Interaction of Heterogeneous Nuclear Ribonucleoprotein C1/C2 with a Novel *cis*-Regulatory Element within p53 mRNA as a Response to Cytostatic Drug Treatment

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ABSTRACT

We describe a novel *cis*-element in the 5′ coding region of p53 mRNA and its interaction with heterogeneous nuclear ribonucleoprotein (hnRNP)C1/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 [*cis*-diamminedichloroplatinum(II)] and the cytostatic transcriptional inhibitor actinomycin D (dactinomycin), both known inducers of apoptosis and p53. Strongly stimulated binding is observed in both nuclear and cytoplasmic compartments, and it is accompanied by a cytoplasmic increase of hnRNPC1/C2. Changes in hnRNPC1/C2 protein levels are not proportional to binding activity, 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 in which 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 preapoptotic signals induced by transcriptional inhibition trigger the appearance of a truncated, exclusively cytoplasmic 43-kDa variant of p53 before apoptosis.

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 that 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 antiapoptotic 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 nonspecific DNA damage has been suggested to function as a so called "lesion dosimeter" that 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; dactinomycin) or 5,6-dichlorobenzimidazole riboside ($C_{12}H_{12}Cl_2N_2O_4$), is known to trigger p53 induction and apoptosis (Ljungman et al., 1999; Blagosklonny et al., 2002). However, the signaling 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 proapoptotic genes and down-regula-

ABBREVIATIONS: Act D, actinomycin D; hnRNP, heterogeneous nuclear ribonucleoprotein; aa, amino acid(s); IRES, internal ribosome entry site; nt, nucleotide(s); UTR, untranslated region; PAGE, polyacrylamide gel electrophoresis; HSP, heat shock protein; PARP, poly(ADP-ribose) polymerase; WT, wild type; Nuc, nuclear; Cyt, cytoplasmic; UVXL, UV cross-linked(ing).

tion of antiapoptotic 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 that bind mRNA cooperatively, although the functional difference between the two variants remains unclear (McAfee et al., 1996). The hnRNPC1/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 internal ribosome entry site (IRES)-dependent translation of proteins implicated in cell division and apoptosis (Holcík et al., 2003; Kim et al., 2003).

In this article, 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 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.

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 containing 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and nonessential amino acids. HeLa and MCF-7 cells were obtained from Dr. Dan Lindholm (Uppsala University, Uppsala, Sweden) and were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Primary human hepatocytes (a gracious gift of Dr. Patrick Maurel, Institut National de la Santé et de la Recherche Médicale U238, Montpellier, France) were isolated and cultured as described previously (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 h. All cell lines were kept in a humidified atmosphere of 5% CO_2. In some cases, the cells were treated with 16 μg/ml cisplatin [*cis*-diamminedichloroplatinum(II)] dissolved in 0.9% NaCl solution, 4 μM Act D in dimethyl sulfoxide, 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 Diagnostics, Basel Switzerland) according to the manufacturer's recommendations. All transfections included a cotransfected plasmid expressing β-galactosidase (pCMV-βgal) for internal control

of transfection. HepG2 cells were plated at a density of 4.5×10^4 cells/cm^2 in either six- or 24-well plates. Treatment was carried out 24 h after transfection, and the cells were harvested using reporter lysis buffer (Promega, Madison, WI) and snap-frozen at $-80°C$ for lysis. Luciferase activities were obtained via luminometer model TD 20/20 (Turner Designs, Sunnyvale, CA) using luciferase assay reagent (Promega) according to the manufacturer's protocol. The resulting values are expressed as relative light units corrected for β-galactosidase (transfection control) activity.

Isolation of Cytoplasmic and Nuclear Proteins. Protein extracts were prepared as described previously (Christian et al., 2004). In short, the cultured cells were scraped into phosphate-buffered saline and centrifuged at 2000g 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 phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.4% IGEPAL), and then it was incubated at 4°C for 1 h. The cells were homogenized and centrifuged at 12,000g at 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 phenylmethylsulfonyl fluoride, and 0.4% IGEPAL), homogenized, and centrifuged at 12,000g 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 Dundee, UK) was amplified in a polymerase chain reaction containing the primers 5′-ATGGAGGAGCCGCAGTCAGAT-3′ (sense) and 5′-GGAGAATGTCAGTCTGAGTCA-3′(antisense), using Phusion high-fidelity thermostable DNA polymerase (Finnzymes, Espoo, Finland) according to the manufacturer's instructions. An initial denaturation at 98°C for 30 s was followed by 35 cycles consisting of 98°C for 10 s, 49°C for 20 s, and 72°C for 20 s. The polymerase chain reaction product was cloned into the pGEM-T vector containing a T7 promoter (Promega) using standard molecular biology techniques. Cloning product and orientation were confirmed using restriction digest, and a series of exonuclease III-digested 3′ truncations were created using the Erase-a-Base system (Promega) according to manufacturer's recommendations (Fig. 1). All truncations were sequenced 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 (800 Ci/mmol; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) according to manufacturer's specifications (Promega). After 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 Corporation, Billerica, MA). A probe corresponding to the 234-nt 3′ untranslated region of human *CYP2A6* mRNA was synthesized as described previously 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. Using standard molecular biology techniques, the full-length firefly luciferase coding region cDNA obtained from the plasmid pGL3 (Invitrogen) was cloned in frame downstream of 258 base pairs of the human p53 cDNA containing the entire 5′-UTR and part of the p53 coding region in the plasmid TC119832 (Origene, Rockville, MD). The resulting plasmid, 5′p53luc (Fig. 9A), was driven via a cytomegalovirus 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 Gene-Editor site-directed mutagenesis kit according to manufacturer's directions (Promega). 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 GenBank sequence NM_000546. All mutations located within the coding region of p53 were designed to be silent, and they were confirmed to lack rare codons.

To create radioactive probes of the mutated sequences, the mutated plasmids were linearized 3′ of the p53 sequence, and overhanging nucleotides were 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. 8A.

One microgram 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) according to the manufacturer's protocol.

Equimolar amounts of the nonmutated and site directed-mutated RNA probes were then used in UV cross-linking reactions using equal amounts of total cytoplasmic proteins from 6-h Act D-treated 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 were taken using Scion Image for Windows (Scion Corporation, Frederick, MD).

RNA Secondary Structure Prediction. The sequence corresponding to probe E (Fig. 1), or this region including the 5′-UTR (data not shown), was input into Mfold 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 described previously (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 nonreducing conditions at 95°C for 10 min, separated via 12% SDS-PAGE, and visualized by autoradiography. In some cases, the cell extract was pretreated with proteinase K or potato acid phosphatase (108227; Roche Applied Science, Indianapolis, IN), for the indicated times before cross-linking was performed. In all cases, the amount of radioactive RNA probe was quantitated via scintillation, and master mixes of components in the UV cross-linking 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. Immunoprecipitation was performed essentially as described previously (Hamilton et al., 1993). In brief, UV cross-linked samples were performed as indicated, and they were added to antibody binding buffer containing 1 μl of 4F4 monoclonal hnRNPC1/C2-specific antibody (Sigma Aldrich AB, Tiresö, Sweden), 1 μl of monoclonal hnRNP I antibody (Zymed Laboratories, South San Francisco, CA), 2 μl of polyclonal anti-hnRNPA1 antiserum, 2 μl of preimmune serum, or no antibody. Immunoprecipitation was carried out with protein A-Sepharose beads (GE Healthcare). The samples were washed five times with phosphate-buffered saline, 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; GE Healthcare), and then they were 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, Inc., Santa Cruz, CA). Detection was performed using enhanced chemiluminescence reagents according to manufacturer's recommendations (GE Healthcare).

Statistical Analysis. Statistical analysis of luciferase data were carried out with MINITAB version 14 statistical software (Minitab Inc., State College, PA) using a general linear model analysis of variance. Statistical significance was assumed at $p < 0.01$ (∗∗) and $p < 0.001$ (∗∗∗).

Results

Several Cellular Proteins Interacted with the Coding Region of Human p53 mRNA. To identify proteins interacting with the coding region of human p53 mRNA, UV cross-linking 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 labeled probe (Fig. 1, probe A).

The results showed several complexes of varying size and subcellular localization (Fig. 2A). A strong, approximately 41-kDa nuclear complex occurred 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 it 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 de-
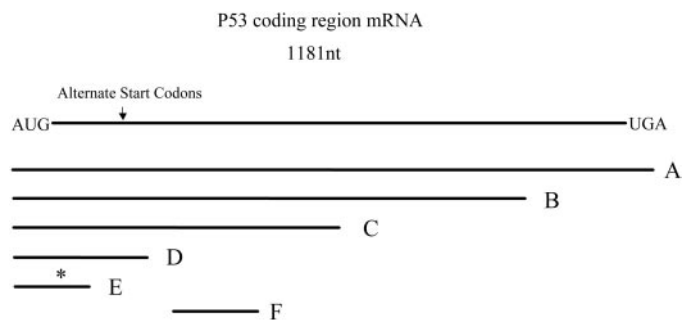
TABLE 1

Sense oligonucleotides used for site-directed mutagenesis

Mutation numbers refer to nucleotide sequence NM_000546, National Center for Biotechnology Information GenBank

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

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 indicated by the asterisk (∗).

stroyed by proteinase K pretreatment of extracts (data not shown), showing that the complexes include proteins.

Formation of the 41- and 44-kDa Complexes Was Influenced by Cisplatin. To determine whether 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 it is known to induce p53 protein levels and result in apoptosis in HepG2 cells (Qin and Ng, 2001).

Treatment for 6 h markedly increased the intensity of the 41- and 44-kDa complexes obtained with both nuclear and cytoplasmic extracts (Fig. 2B). The increase seemed to be specific to these protein complexes, because 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 Induced 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 Act D, a potent transcriptional inhibitor widely used in the treatment of cancer (Green et al., 2007; Hauer et al., 2007). 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 h of exposure, a massive increase of both the 41- and 44-kDa complexes took place (Fig. 3A). The induction was much greater than that observed with cisplatin treatment, and it was most marked with cytoplasmic, but it also was clearly evident with nuclear extracts. As with cisplatin, complexes other than the 41/44-kDa complexes remained apparently unaffected by Act D. Maximal binding intensity in the cytoplasm was reached at 24 h after treatment, followed by a decrease at 48 h. In contrast, the corresponding nuclear complex remained at maximal level at 48 h. 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.

Binding of the Complexes to p53 mRNA Was Phosphorylation-Dependent. To test the phosphorylation dependence of the 41/44-kDa complexes, protein extracts from Act D-treated cells were pretreated with increasing concentrations of potato acid phosphatase and then subjected to UV cross-linking.

As shown in Fig. 3B, pretreatment of extracts with amounts of phosphatase above 0.2 U for 20 min resulted in a
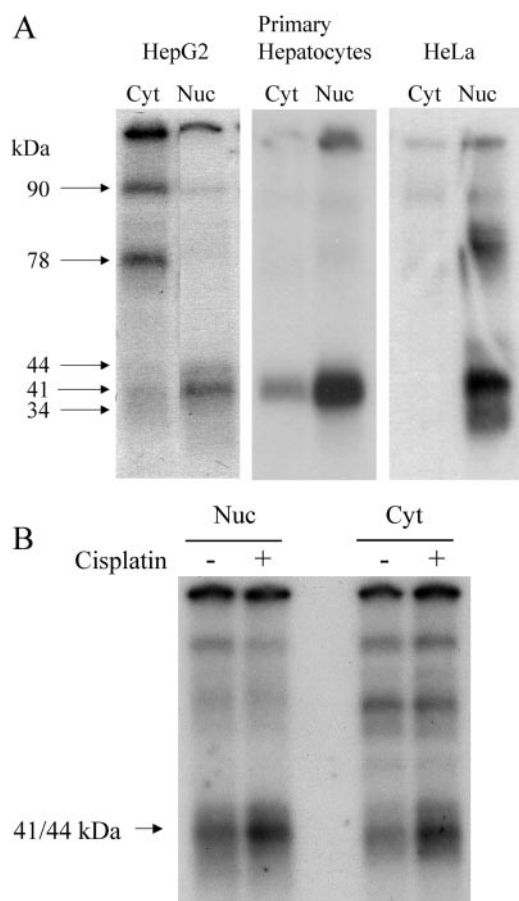

Fig. 2. Formation of RNA-protein complexes with the coding region of p53 mRNA. A, UV cross-linking experiments showing proteins binding to the human p53 coding region in various cell types. The radioactive probe A (full-length coding region) was cross-linked to untreated cellular extracts from the indicated cell types. Cyt, cytoplasmic extracts; Nuc, nuclear extracts. The apparent molecular masses 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 h with 16 μg/ml cisplatin. Five micrograms of Nuc and 10 μg of Cyt extracts were used in the indicated UV cross-linking experiments with probe A. The 41/44-kDa complexes are indicated.
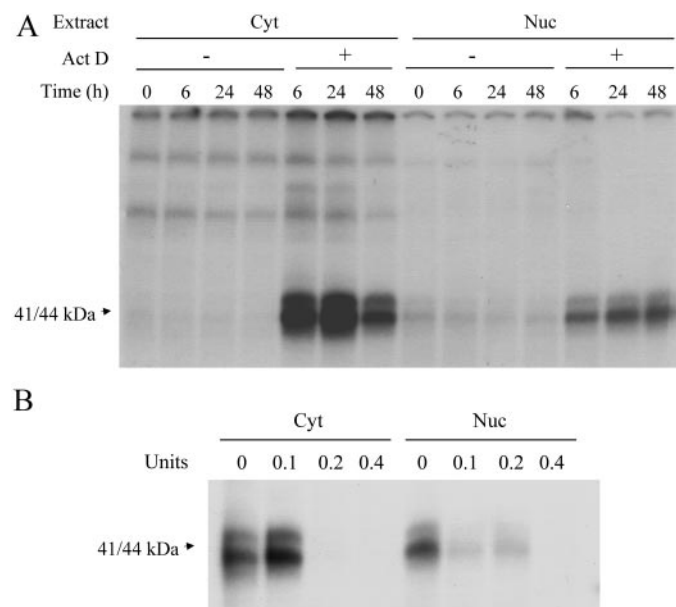

Fig. 3. A, effect of Act D on the 41- and 44-kDa complexes. The cells were harvested after exposure to 4 μM Act D (+) or carrier only (−) for the times indicated. UV cross-linking was performed using 5 μg of total protein from the indicated cell extract and identical amounts of the full-length p53 coding region probe (probe A). B, Act D-induced binding of the complexes to p53 mRNA is phosphorylation-dependent. Cyt or Nuc extracts from Act D-treated HepG2 cells (24 h) were preincubated for 20 min with the indicated amounts of potato acid phosphatase (PAP), and then they were UV cross-linked to probe A. The lanes marked 0 represent conditions with no phosphatase activity, and they are provided as negative controls.

complete loss of binding activity, suggesting that the interaction is highly phosphorylation-dependent in both the cytoplasmic and nuclear compartments. It is noteworthy that 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. Thus, 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, immunoprecipitation with the monoclonal antibody 4F4 specific to hnRNPC1/C2 was performed. The UV cross-linked nuclear or cytoplasmic complexes were incubated with monoclonal antibodies against hnRNPC1/C2, hnRNP I (polypyrimidine tract binding protein), polyclonal anti-hnRNPA1 antiserum, preimmune serum, or no antibody. We observed that only the use of the hnRNPC1/C2-specific antibody resulted in an immunoprecipitation of both the 41- and the 44-kDa complexes (Fig. 4A).

These results suggest that the 41-kDa protein contained within nuclear and cytoplasmic extracts of HepG2 cells is in fact hnRNPC1 and that 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 Was Not Cell-Specific. To investigate whether 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 cross-linking assay (Fig. 4B).

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 it was identified as the 41-kDa protein (data not shown). This protein was identified by immunoprecipitation as hnRNPC1 (Fig. 4B).

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 it is conserved in primary, nontransformed, human cells.

Inhibition of Transcription Led to an Apparent Partial Nucleocytoplasmic Translocation of hnRNPC and Enhances Its Affinity toward p53 mRNA. The next series of experiments were designed to investigate whether 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. 5A) and cytoplasmic (Fig. 5B) proteins from untreated and Act D-treated HepG2 cells. Monoclonal antibodies, specific for hnRNPC1/C2 as well as for HSP-90 and PARP were used. HSP-90 and PARP-1 served as both internal loading and subcellular fractionation controls.

The results showed that although the nuclear levels of hnRNPC1/C2 were unchanged or in some experiments slightly decreased upon treatment, the cytoplasmic levels
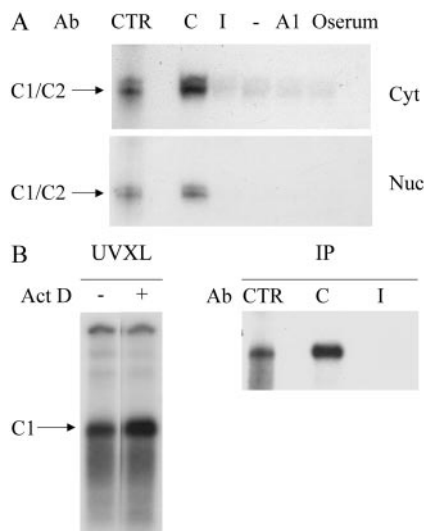

Fig. 4. Identification of the 41- and 44-kDa complexes as hnRNPC1/C2 in transformed and normal cells. A, 41- and 44-kDa complexes formed with probe A in HepG2 Act D-treated (24-h) Cyt or Nuc extracts were immunoprecipitated using monoclonal antibodies against hnRNPC1/C2 (C), hnRNP I (I), no antibody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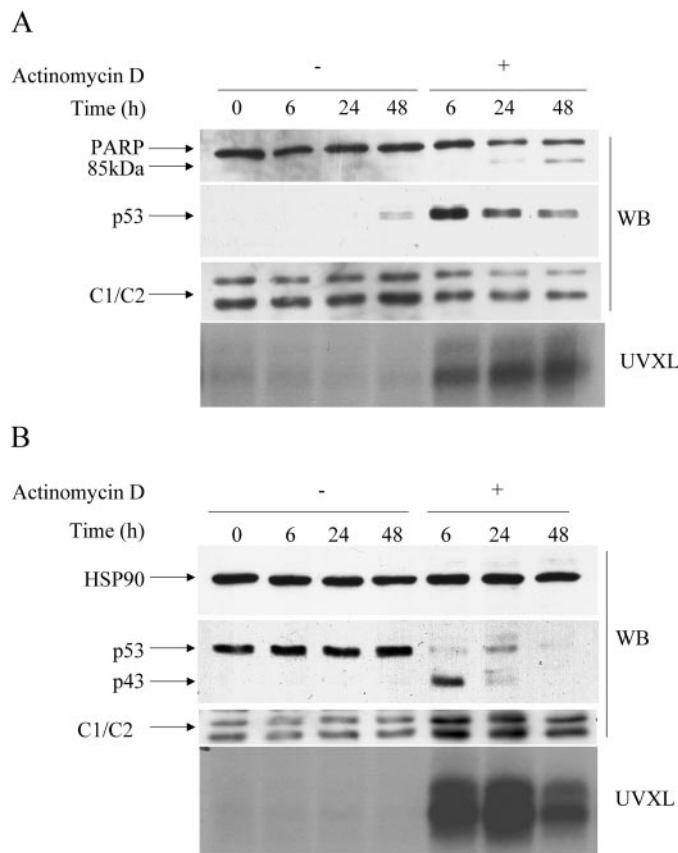

Fig. 5. Effects of Act D on p53 and hnRNPC1/C2 levels in the nucleus and cytoplasm of HepG2 cells. Shown are Western blots (WB) and comparison with UVXL reactions from cells treated with Act D (+) or carrier only (−) for the indicated times. The proteins are indicated. A, nuclear compartment. The 85-kDa fragment of PARP-1 cleavage with caspase-3 is indicated. B, cytoplasmic compartment. The p53-related protein is indicated by p43.

were increased. The initial increase in the cytoplasmic hnRNPC1/C2 levels began to subside by 48 h (Fig. 5B). This decrease correlated with the appearance of an 85-kDa fragment of PARP (Fig. 5A) in the nucleus at 24 and 48 h. 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.

It is noteworthy that 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 with 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 Occurred in the Cytoplasm of Act D-Treated Cells. 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 to 25 of the N terminus of human p53 (Fig. 6).

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

It is noteworthy that Act D treatment resulted in the transient appearance of a 43-kDa protein in the cytoplasm at 6 h after treatment, recognized by the monoclonal antibody DO-1 (Fig. 5B). The 43-kDa protein was not visible in nuclear extract, and it was not visible in the cytoplasm by 24 to 48 h of Act D treatment.

Mapping and Specificity of the hnRNPC1/C2 Binding Site on the Coding Region of p53 mRNA. To determine the binding site(s) of hnRNPC1/C2 on the p53 mRNA, UV cross-linking 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 h.

The binding activity of hnRNPC1/C2 was retained in all truncations (data not shown for larger truncations) as shown in Fig. 7A. These data suggest that the main binding site for hnRNPC1/C2 is located within the region of p53 mRNA covered by probe E, a 101-nt 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).

To investigate the specificity of hnRNPC1 binding to p53 mRNA during Act D-induced conditions, a UV cross-linking 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-induced

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

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 nonradioactive competitors E or F (Fig. 7C), and it was UV cross-linked with Act D-treated HepG2 cytoplasmic extracts. Probe F corresponds to nucleotides 435 to 536, downstream of probe E within the p53 mRNA (Fig. 1). The results presented in Fig. 7C show that equimolar concentrations of the nonradioactive probe E are superior to probe F at competing the hnRNPC1/C2 signal, suggesting binding specificity to probe E.

The Primary Binding Site for hnRNPC1/C2 Was a U-Rich Sequence 55 nt Downstream from 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. 8A). The mutated probes were radiolabeled, and then they were used in UV cross-linking experiments using Act D-treated extracts.

Cytoplasmic hnRNPC1/C2. As shown in Fig. 8B, 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 abolished binding of hnRNPC1 to p53 mRNA. It is noteworthy that the binding of
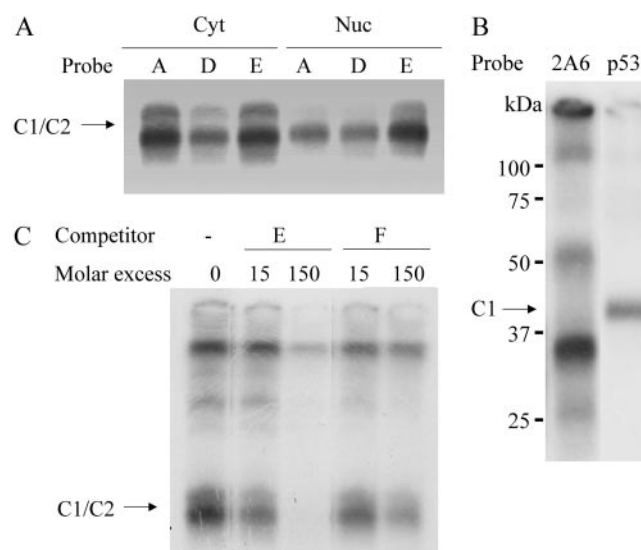

Fig. 7. A, mapping of the primary binding site of hnRNPC1/C2 to p53 mRNA. Two truncations of the full-length probe are shown. The smallest truncation, probe E, shows unchanged binding characteristics toward hnRNPC1/C2 in UV cross-linking experiments using Cyt or Nuc extracts from Act D-treated HepG2 cells (24 h). B, an unrelated sequence does not bind Act D-induced hnRNPC1. A UV cross-linking 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 mass standards are shown. C, 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 cross-linking reactions in the molar excess indicated as competitors in the presence of radiolabeled probe A. UVXL was carried out using Act D-activated (24-h) cytoplasmic extracts.

Start   Epitope DO-1   Alternative Start Sites

MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMDDLM

LSPDDIEQWFTEDPGPDEAPRMPEAAPRVAPAPAAPTPAA/

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.
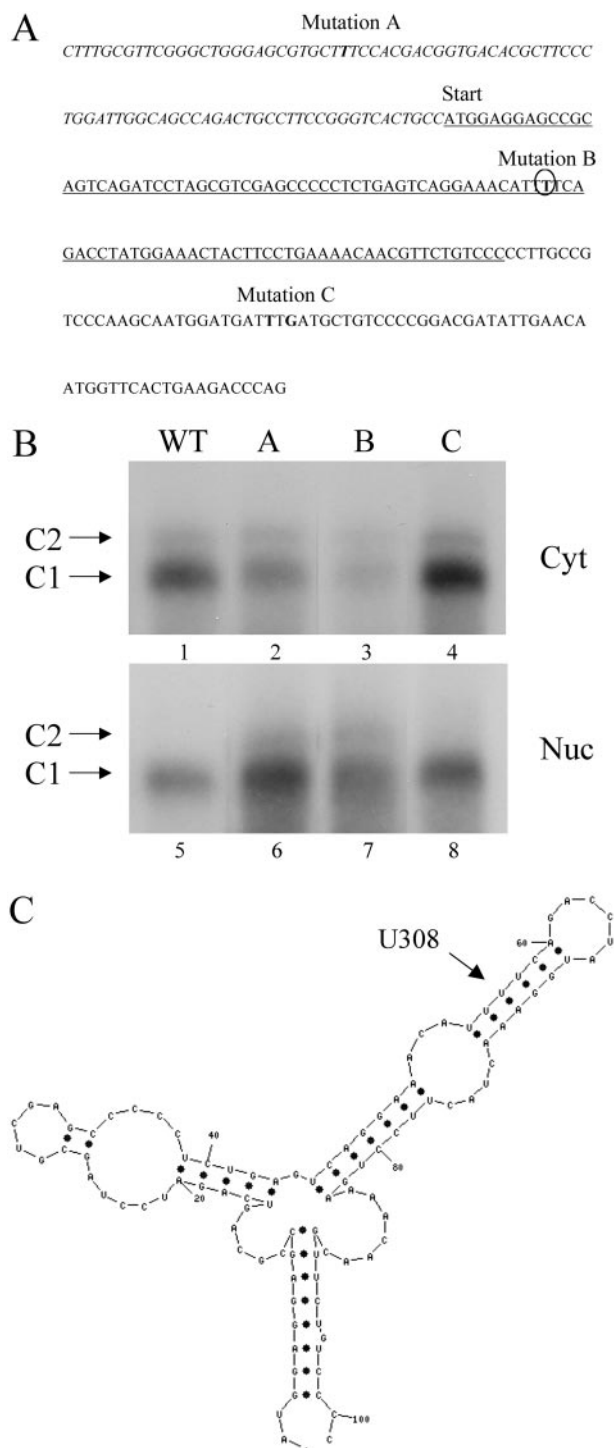
Fig. 8. Fine mapping of the hnRNPC1/C2 binding sites. A, 258-nt sequence used to make the probes in UV cross-linking experiments. The 5′-UTR is in italics, and the first known start codon for p53 is indicated. The locations of mutations A, B, and C are indicated in bold. All numbers refer to the corresponding nucleotides in the GenBank 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 cross-linking experiments using the mutated sequences and Act D-induced Cyt or Nuc extracts. The complexes formed with equimolar amounts of the radioactive nonmutated 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.

cytosolic hnRNPC2 toward p53 mRNA seemed 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 seemed to be particularly strong in the nuclear compartment to mutant A, but it 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 (Fig. 1), and it consists of four U nucleotides downstream of the start codon (Fig. 8A, site B). In particular, nucleotide U308 is pivotal for binding of hnRNPC1/C2. Another, minor, site contains three U nucleotides, and it is situated upstream of the start codon (Fig. 8A, site A). In addition, the binding characteristics of the two proteins toward the mutated sequences seem 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. 8C). 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 hnRNPC1/C2 Binding Site Down-Regulates Gene Expression. The sequence denoted in Fig. 8A, 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. 9A). The constructs were transfected into HepG2 cells, and 24 h after transfection Act D was added to the cells for 6 h. The point mutations were designed to be silent; therefore, the amino acid sequence of the resulting chimeric protein was unchanged. In addition, codon use tables were consulted to ensure the lack of rare codons in the silent mutations.

Figure 9B 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%, whereas a mutation (mutation A) upstream of this critical site resulted in a less pronounced but still significant ($p < 0.01$) decrease in luciferase expression 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 toward p53 mRNA Was Correlated with Luciferase Expression. The two hnRNPC proteins are known to interact, and they bind cooperatively (McAfee et al., 1996). Therefore, 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. 8B) in both 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. 9C). 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.

Discussion

This article 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.
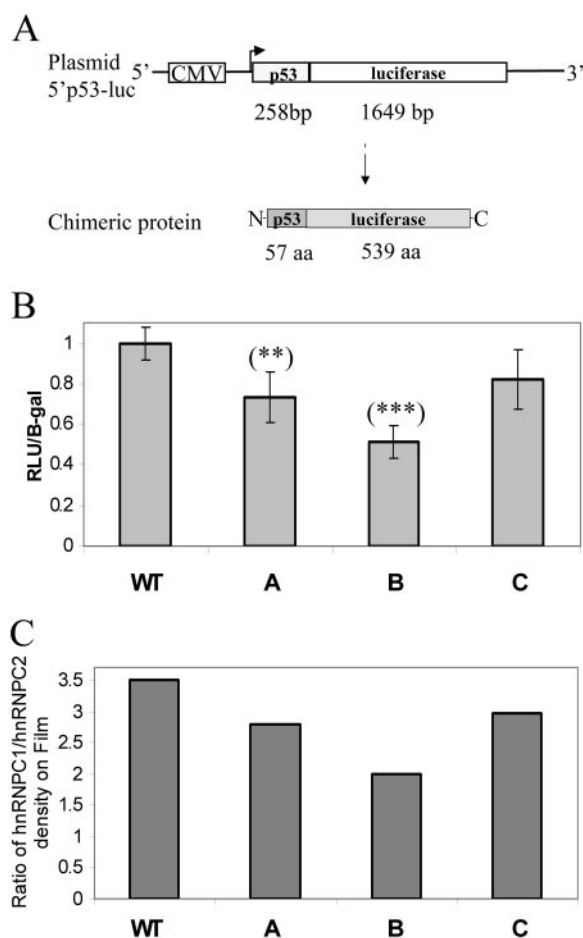

Fig. 9. An element essential for expression of p53 is located at site B. A, schematic of the 5'p53luc (WT) reporter gene. The transcription start site, location, and size of the cDNA sequences, and the resulting chimeric gene product are indicated. The p53 cDNA sequence indicated in the reporter gene corresponds to that given in Fig. 8A. B, expression of the mutants is given as relative light units (RLU) corrected for β-galactosidase (transfection control) activity. Twenty-four hours after transfection, HepG2 cells were treated with 4 μM Act D for 6 h. The nonmutated reporter gene 5'p53luc is indicated by WT. A, B, and C refer to the previously described mutants (see Fig. 8A). Error bars indicate standard deviation of the mean for the experiment ($n = 8$). **, $p < 0.01$ and ***, $p < 0.001$. C, relative binding activity of hnRNPC1 to hnRNPC2 in the cytoplasm correlates with expression of the site directed mutations. The film from Fig. 8B 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.

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 toward 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 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, reduction of normal cap-dependent translation is 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 IRES-dependent mechanisms. One such IRES segment in the 5'-UTR of p53 mRNA has been 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.

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 preapoptotic and apoptotic conditions in which transcription is disturbed.

The doses of cisplatin used in this study are known to create inter- and intrastrand cross-links and adducts on DNA, thus interfering with transcription (Jung and Lippard, 2006). Conversely, Act D was used in concentrations known to shut down global cellular transcription, yet it did not induce discernible 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 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, is affected by changes in global RNA synthesis.

We observed an apparent partial nucleocytoplasmic translocation of hnRNPC1/C2 in response to Act D treatment, concurrent with a massively increased affinity toward p53 mRNA. Until recently, hnRNP C1/C2 has been thought to be strictly nuclear in nondamaged 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 it affects the translation of XIAP and c-myc mRNA (Holčík 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 phorbol 12-myristate 13-acetate or tumor necrosis factor-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 before any cleavage of PARP-1 via caspase 3 (Fig. 5, A and B). It is noteworthy that we observed 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 preapoptotic 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 casein kinase 1-α in response to physiological levels of hydrogen peroxide (Kattapuram et al., 2005) composing survival signals for the cell; and DNA-protein kinase, a well known DNA damage control kinase (Zhang et al., 2004). It is clear that changes in the phosphorylation state of hnRNPC1/C2 are important for its function in response to survival or stress stimuli.

We observed an exclusively cytoplasmic 43-kDa protein, recognizable by the anti-p53 antibody DO-1. The protein, referred to by us as p43, occurs transiently upon 6 h 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 that are a result of both N- and C-terminal truncations of the protein. Although N-terminal truncations seem 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 seems 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 described previously (Mills, 2005). It is noteworthy that hnRNPC1/C2 is known to affect splicing (Dreyfuss et al., 1993). Given the highly changed binding activity of hnRNPC toward p53 mRNA in the nucleus, it can be predicted 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 IRES-dependent translation (Holčík 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. Furthermore, hnRNPC1/C2 was identified to bind PARP-1, a protein necessary for the translocation of the apoptosis-inducing factor 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 "hot spot" 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. It is noteworthy that when the ratio of hnRNPC1 to hnRNPC2 binding toward 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, possibly providing an explanation as to why the relative amounts of the proteins 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, results 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 small interfering RNA knockdown experiments against hnRNPC1/C2 confirm an important role of hnRNPC in regulating the expression of p53, and they 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 preapoptotic 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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