcik et al., 2003; Kim et al., 2003), suggesting cell cycle and apoptotic roles in the cytoplasm. Recent studies indicate that this protein is actively translocated to the cytoplasm in response to apoptotic signals in leukemia cell lines induced with PMA (phorbol-12myristate-13-acetate) or TNF-α (Lee et al., 2004). Similar to these studies, we observe a cytoplasmic increase of hnRNPC1/C2 in HepG2 cells in response to Act D prior to any cleavage of PARP-1 via caspase 3 (Fig 5 A and B). Importantly, we observe no contamination of nuclear proteins in the cytoplasmic extracts (data not shown), showing that the nuclear envelope integrity has been maintained. Our observations suggest that inhibition of transcription or DNA damage induced pre-apoptotic signals may be a caspase-independent trigger for the partial translocation of hnRNPC1/C2 to the cytoplasm.

The presence of hnRNPC1/C2 in the cytoplasm is a prerequisite for cytoplasmic binding to p53 mRNA. Nevertheless, our results indicate that it is the massive increase in binding affinity for p53 mRNA that is central for the observed response to transcriptional arrest.

Our experiments indicate that the stress response shown by hnRNPC1/C2 is phosphorylation dependent, and that the pattern of phosphorylation most likely differs between the cytoplasmic and nuclear compartments. Several kinases are known to phosphorylate hnRNPC, including CK1-α in response to physiological levels of hydrogen peroxide (Kattapuram et al., 2005) composing survival signals for the cell, and DNA-PK, a well known DNA damage control kinase (Zhang et al., 2004). Clearly, changes in the phosphorylation state of hnRNPC1/C2 are important for its function in response to survival or stress stimuli.

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We observed an exclusively cytoplasmic 43 kDa protein, recognizable by the anti-p53 antibody DO-1. The protein, referred to by us as p43, appears transiently upon 6 hours of Act D treatment, simultaneously with the massively increased mRNA binding activity of hnRNPC1/C2, and concurrent with the expected full-length p53 translocation to the nucleus. Several isoforms of p53 have been described which are a result of both N-terminal and C terminal truncations of the protein. While N-terminal truncations appear to be the result of alternative translation start sites (Fig. 6), the known C terminal truncations are thought to be due to alternative splicing of the transcript within intron 9. For a comprehensive review of known isoforms, see (Mills, 2005).

The antibody DO-1 used in immunoblots presented here recognizes the N-terminus of p53. Thus, the observed protein p43 cannot be any of the known N-terminal truncations. Therefore, the p53-related protein appears to be either a result of internal, or possibly C-terminal truncation of p53.

Several alternatively spliced isoforms with similar sizes as the present p53 variant resulting from truncated and modified C terminal amino acid sequences have been previously described (Mills, 2005). Of note, hnRNPC1/C2 is known to affect splicing (Dreyfuss et al., 1993). Given the highly changed binding activity of hnRNPC towards p53 mRNA in the nucleus, it can be envisaged that hnRNPC is involved in altered splicing of p53 pre-mRNA. However more work is needed to elucidate this possibility.

The hnRNP C1/C2 protein has multiple functions within the eukaryotic cell. It is involved in splicing, stabilization of mRNA (Rajagopalan et al., 1998; Shetty, 2005), and in IRES-dependent translation (Holcik et al., 2003; Kim et al., 2003). In addition, hnRNPC has been found to bind chromatin in response to DNA damage (Lee et al.,

2005), showing that hnRNPC1/C2 responds to cellular perturbation, in accordance with our results. Further, hnRNPC1/C2 was identified to bind PARP-1, a protein necessary for the translocation of the apoptosis-inducing factor (AIF) from the mitochondria to the nucleus (Yu et al., 2002), and previously known to bind to p53. These results anchor hnRNPC1/C2's role in apoptotic processes.

We identified a "hotspot" for hnRNPC1/C2 binding activity where a single point-mutation (U308C) abolished hnRNPC1 binding in the cytoplasm of Act D-treated cells resulting in an approximate 50% decrease in reporter gene luciferase expression. Notably, when the ratio of hnRNPC1 to hnRNPC2 binding towards p53 RNA in the cytoplasm was measured, a correlation between these data and luciferase expression of the mutated reporter genes was found. The binding activity of hnRNPC1 and hnRNPC2 is known to be cooperative in nature, possibly providing an explanation as to why the relative amounts of the proteins may affect binding.

The evidence presented here also indicates an upstream binding site in the 5'UTR of p53, located at the site of mutation A, that also contributes, albeit less markedly, to both hnRNPC1 binding in the cytoplasm, and a corresponding decrease in luciferase expression. We therefore propose that the critical mutation U308C, and to a lesser extent the 5'UTR mutation, T192G, result in a perturbation of the natural hnRNPC1 and hnRNPC2 interaction on p53 mRNA, thus altering the expression of p53.

In summary, the data presented here suggest a cytoplasmic role for hnRNPC1/C2 in the expression of p53. Our preliminary siRNA knockdown experiments against hnRNPC1/C2 confirm an important role of hnRNPC in regulating the expression of p53,

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and suggest a complex mechanism involving at least two distinct regulatory pathways. The molecular mechanisms involved are currently under investigation in our laboratory.

Our report presents hnRNPC1/C2 as a novel link between transcriptional arrest and p53 activation. It seems, therefore, that hnRNPC1 and its splice variant hnRNPC2 can now be considered as cytostatic drug activated "stress responsive multifunctional proteins". In particular, our data support a model where hnRNPC1/C2 respond to pre-apoptotic signals induced by inhibition of global cellular transcription, by partially translocating to the cytoplasm and specifically binding p53 mRNA at a regulatory cis-site.

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Footnotes

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Figure Legends

Fig. 1. Truncations of the full-length human p53 coding region cDNA. The probes resulting from in vitro transcription are indicated. Probe A corresponds to the full-length coding region. The location of the U rich sequence within probe E, identified as the primary binding site for hnRNPC1/C2, is shown as "*".

Fig. 2. Formation of RNA-protein complexes with the coding region of p53 mRNA **(A)** UV- crosslinking experiments showing proteins binding to the human p53 coding region in various cell types. The radioactive probe A (full-length coding region) was crosslinked to untreated cellular extracts from the indicated cell types. Cyt, cytoplasmic extracts, Nuc, nuclear extracts. The apparent molecular weights of the protein/RNA complexes are indicated. **(B)** Effect of cisplatin on the 41 and 44 kDa complexes. HepG2 cells were treated (+) or untreated (-) for 6 hours with 16 μg/ml cisplatin. 5 μg of nuclear (Nuc), and 10 μg of cytoplasmic (Cyt) extracts are used in the indicated UV-crosslinking experiments with probe A. The 41/44 kDa complexes are indicated.

Fig. 3. (A) The Effect of Act D on the 41 and 44 kDa complexes. The cells were harvested after exposure to 4 μM Act D (+) or carrier only (-) for the times indicated. UV-crosslinking was performed using 5 μg total protein from the indicated cell extract, and identical amounts of the full-length p53 coding region probe (probe A). **(B)** The Act D-induced binding of the complexes to p53 mRNA is phosphorylation-dependent. Cytoplasmic (Cyt) or nuclear (Nuc) extracts from Act D-treated HepG2 cells (24h) were pre-incubated for 20 min with the indicated amounts of potato acid phosphatase (PAP)

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and UV-crosslinked to probe A. The lanes marked "0" represent conditions with no phosphatase activity and are provided as negative controls.

Fig. 4. Identification of the 41 and 44 kDa complexes as hnRNPC1/C2 in transformed and normal cells. **(A)** The 41 and 44 kDa complexes formed with probe A in HepG2 Act D-treated (24h) cytoplasmic (Cyt) or nuclear (Nuc) extracts were immunoprecipitated using monoclonal antibodies against hnRNPC1/C2 (C), hnRNP I (I), no antibody (-), polyclonal immunoserum against hnRNPA1 (A1), or pre-immune serum (0 serum). A standard UV-crosslinking reaction carried out with no IP is shown (CTR). **(B)** Nuclear extracts from primary human hepatocytes treated with Act D for 24h (+), or carrier only (-) were UV- crosslinked (UVXL) to probe A. The UV-crosslinked complexes were identified by immunoprecipitation (IP) using monoclonal antibodies against either hnRNPC1/C2 (C) or hnRNP I (I).

Fig. 5. Effects of Act D on p53 and hnRNPC1/C2 levels in the nucleus and cytoplasm of HepG2 cells. Shown are western blots (WB) and comparison to UV-crosslinking reactions (UVXL) from cells treated with Act D (+) or carrier only (-) for the indicated times. The proteins are indicated. **(A)** Nuclear compartment. The 85 kDa fragment of PARP-1 cleavage with Caspase 3 is indicated. **(B)** Cytoplasmic compartment. The p53-related protein is indicated by "p43".

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Fig. 6. Partial amino acid sequence of human p53. The epitope for the monoclonal antibody DO-1 is underlined. The known primary and alternative translational start sites for p53 are indicated as bold "**M"**.

Fig 7. (A) Mapping of the primary binding site of hnRNPC1/C2 to p53 mRNA. Two truncations of the full-length probe are shown. The smallest truncation, probe E, shows unchanged binding characteristics towards hnRNPC1/C2 in UV-crosslinking experiments using cytoplasmic (Cyt) or nuclear (Nuc) extracts from Act D-treated HepG2 cells (24h). **(B)** An unrelated sequence does not bind Act D induced hnRNPC1. A UV-crosslinking reaction was performed using a probe corresponding to the human CYP2A6 3'UTR (2A6), or p53 probe A (p53) and nuclear extracts from Act D treated primary human hepatocytes. The expected location of hnRNPC1 is indicated. The locations of the protein molecular weight standards are shown. **(C)** The binding of hnRNPC1/C2 to the full-length probe A is specific to region E. The unlabeled probes E or F were added to UV-crosslinking reactions in the molar excess indicated as competitors in the presence of radiolabeled probe A. UVXL was carried out using Act D-activated (24 hrs) cytoplasmic extracts.

Fig. 8. Fine mapping of the hnRNPC1/C2 binding sites. **(A)** The 258 nt sequence used to make the probes in UV-crosslinking experiments. The 5'UTR is in italics, and the first known start codon for p53 is indicated. The locations of mutations A, B, and C are indicated in bold. All numbers refer to the corresponding nucleotides in the GeneBank reference sequence NM_000546. The critical point mutation for cytoplasmic binding,

mutation B, is circled. The sequence corresponding to probe E is underlined. **(B)** UV-crosslinking experiments using the mutated sequences and Act D-induced cytoplasmic (Cyt) or nuclear (Nuc) extracts. The complexes formed with equimolar amounts of the radioactive non-mutated probe (WT), and probes containing mutation A, B or C are indicated. **(C)** Probe E forms a stable predicted secondary structure. The critical nucleotide for cytoplasmic hnRNPC1/C2 binding (U308), identified via site-directed mutagenesis is indicated.

Fig. 9. An element essential for expression of p53 is located at site B. **(A)** A schematic of the 5'p53luc (WT) reporter gene. The transcription start site, location and size of the cDNA sequences, and the resulting chimeric gene product are indicated. The p53 cDNA sequence indicated in the reporter gene corresponds to that given in Fig. 8A.
(B) Expression of the mutants is given as relative light units (RLU) corrected for ß-galactosidase (transfection control) activity. 24 hrs after transfection, HepG2 cells were treated with 4μM Act D for 6hrs. The non-mutated reporter gene 5'p53luc is indicated by "WT". "A", "B", and "C" refer to the previously described mutants (see Fig. 8 A). Error bars indicate standard deviation of the mean for the experiment (n = 8). Statistical significance of $p < 0.01$ is denoted as "**" while $p < 0.001$ is "***" **(C)** The relative binding activity of hnRNPC1 to hnRNPC2 in the cytoplasm correlates with expression of the site directed mutations. The film from figure 8 B was read for signal density and the ratio of cytoplasmic hnRNPC1/hnRNPC2 complex intensity was calculated for each mutant and plotted on the y-axis.

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Tables

Table 1.

Sense oligonucleotides used for site directed mutagenesis

Mutant	**Mutation (*)**	**Sense Mutagenic Oligonucleotide (5'→3')**
A	T192G	GGAGCGTGCTGTCCACGACGG
B	T308C	CAGGAAACATTCTCAGACCTATG
C	T378C, G380A	GCAATGGATGATCTAATGCTGTCCCCGG

(*) numbers refer to nucleotide sequence NM_000546, NCBI GeneBank

Fig. 1

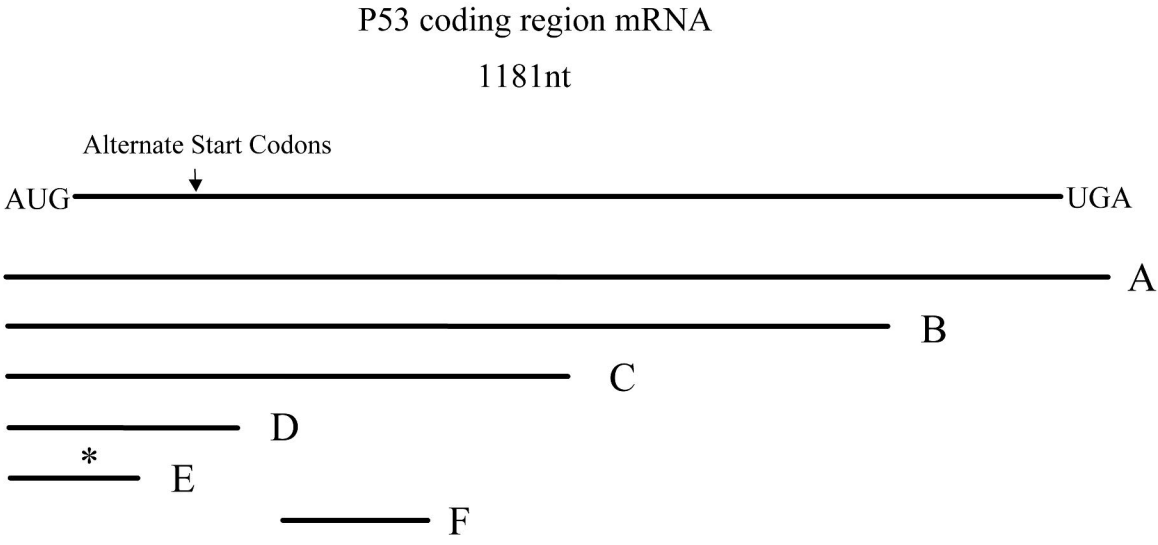

P53 coding region mRNA
1181nt
Alternate Start Codons
AUG
UGA
A
B
C
D
*
E
F

Fig. 2

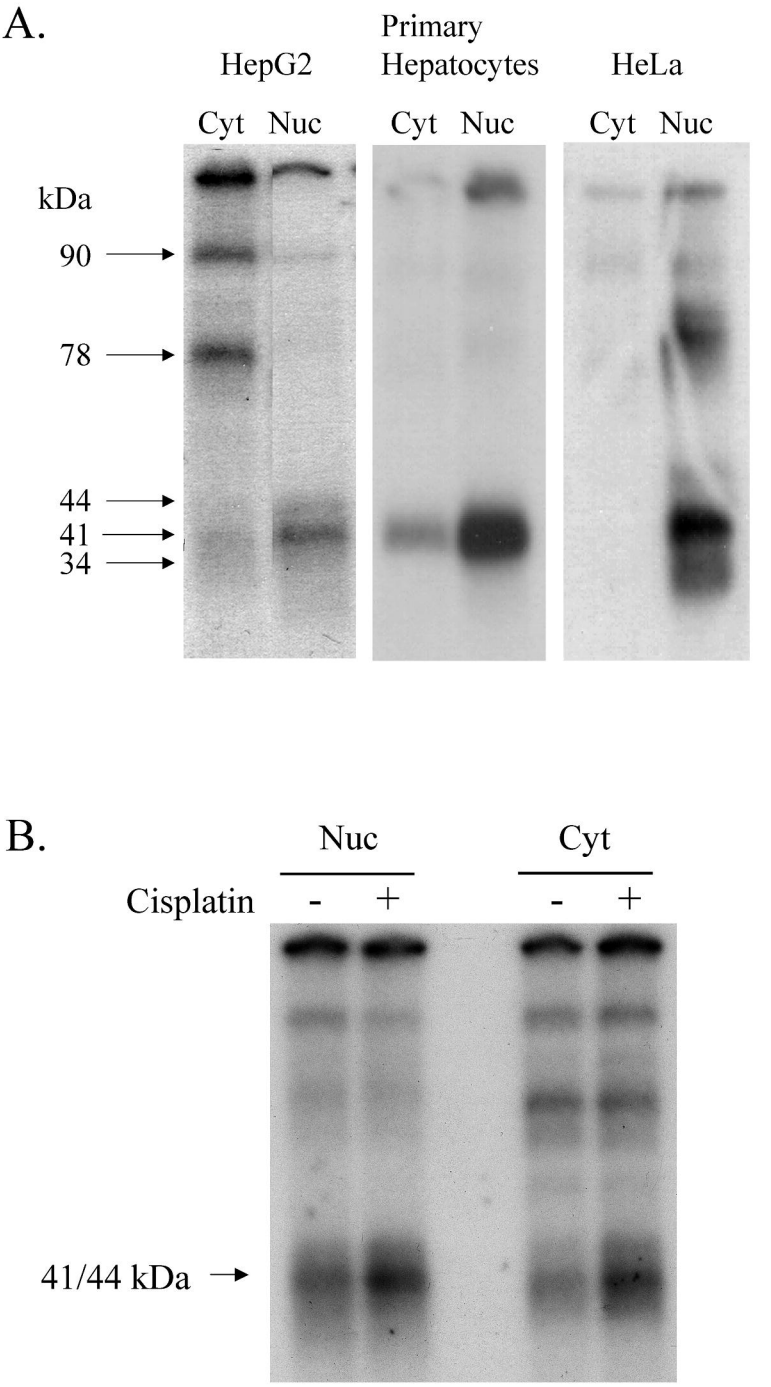
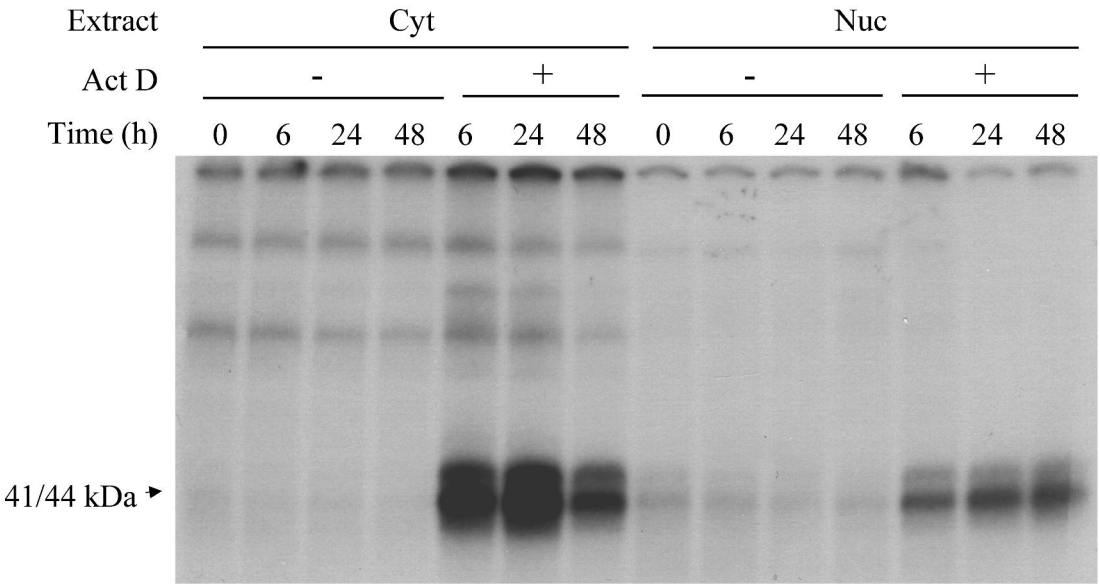
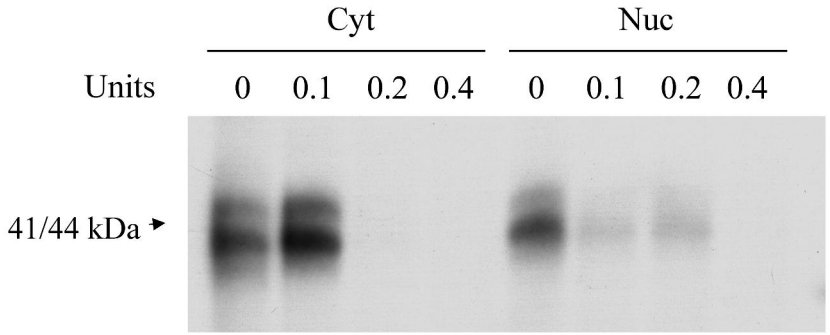

A.
HepG2
Primary Hepatocytes
HeLa
Cyt Nuc
Cyt Nuc
Cyt Nuc
kDa
90
78
44
41
34
B.
Nuc
Cyt
Cisplatin
-
+
-
+
41/44 kDa

Fig. 3

A.


Extract
Cyt
Nuc
Act D
-
+
-
+
Time (h)
0 6 24 48 6 24 48 0 6 24 48 6 24 48
41/44 kDa

B.


Cyt
Nuc
Units
0 0.1 0.2 0.4 0 0.1 0.2 0.4
41/44 kDa

Molecular Pharmacology Fast Forward. Published on February 22, 2008 as DOI: 10.1124/mol.107.042507
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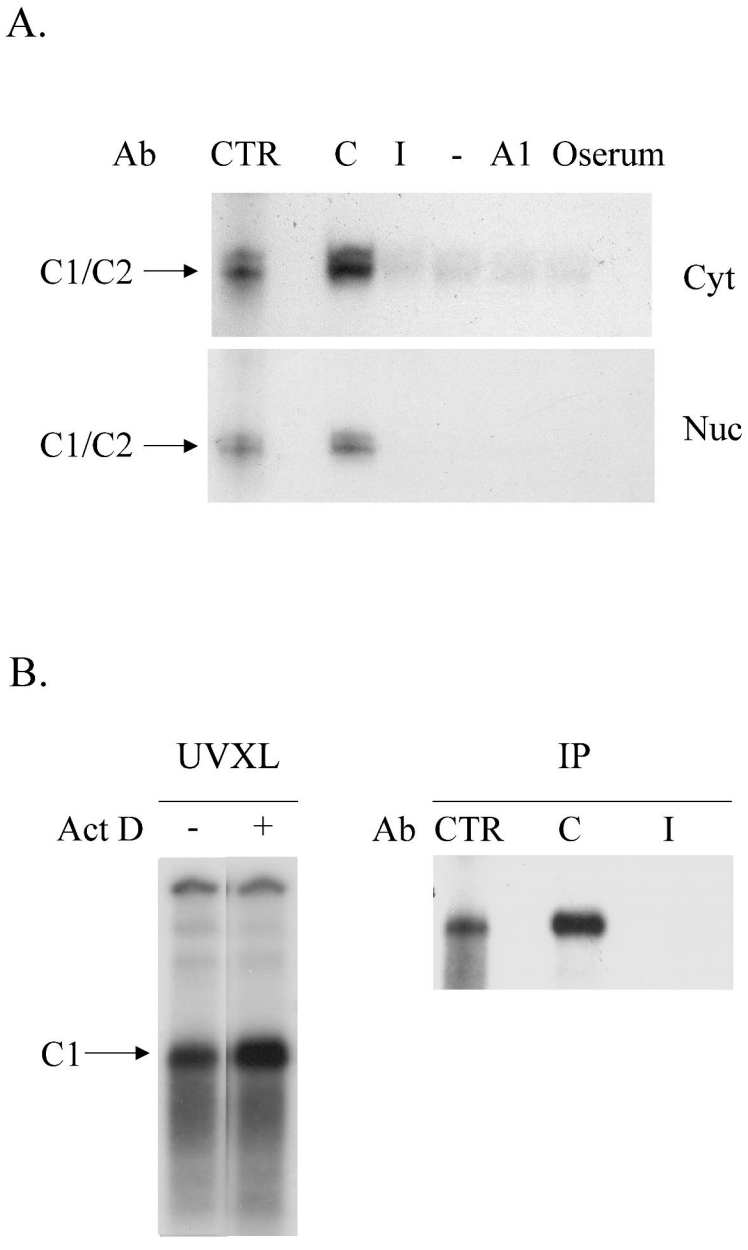
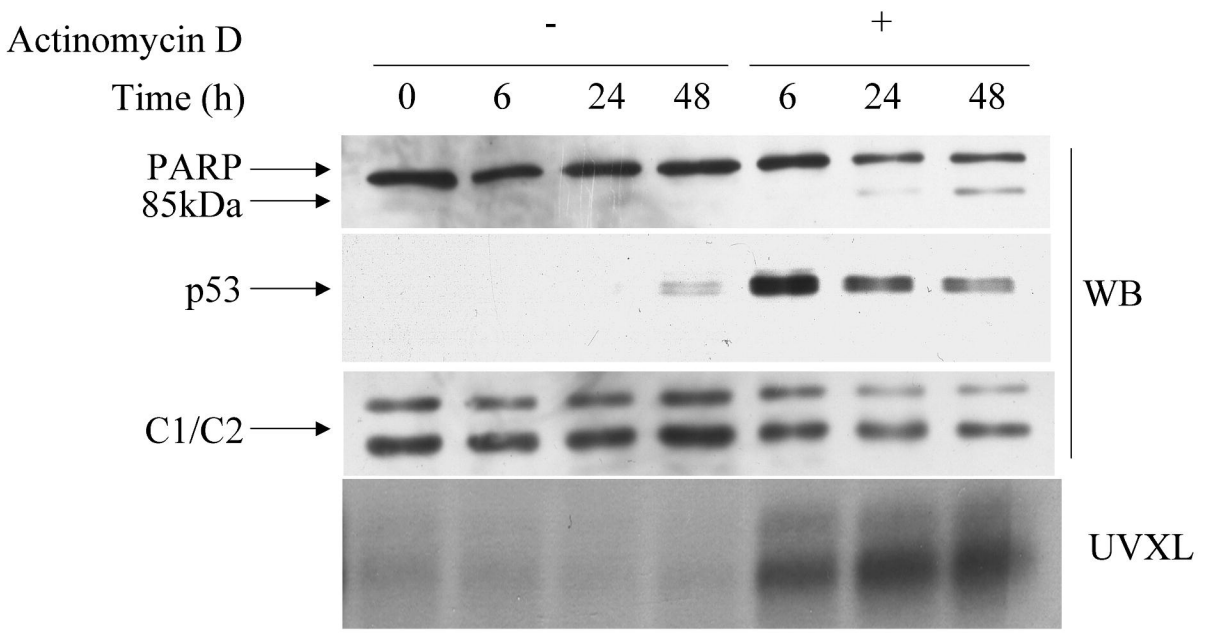
Fig. 4



A.


Actinomycin D
-
+
Time (h)
0
6
24
48
6
24
48
PARP
85kDa
p53
C1/C2
WB
UVXL

B.

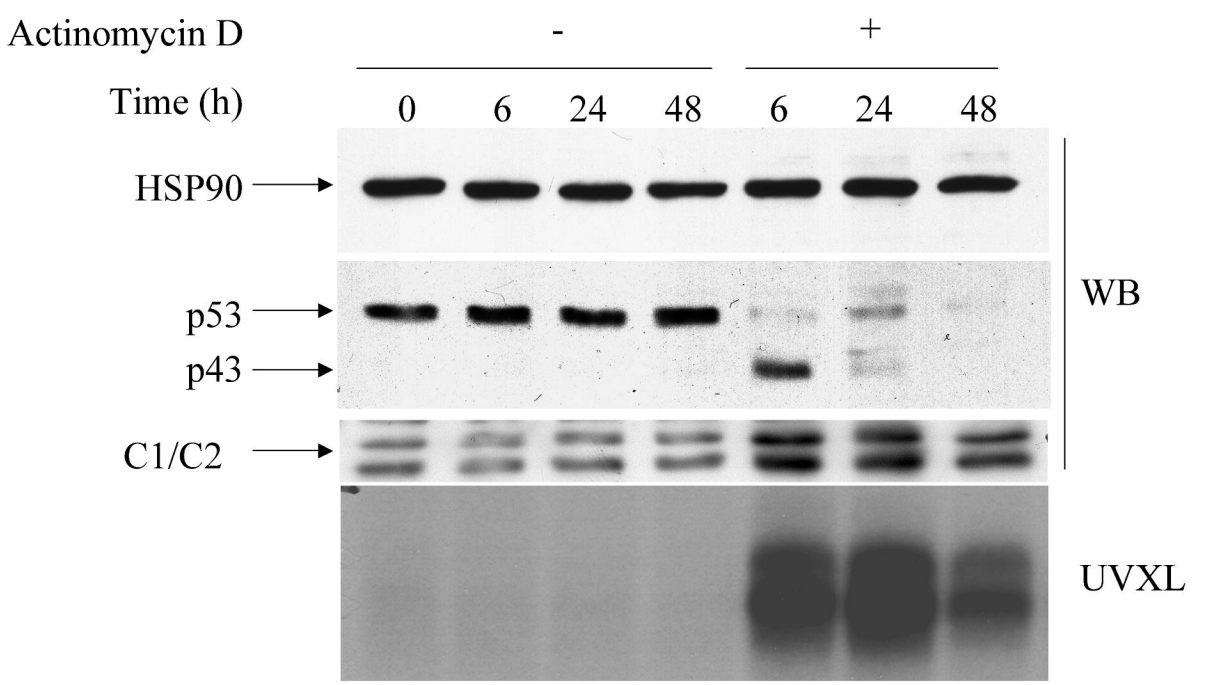

Actinomycin D
-
+
Time (h)
0
6
24
48
6
24
48
HSP90
p53
p43
C1/C2
WB
UVXL

Fig. 6

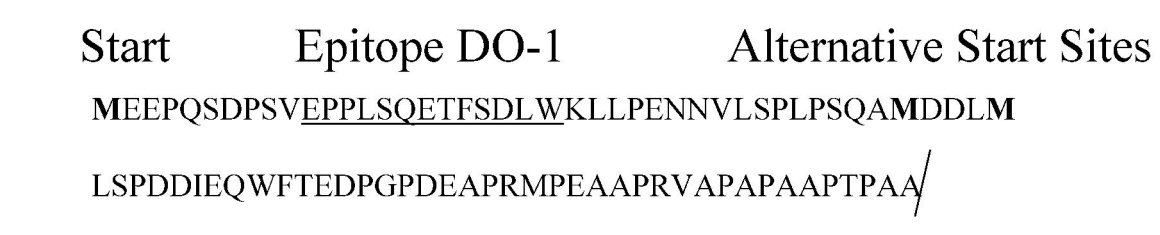
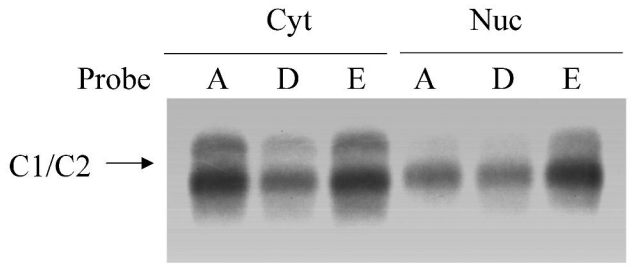
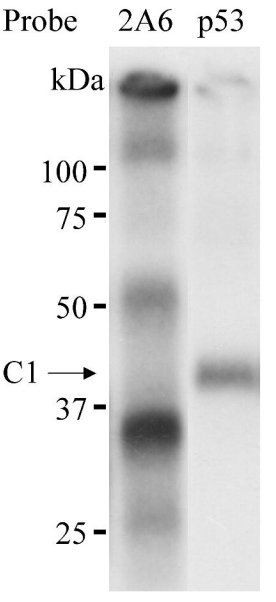

Start
Epitope DO-1
Alternative Start Sites
MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMDDLM
LSPDDIEQWFTEDPGPDEAPRMPEAAPRVAPAPAAPTPAA

Fig. 7

A.


Cyt
Nuc
Probe
A
D
E
A
D
E
C1/C2

B.


Probe
2A6
p53
kDa
100
75
50
C1
37
25

C.

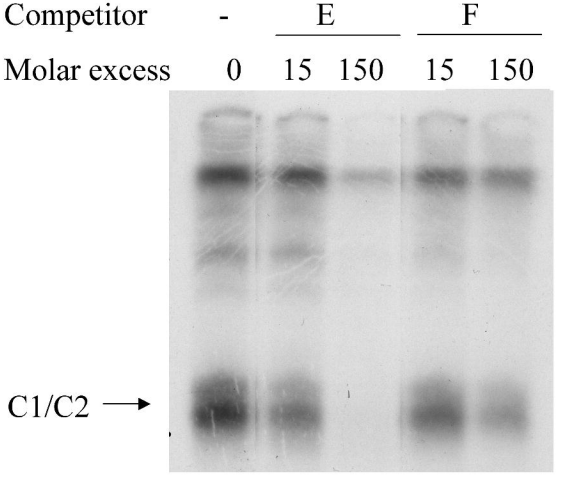
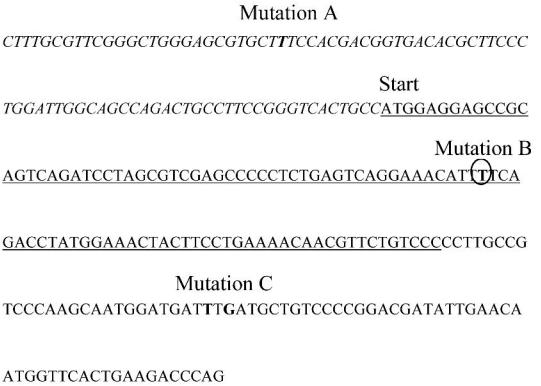
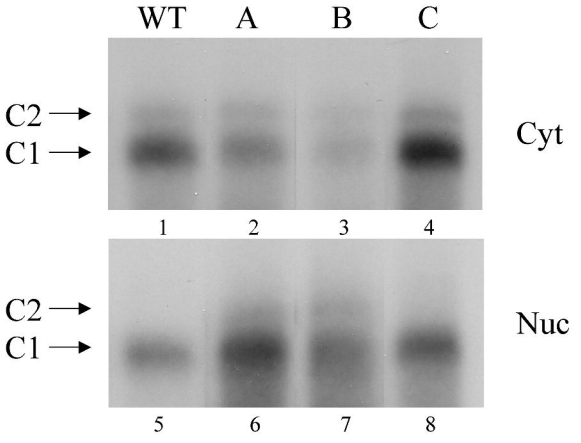

Competitor
-
E
F
Molar excess
0
15
150
15
150
C1/C2

Fig. 8

A.


Mutation A
CTTTGCGTTCGGGCTGGGAGCGTGCTTTCCACGACGGTGACACGCTTCCC
Start
TGGATTGGCAGCCAGACTGCCTTCCGGGTCACTGCCATGGAGGAGCCGC
Mutation B
AGTCAGATCCTAGCGTCGAGCCCCCTCTGAGTCAGGAAACATTTTCA
GACCTATGGAAACTACTTCCTGAAAACAACGTTCTGTCCCCCTTGCCG
Mutation C
TCCCAAGCAATGGATGATTTGATGCTGTCCCCGGACGATATTGAACA
ATGGTTCACTGAAGACCCAG

B.


WT
A
B
C
C2
C1
Cyt
1
2
3
4
C2
C1
Nuc
5
6
7
8

C.

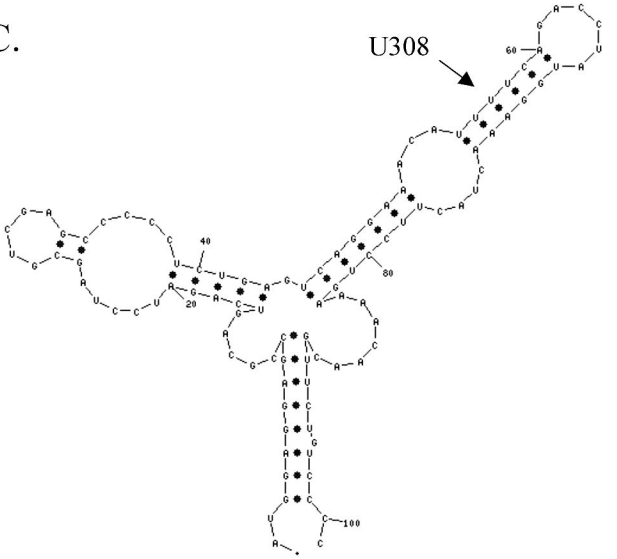
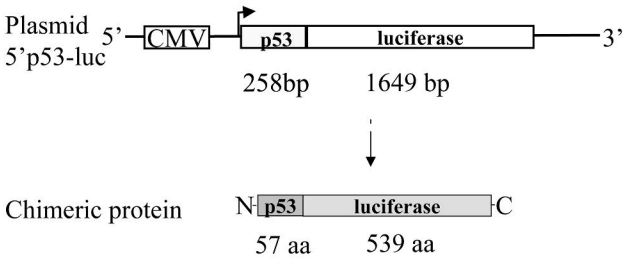
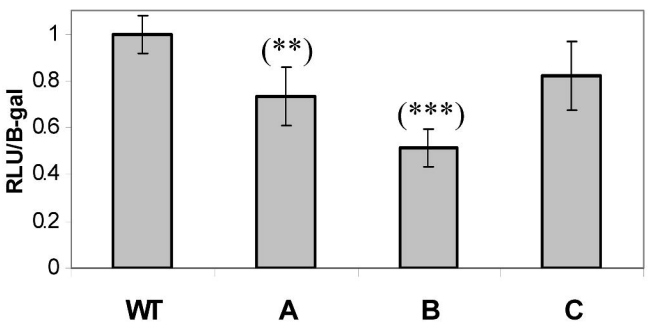

U308
60
40
80
20
100

Fig. 9

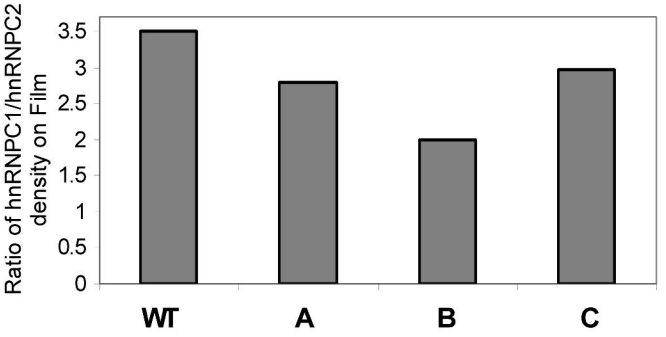
A.


Plasmid
5'p53-luc
5'
CMV
p53
luciferase
3'
258bp
1649 bp
Chimeric protein
N
p53
luciferase
C
57 aa
539 aa

B.


RLU/B-gal
1
0.8
0.6
0.4
0.2
0
(**)
(***)
WT
A
B
C

C.


Ratio of hnRNPC1/hnRNPC2 density on Film
3.5
3
2.5
2
1.5
1
0.5
0
WT
A
B
C