The EMBO Journal Vol. 20 No. 15 pp. 4163–4172, 2001

Integrity of the N-terminal transcription domain of p53 is required for mutant p53 interference with drug-induced apoptosis

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The present study examined whether the ability of mutant p53 to block apoptosis depended on its transcriptional activity. A core domain mutant p53 (143 Val to Ala), in which two N-terminal residues (22 and 23) essential for transactivation were also mutated (Leu to Glu and Trp to Ser, respectively), was examined. While p53 containing only the core mutation efficiently interfered with drug-induced apoptosis, further modification at the N-terminus abolished this blocking activity. Furthermore, expression of *c-myc*, a suggested target for core mutant p53 transactivation, was elevated in the core mutant p53-expressing cells, but was abolished in the presence of the transcription-deficient p53 core mutant. In addition, wild-type p53, mutated in the N-terminus (residues 22 and 23), was unable to induce apoptosis by itself. Nevertheless, it synergized with drugs in the induction of apoptosis. This suggests that the integrity of the N-terminus is essential for both the activity of wild-type p53 in apoptosis and for mutant p53-mediated block of drug-induced apoptosis. This supports the notion that core p53 mutants act via a gain of function mechanism.

Keywords: apoptosis/c-myc/mutant p53/transcription activity

Introduction

The p53 tumor suppressor gene plays a central role in the regulation of the cell cycle. Once activated by genotoxic stress, wild-type p53 may induce a variety of cellular processes including growth arrest and apoptosis (Ko and Prives, 1996; El-Deiry, 1998; Gottlieb and Oren, 1998). Numerous stimuli can cause p53-dependent apoptosis, including DNA damage (Maltzman and Czyzyk, 1984; Kastan *et al*., 1991), activation of dominant oncogenes (Serrano *et al*., 1997) and hypoxia (Graeber *et al*., 1996).

p53-induced apoptosis has been shown by several studies to be dependent on the sequence-specific transactivation (SST) function of the p53 protein (Sabbatini *et al*., 1995; Attardi *et al*., 1996; Yonish-Rouach *et al*., 1995; Chao *et al*., 2000; Jimenez *et al*., 2000). Other reports have shown that p53-mediated apoptosis may also occur through SST-independent mechanisms (Caelles *et al*., 1994; Wagner *et al*., 1994; Haupt *et al*., 1995; Roemer and Mueller-Lantzsch, 1996; Ding *et al*., 2000; Kokontis *et al*., 2001). The contribution of each of these pathways to p53-induced apoptosis, and the interactions between the two, are poorly understood. Nevertheless, the physiological and clinical importance of the p53 apoptotic pathway have been clearly demonstrated. p53-induced apoptosis acts to protect the organism from cancer development by eliminating potential tumor precursor cells. Additionally, p53-dependent apoptosis can affect the outcome of cancer therapy. Experiments comparing transformed murine fibroblasts from normal and p53-deficient mice demonstrated that disruption of p53 function reduces apoptosis induced by anticancer agents (Lowe *et al*., 1993). Subsequent studies using an *in vivo* model have provided further support for this notion (Lowe *et al*., 1994).

Given the major role of p53 as a tumor suppressor, it is not surprising that mutations in the p53 gene are the most frequent genetic alterations in human cancer (Hussain and Harris, 1998). Most p53 mutations are clustered within the DNA-binding domain of the protein, resulting in mutant proteins expressed at high levels (Rotter, 1983). Typically, a mutation in one of the p53 alleles leads to inactivation of the remaining wild-type allele by a dominant-negative mechanism. Transdominance of p53 mutants over the wild-type p53 form and repression of wild-type p53 activity were demonstrated in various experimental systems (Kern *et al*., 1992; Shaulian *et al*., 1992; Srivastava *et al*., 1993; Unger *et al*., 1993; Chene, 1998). Additionally, it appears that at least some mutant p53 proteins may exert oncogenic effects by a gain of function mechanism, independent of the inhibition of wild-type p53. Supporting this model are observations that expression of mutant p53 protein in p53-null transformed cells increases tumorigenicity (Wolf *et al*., 1984; Dittmer *et al*., 1993; Hsiao *et al*., 1994; Lanyi *et al*., 1998), mutation frequency (Iwamoto *et al*., 1996) and metastatic potential (Crook and Vousden, 1992; Hsiao *et al*., 1994). Resistance to chemotherapy is a major obstacle to cancer treatment. The relationship between mutant p53 expression and the resistance of tumors to apoptosis is not clear. While several studies have demonstrated that expression of mutant p53 results in chemoresistance, others have reported an opposite correlation (reviewed in Hickman and Dive, 1999).

In our previous studies we have found that in M1/2, a p53 non-producer murine myeloid cell line, wild-type p53 synergizes with p53-independent apoptosis induced by removal of growth factors, while the expression of the p53 135 (Ala to Val) mutant interferes with this apoptotic pathway (Peled *et al*., 1996). We reported that the same murine mutant, expressed in M1/2 cells, can protect against p53-independent apoptosis mediated by γ-irradiation and several chemotherapeutic agents (Li *et al*., 1998).

To further expand these observations, we introduced into the M1/2 cell line human p53 mutants, frequently expressed in human tumors, and examined their ability to suppress the induction of apoptosis by chemotherapeutic agents. We found that these mutant proteins confer anti-apoptotic function against cisplatin, α-amanitin and etoposide, but do not interfere with doxorubicin-, DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole)- or actinomycin D-induced apoptosis. The observation that the p53 N-terminal domain is rarely modified in human tumors (Hussain and Harris, 2000), coupled with the characterization of this domain as the transcription activation domain of wild-type (Unger *et al*., 1992; Subler *et al*., 1994) and mutant p53 (Lin *et al*., 1995; Frazier *et al*., 1998), prompted us to investigate whether the transcriptional activity of mutant p53 is required for the anti-apoptotic function of this protein. For this purpose we generated cell lines that express N-terminally modified p53 core mutant and compared their apoptosis blocking activity with that mediated by cell lines expressing the p53 core mutant. We found that transcriptional activation is necessary for the interference of mutant p53 with drug-induced apoptosis. As it is expected that mutant p53 will transactivate target genes other then those transactivated by wild-type p53, we focused our analysis on the expression of *c-myc*, which is suggested to be a target gene for mutant p53 (Frazier *et al*., 1998). We found that cells express higher levels of c-myc in the presence of mutant p53, yet this elevation in c-myc is abolished when mutant p53 is transcriptionally inactive and unable to induce drug resistance. In addition, we addressed the need for transcription in wild-type p53-induced apoptosis by using the same modification. We show that p53-dependent apoptosis may occur without the involvement of SST, but unlike apoptosis mediated by SST-competent p53, SST-independent apoptosis occurs only upon genotoxic stress.

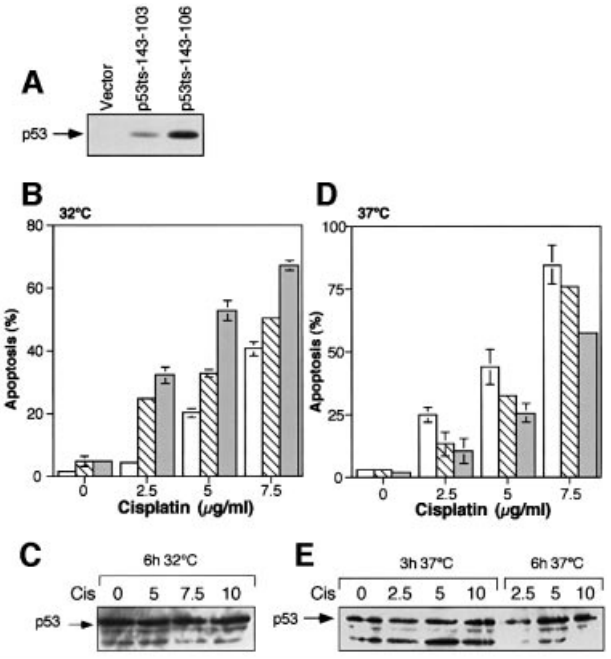

Fig. 1. Cisplatin-induced apoptosis in p53 143 (Val to Ala) expressing M1/2 cells. (**A**) A quantitative western blot analysis of M1/2-derived clones expressing p53 143 (Val to Ala). Vector, a clone expressing the empty vector. (**B**) Wild-type p53 synergizes with cisplatin-induced apoptosis. A comparison of apoptosis induction in M1/2-derived clones expressing the p53 143 (Val to Ala) protein or the empty vector after cisplatin treatment and incubation at 32°C. Open bars represent a clone generated by infection with empty vector, hatched bars represent clone p53ts-143-103 and gray bars represent clone p53ts-143-106. (**C**) p53 143 (Val to Ala) protein expression was quantitated in clone p53ts-143-106 after 6 h of cisplatin treatment at 32°C. Cis, cisplatin. (**D**) Mutant p53 143 (Val to Ala) protein expression interferes with cisplatin-induced apoptosis. M1/2-derived clones expressing p53 143 (Val to Ala) protein were incubated with cisplatin at 37°C for 24 h and harvested for FACS. Open bars represent a clone generated by infection with empty vector, hatched bars represent clone p53ts-143-103 and gray bars represent clone p53ts-143-106. (**E**) Cells treated as in (D) were harvested for western blot analysis after 3 and 6 h of treatment. Western blotting was performed with p53ts-143-106 cells. Cis, cisplatin.

Results

In our previous studies we have observed that expression of the murine mutant p53 protein, p53 135 (Ala to Val), protects cells from apoptosis induced by several chemotherapeutic agents (Li *et al*., 1998). Our present experiments were aimed at determining whether a similar phenomenon is also exhibited by human p53 mutants. Using a retroviral infection procedure (see Materials and methods), we established a series of stable clones expressing human p53 mutant proteins, derived from M1/2, a myeloid p53 non-producer parental cell line. The clones generated expressed various levels of either p53 143 (Val to Ala), which is known to be a temperature-sensitive (ts) mutation (Zhang *et al*., 1994), or p53 248 (Arg to Trp) or p53 273 (Arg to His), which are the most frequent mutations in p53 found in human cancer cells.

Analysis of the effect of the p53 143 (Val to Ala) mutant protein on drug-induced apoptosis

Cell cycle and apoptosis analysis of clones expressing the p53 143 (Val to Ala) protein, following a shift from 37 to 32°C, confirmed that the p53 protein expressed behaves in a ts manner. However, the induction of apoptosis in these clones at 32°C was slower, compared with that observed previously with established clones expressing the mouse ts protein p53 135 (Ala to Val) (data not shown). We analyzed the effect of p53 143 (Val to Ala) protein expression on p53-independent apoptosis induced by anti-cancer agents. Two representative clones were selected, p53ts-143-103 and p53ts-143-106, expressing lower and higher levels of the p53 143 (Val to Ala) protein, respectively (see Figure 1A). Cells were treated with increasing concentrations of various drugs and maintained for 24 h either at 37°C, the permissive temperature for the mutant conformation of p53 143 (Val to Ala), or at 32°C, the permissive temperature for the wild-type p53 conformation. Apoptosis was mostly analyzed by the fluorescence-activated cell sorter (FACS)-based acridine orange (AO) DNA denaturability assay. This assay is based on the differential denaturability of interphase, mitotic and apoptotic DNA at low pH. Chromatin degradation, which is specific for apoptosis, renders the DNA sensitive to denaturation (Darzynkiewicz *et al*., 1994). This is a very reliable and reproducible assay for apoptosis. Some of the experiments were also performed in parallel by the propidium iodide (PI) exclusion assay. Both assays gave similar results. It should be mentioned that since the M1/2

cell line and its derived clones are dependent on conditioned medium (CM) (Peled *et al*., 1996), each experiment was performed with the same CM batch. Variations observed in the percentage of apoptosis in different experiments are probably due to the use of different CM batches.

Cisplatin-mediated apoptosis

Cisplatin causes DNA damage by intra- and interstrand cross-links in the DNA, which may induce cell cycle arrest and apoptosis (Zamble and Lippard, 1995). Figure 1 depicts the apoptotic patterns observed for cisplatin treatments. Upon a shift to 32°C, p53 143 (Val to Ala)-expressing cells underwent low levels of apoptosis in the absence of any treatment, due to the expression of p53 in the wild-type conformation. However, following treatment with cisplatin, the fraction of cells undergoing apoptosis was enhanced significantly (Figure 1B). This is in agreement with our previous observation, in which a synergy between murine wild-type p53-mediated apoptosis and cisplatin-mediated apoptosis was evident (Li *et al*., 1998). A similar set of samples was utilized to compare the levels of p53 143 (Val to Ala) protein before and after cisplatin treatment. As can be seen in Figure 1C, drug treatment at 32°C did not affect the levels of the p53 143 (Val to Ala) protein.

We next analyzed the effect of p53 143 (Val to Ala) protein expression at 37°C (the permissive temperature for the mutant conformation) on cisplatin-mediated apoptosis. Twenty-four hours after cisplatin treatment, cells were fixed and subjected to the AO assay. FACS analysis revealed that p53 143 (Val to Ala) expressing clones experienced lower levels of apoptosis, compared with that of the vector expressing clone (Figure 1D). Furthermore, the degree of protection from cisplatin-induced apoptosis seemed to correlate directly with mutant p53 protein levels (see Figure 1A). To further confirm this pattern of apoptosis, we exposed cells in parallel to the PI assay. Twenty-four hours after treatment of the various clones with 10 μg of cisplatin, cells were subjected to PI labeling. We found that clones p53ts-143-103 and p53ts-143-106 exhibited 33 and 43% less apoptosis, respectively, compared with the empty vector expressing clone. These data are consistent with the cisplatin resistance that we had previously observed in murine mutant p53 135 (Ala to Val) expressing cells (Li *et al*., 1998). We examined mutant p53 protein levels to ensure its presence after cisplatin treatment, and found that expression not only was not reduced but was slightly increased (Figure 1E).

α-amanitin-mediated apoptosis

We then utilized α-amanitin, a specific inhibitor of RNA polymerase II (Cochet-Meilhac and Chambon, 1974; De Mercoyrol *et al*., 1989), which has been shown to induce apoptosis in certain cell lines (Leist *et al*., 1994; Andera and Wasylyk, 1997; Koumenis and Giaccia, 1997; Ljungman *et al*., 1999). Figure 2A depicts the levels of apoptosis in control and p53 143 (Val to Ala) expressing clones, after 24 h at 32°C with or without α-amanitin. As with cisplatin, a clear cooperation was observed between wild-type p53 expression and α-amanitin-induced apoptosis.

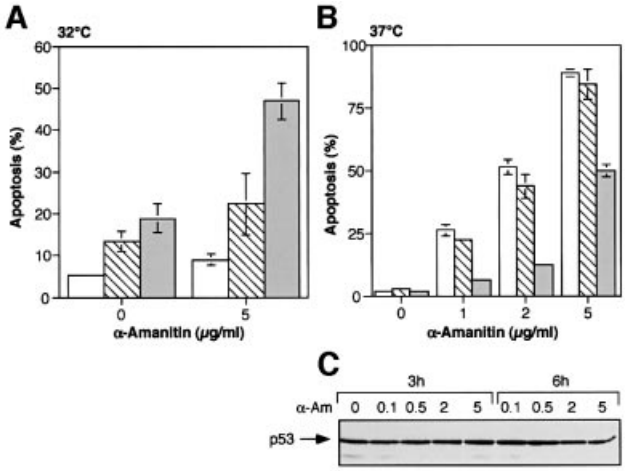

Fig. 2. α-amanitin-induced apoptosis in p53 143 (Val to Ala) expressing M1/2 cells. (**A**) Synergism between wild-type p53 and α-amanitin in the induction of apoptosis. Representation of apoptosis of p53 143 (Val to Ala) expressing cells at 32°C, after exposure to 5 μg of α-amanitin. Open bars represent a clone generated by infection with empty vector, hatched bars represent clone p53ts-143-103 and gray bars represent clone p53ts-143-106. (**B**) Mutant p53 143 (Val to Ala) protein expression interferes with α-amanitin-induced apoptosis. Measurement of apoptosis in p53 143 (Val to Ala) expressing cells after a 24 h treatment with α-amanitin at 37°C. Open bars represent a clone generated by infection with empty vector, hatched bars represent clone p53ts-143-103 and gray bars represent clone p53ts-143-106. (**C**) p53 protein expression of p53ts-143-106 cells grown at 37°C and treated with α-amanitin (α-Am).

Upon treatment with increasing concentrations of α-amanitin at 37°C, the clone that expresses high levels of mutant p53 143 (Val to Ala) protein, p53ts-143-106, exhibited lower levels of apoptosis, compared with the control clone (Figure 2B). Thus, expression of the mutant protein p53 143 (Val to Ala) can also protect the cells from α-amanitin-induced apoptosis. Exposure to α-amanitin did not change the levels of the p53 143 (Val to Ala) protein. A typical western blot using the p53 143 (Val to Ala) expressing clone p53ts-143-106 is represented in Figure 2C. It should be mentioned that the difference in the levels of apoptosis in the presence of 5 μg of α-amanitin at 32 versus 37°C is most likely the result of the different temperatures, and of the use of different CM batches in the individual experiments.

Doxorubicin-mediated apoptosis

Doxorubicin is frequently used in the treatment of a variety of human malignancies (Hortobagyi, 1997). Suggested mechanisms for the cytotoxic effect of doxorubicin include intercalation into DNA, inhibition of enzymes such as topoisomerase II and the generation of free radicals. This drug has also been shown to act as an apoptosis inducer (Gewirtz, 1999). To measure the effects of p53 in the wild-type conformation on doxorubicin-mediated apoptosis, cells maintained at 37°C were treated with increasing concentrations of doxorubicin and shifted to 32°C for 24 h. As can be seen in Figure 3A, wild-type p53 and doxorubicin synergize to induce apoptosis.

At 37°C (the permissive temperature for the mutant conformation) the percentage of apoptosis in p53 143 (Val to Ala) expressing clones following doxorubicin treatment was similar to, or even higher than the control clone

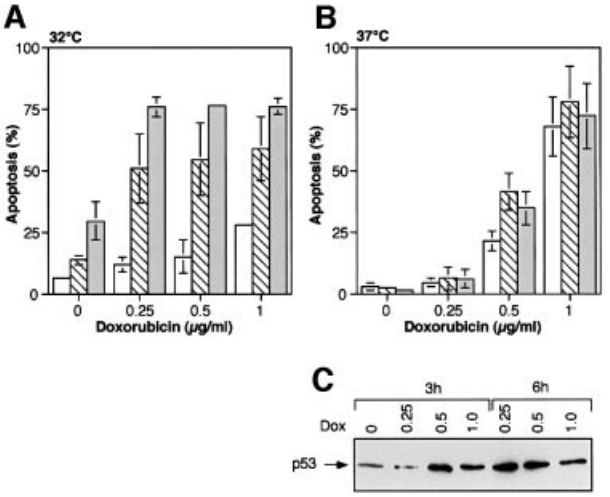

Fig. 3. Doxorubicin-induced apoptosis in p53 143 (Val to Ala) expressing M1/2 cells. (**A**) Wild-type p53 protein expression cooperates with doxorubicin in the induction of apoptosis. FACS analysis of M1/2 clones expressing p53 143 (Val to Ala) protein after a 24 h exposure to increasing concentrations of doxorubicin at 32°C. Open bars represent a clone generated by infection with empty vector, hatched bars represent clone p53ts-143-103 and gray bars represent clone p53ts-143-106. (**B**) Expression of mutant p53 143 (Val to Ala) protein does not affect doxorubicin-mediated apoptosis. Graphical representation of the percentage of apoptosis in p53 143 (Val to Ala) expressing clones following doxorubicin treatment at 37°C. Open bars represent a clone generated by infection with empty vector, hatched bars represent clone p53ts-143-103 and gray bars represent clone p53ts-143-106. (**C**) Western blot analysis for p53ts-143-106 cells grown at 37°C and treated for 3 and 6 h with doxorubicin (Dox).

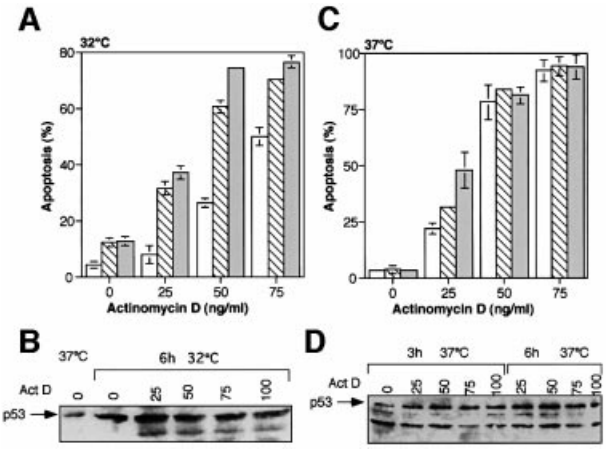

Fig. 4. Actinomycin D-induced apoptosis in p53 143 (Val to Ala) expressing M1/2 cells. (**A**) Cooperation between wild-type p53 and actinomycin D in the induction of apoptosis. Results of FACS analysis of M1/2 p53 143 (Val to Ala) expressing clones following treatment with actinomycin D and a temperature shift to 32°C. Open bars represent a clone generated by infection with empty vector, hatched bars represent clone p53ts-143-103 and gray bars represent clone p53ts-143-106. (**B**) Western blot analysis for p53 for p53ts-143-106 cells exposed to the same treatments as in (A) for 6 h. Act D, actinomycin D. (**C**) Expression of mutant p53 143 (Val to Ala) protein does not affect actinomycin D-mediated apoptosis. Percentage of apoptotic cells of the M1/2-derived clones following a 24 h exposure to actinomycin D. Open bars represent a clone generated by infection with empty vector, hatched bars represent clone p53ts-143-103 and gray bars represent clone p53ts-143-106. (**D**) Western blot analysis of p53ts-143-106 cells for the same treatments as in (C). Act D, actinomycin D.

(Figure 3B). To exclude the possibility that the drug treatment merely abolished expression of mutant p53 protein in these cells, we analyzed mutant p53 protein levels before and after doxorubicin treatment. Western blot analysis of mutant p53 protein levels in the p53 143 (Val to Ala) expressing clone p53ts-143-106 revealed that protein expression was elevated upon doxorubicin treatment (Figure 3C). Hence, the lack of mutant p53-mediated suppression of apoptosis was not due to a reduction in its protein level. In agreement with our observations with cisplatin and α-amanitin, wild-type p53 synergizes with doxorubicin-induced apoptosis. Unlike cisplatin and α-amanitin, where mutant p53 blocked apoptosis, in the case of treatment with doxorubicin, such blocking activity was not evident.

Actinomycin D-induced apoptosis

We next investigated the effect of p53 expression on apoptosis induced by the intercalating agent actinomycin D. Intercalation of actinomycin D into DNA creates a physical block to the various RNA polymerases, thus inhibiting transcription (Pratt and Ruddon, 1979). Actinomycin D has been shown to induce apoptotic cell death, probably as an outcome of transcription inhibition. At 32°C, the permissive temperature for the wild-type conformation, p53 expression and actinomycin D clearly cooperate in the induction of apoptosis (Figure 4A). The p53 143 (Val to Ala) protein level was elevated by the exposure to the drug (Figure 4B).

As we have previously demonstrated for the murine p53 135 (Ala to Val) mutant (Li *et al*., 1998), the expression of p53 143 (Val to Ala) at 37°C does not interfere with actinomycin D-induced apoptosis (see Figure 4C). This lack of protective effect against actinomycin D-induced apoptosis by mutant p53 is not due to a reduction in the levels of the mutant p53 protein, as actinomycin D treatments result in the stabilization of mutant p53 143 (Val to Ala) (Figure 4D). Thus, p53 143 (Val to Ala) expression does not affect actinomycin D-induced apoptosis.

M1/2 clones expressing the human p53 248 (Arg to Trp) mutant or p53 273 (Arg to His) mutant were examined for sensitivity to the same array of drugs as p53 143 (Val to Ala). We also investigated the response of M1/2 clones expressing the different mutations to etoposide, a topoisomerase II inhibitor (Hande, 1998), and to DRB, a specific inhibitor of RNA polymerase II function (Dubois *et al*., 1994; Yankulov *et al*., 1995). The results obtained with the various p53 mutations and the different drugs tested are summarized in Table I. As can be seen, similar patterns of protection were observed with all p53 mutations. The various p53 core domain mutants interfered with cisplatin-, α-amanitin- and etoposide-induced apoptosis, but exerted no blocking activity on doxorubicin-, actinomycin D- or DRB-mediated apoptosis.

The effect of loss of transcriptional competence on the apoptotic activity of p53

We next investigated the mechanism that underlies the protective effect of mutant p53 against cisplatin-, α-amanitin- and etoposide-induced apoptosis. The observation that the p53 N-terminal domain is rarely modified in human tumors (Hussain and Harris, 2000), coupled with

Table I. Specific interference with apoptosis-inducing agents by various human p53 mutations

Mutation	Cisplatin	α-amanitin	Actinomycin D	Doxorubicin	Etoposide	DRB
143Val → Ala	+	+	–	–	+	–
248Arg → Trp	+	+	–	–	+	–
273Arg → His	+	+	–	–	+	–

–, no blocking activity; +, protective effect.

the characterization of this domain as the transcription activation domain of p53 (Unger *et al*., 1992; Subler *et al*., 1994), prompted us to investigate the possibility that transcription activation by mutant p53 is required for the rescue from apoptosis. It has been shown previously that a double mutation changing Leu to Gln at position 22 and Trp to Ser at position 23 (Gln22, Ser23) in the p53 transactivation domain impaired the ability of the wild-type p53 protein to activate transcription (Lin *et al*., 1994). Furthermore, the p53 281 (Asp to Gly) mutant, which was shown to transactivate MDR-1 and *c-myc*, lost this ability upon insertion of these two N-terminal mutations (Lin *et al*., 1995; Frazier *et al*., 1998). Thus, it appears that the transcription domain of mutant p53 is similar to that identified in the wild-type p53 protein. Therefore, we generated M1/2 stable clones, which express a p53 molecule harboring the 22 Gln and 23 Ser mutations in conjunction with the 143 Ala core mutation (referred to as the 22,23,143 triple mutant). M1/2 clones expressing wild-type p53 harboring the same N-terminal mutations [hereafter termed p53 (22-23)] were generated in parallel.

Effect of the 22-23 mutations on the apoptotic activity of wild-type p53

We next examined the p53 (22-23) expressing clones. Our infections yielded a series of M1/2-derived clones expressing various levels of p53 (22-23). Western blot analysis of the clones that were selected for further study is presented in Figure 5A. We found that the p53 (22-23)-expressing clones exhibit a normal cell cycle over a prolonged incubation. The percentage of apoptotic cells in these clones is very low and is comparable to that in the parental cells (2–5%). The fact that we have generated viable clones harboring a plasmid coding for a transcription-deficient p53 but otherwise wild type in structure, further confirms the notion that apoptosis mediated by wild-type p53 is transcription dependent (Sabbatini *et al*., 1995; Attardi *et al*., 1996; Yonish-Rouach *et al*., 1995; Chao *et al*., 2000; Jimenez *et al*., 2000). To confirm that the p53 (22-23) protein expressed in our M1/2 clones is transcriptionally defective, we analyzed the expression of p21/WAF1, a p53 downstream gene (El-Deiry *et al*., 1993) involved in growth arrest, before and following DNA damage with cisplatin. p53 (22-23) expressing cells were treated with various concentrations of cisplatin, and RNA was extracted following 4 h of treatment. RT–PCR analysis revealed that without treatment WAF1 is expressed at very low levels (Figure 5B). Furthermore, after treatment with cisplatin, WAF1 expression could not be detected. As a positive control, we demonstrated WAF1 induction by p53 143 (Val to Ala) expressed as its wild-type p53 conformation (see Figure 5B). We therefore concluded that the p53 (22-23) in our experimental system is indeed transcriptionally inactive.

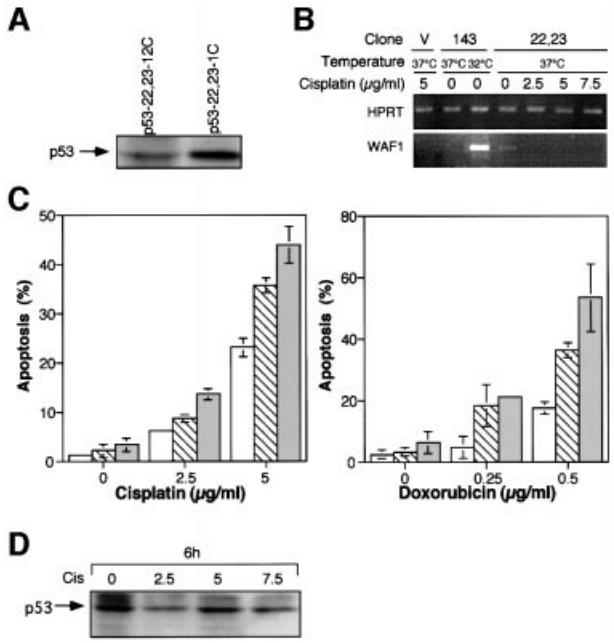

Fig. 5. Characterization of M1/2 clones expressing the p53 (22-23) molecule. (**A**) Western blot analysis of representative M1/2 clones expressing the p53 (22-23) transactivation-deficient mutant. (**B**) p53 (22-23) expressed in M1/2-derived clones is transactivation-defective. RT–PCR analysis to examine WAF1 gene expression in p53-22,23-1C clone, before and after treatment with cisplatin. cDNAs from clone p53ts-143-106, expressing the p53 143 (Val to Ala) ts mutant, at 37 and 32°C, were used as negative and positive controls, respectively. HPRT gene expression was used for all cDNAs as a quantity control. V, vector expressing cells. (**C**) Enhancement of DNA damage-induced apoptosis in p53 transactivation-deficient expressing clones. FACS analysis of cisplatin and doxorubicin treatments of p53 (22-23) expressing clones. Open bars represent a clone generated by infection with empty vector, hatched bars represent clone p53-22,23-12C and gray bars represent clone p53-22,23-1C. (**D**) Western blot for p53 expression in p53-22,23-1C cells treated with cisplatin (Cis).

We next examined whether the p53 transcription-deficient mutant, p53 (22-23), can synergize with drug-induced apoptosis, as we observed for intact wild-type p53 (Li *et al*., 1998). As can be seen in Figure 5C, treatment with cisplatin or doxorubicin induced significant enhancement in apoptosis in the p53 (22-23) expressing clones, compared with the control clone. Western blot analysis confirmed that p53 (22-23) is expressed during cisplatin treatment (Figure 5D). These observations suggest that synergism of wild-type p53 with drug-induced apoptosis involves a transcription-independent mechanism, supporting the notion that wild-type p53-mediated apoptosis may be transcription independent (Caelles *et al*., 1994; Wagner *et al*., 1994; Haupt *et al*., 1995; Roemer and Mueller-Lantzsch, 1996; Ding *et al*., 2000; Kokontis *et al*.,

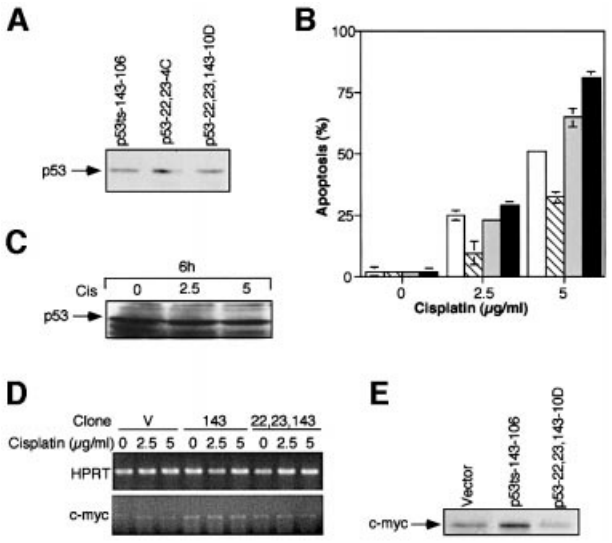

Fig. 6. Protection of mutant p53 from cisplatin-induced apoptosis requires a transcriptionally active mutant p53. (**A**) A representative western blot analysis of selected clones expressing similar levels of p53 143 (Val to Ala), p53 (22-23) or p53-22,23,143. (**B**) M1/2 stable clones expressing comparable levels of mutated p53, 143Ala or 22Gln and 23Ser, or these three mutations all together, were treated with cisplatin at 37°C and assayed by AO for percentage of apoptosis. Open bars represent a clone generated by infection with empty vector, hatched bars represent clone p53ts-143-106, light gray bars represent clone p53-22,23-4C and dark gray bars represent clone p53-22,23,143-10D. (**C**) Cell lysates from p53-22,23,143-10D cells, before and after treatment with cisplatin, were analyzed by immunoblotting for p53 triple mutant expression. Cis, cisplatin. (**D**) p53 (22-23),143 expressed in M1/2-derived clones is transactivation defective. RT–PCR analysis to examine *c-myc* gene expression in clones expressing the empty vector (V), the p53 single mutant or the p53 triple mutant, before and after treatment with cisplatin. HPRT gene expression was used for all cDNAs as a quantity control. (**E**) Western blot analysis of c-myc protein expression in the various clones. Vector, vector expressing cells.

2001). Based on these observations, we propose that the capacity of p53 to initiate apoptosis is dependent on the activation of p53 downstream genes. However, in drug-induced apoptosis, p53 may facilitate this process in a manner that does not require p53 to be transcriptionally active.

Effect of the 22-23 mutations on mutant p53-mediated interference with apoptosis

We then analyzed the triple mutant clones expressing the p53 143 (Val to Ala) core mutation in conjunction with the N-terminal 22Gln and 23Ser mutations. We sought to determine the response of these clones to treatment with cisplatin, for which we found the single mutant p53 143 (Val to Ala) protective. To that end, we chose a set of clones expressing comparable levels of either the p53 143 (Val to Ala) mutant, the p53 (22-23) double mutant or the p53 (22-23),143 triple mutant. Protein levels in the various clones were determined by western blot analysis and immunoprecipitations. A typical western blot analysis for the clones selected, p53ts-143-106, p53-22,23-4C and p53-22,23,143-10D, is represented in Figure 6A. We subjected the set of chosen clones to treatment with cisplatin at 37°C, the permissive temperature for the mutant conformation. As can be seen in Figure 6B, addition of the 22Gln and 23Ser mutations to the p53 143 (Val to Ala) mutant abolished the protective effect against cisplatin-induced apoptosis. It should be mentioned that the percentage of apoptosis in the p53-22,23,143-10D clone, expressing the triple mutant, was even higher than in the p53 (22-23) expressing clone. Western blot analysis of p53-22,23,143-10D cells revealed that the cisplatin treatment did not affect the levels of the p53 triple mutant protein (Figure 6C). Thus, the loss of protection from drug-induced apoptosis in these cells cannot be attributed to a loss of expression of the mutant p53 protein, and is most likely directly related to the lack of mutant p53 transcription activity. Protection of mutant p53 against cisplatin-induced apoptosis requires a transcriptionally active mutant p53.

Modulations in c-myc expression pattern in the various mutant p53-expressing clones

To further resolve the pathway that controls the activity of mutant p53 in drug resistance, it was important to characterize specific changes in the expression of downstream genes. At present, several candidates have been suggested (reviewed in Sigal and Rotter, 2000). We have focused our experiments on c-myc, which has been suggested as a specific target gene of mutant p53 (Frazier *et al*., 1998). In our experiments we examined whether activation of c-myc correlated with mutant p53 activity in drug resistance. As it has been shown that c-myc induction by mutant p53 is dependent on the integrity of the transcription domain (Frazier *et al*., 1998), it was important to examine whether the p53 triple mutant protein, which lost the ability to block cisplatin-induced apoptosis, is also deficient in the ability to activate the c-myc promoter. We therefore used RT–PCR analysis to compare the RNA levels of c-myc in the empty vector expressing clone, the p53ts-143-106 clone and the p53-22,23,143-10D clone, before and after a 4 h treatment with cisplatin. We noticed that while c-myc is upregulated in the single mutant expressing clone, this upregulation is absent in the triple mutant-expressing clone (Figure 6D). Analysis of c-myc protein levels in these clones indicated that, like the RNA, the elevation in c-myc expression observed in the p53 core domain mutant was abolished in the p53 triple mutant expressing clone (Figure 6E). We therefore conclude that the p53 (22-23),143 expressed in M1/2 is transcriptionally inactive in this pathway.

Discussion

p53, 'the guardian of the genome', has been shown to exert tumor suppressor activity by the activation of cell cycle checkpoints (Schwartz and Rotter, 1998; Sionov and Haupt, 1999), involvement in DNA repair (Tlsty, 1997; Wahl *et al*., 1997; Janus *et al*., 1999), induction of apoptosis (Levine, 1997; Gottlieb and Oren, 1998) and cell differentiation (Almog and Rotter, 1997). In cancerous cells, however, it appears that not only the loss of the wild-type p53 form, but also the accumulation of mutant p53 forms, plays a critical role in the process of establishing the malignant phenotype (Sigal and Rotter, 2000).

In our previous studies using a murine model we observed that while the wild-type p53 form synergizes

with p53-independent apoptosis induced by DNA-damaging agents, mutant p53 interferes with these apoptotic pathways (Peled *et al*., 1996; Li *et al*., 1998).

The mechanism by which wild-type p53 mediates apoptosis is still unclear. In particular, the question of whether p53-induced apoptosis requires transcriptional activity is debatable. Several studies suggest that the human p53 (22-23) transcription-deficient mutant is capable of inducing apoptosis (Haupt *et al*., 1995; Ding *et al*., 2000; Kokontis *et al*., 2001). Others have shown that the same p53 mutant was incompetent for the induction of apoptosis (Sabbatini *et al*., 1995; Attardi *et al*., 1996; Yonish-Rouach *et al*., 1995). Findings that support the latter possibility were published recently for the analogous murine p53 mutant (Chao *et al*., 2000; Jimenez *et al*., 2000).

Here we show that p53 may mediate apoptosis by both transcription-dependent and -independent pathways. Our success in establishing M1/2 stable clones expressing the transcriptionally deficient p53, p53 (22-23), with a very limited apoptotic population, argues for a transcription-dependent mechanism for initiating p53-mediated apoptosis. On the other hand, the ability of the p53 (22-23) to synergize with drug-induced apoptosis supports the notion that p53-dependent apoptosis may occur in the absence of transcription.

It appears that in these clones the transcription-independent pathway for apoptosis by p53 is silent in normal growing conditions and is turned on only following exposure to DNA damage. Further support for the idea that a transcription-independent pathway is induced by p53 following stress is provided by the observations presented in this report, and previously shown by others (Caelles *et al*., 1994), that treatment with the transcription inhibitors actinomycin D or α-amanitin does not interfere with wild-type p53-induced apoptosis. It is conceivable that during the normal cell cycle transcription may take place to execute programmed cell death. However, the presence of damaged DNA requires an immediate response. Thus, a transcription-coupled pathway, which is time consuming, might be less appropriate. It may well be that the mechanism chosen for apoptosis by p53 is also dependent on the cell type, the genetic background and the type of damage. Taken together, this may explain the controversial findings in this field. We suggest that the net result of this complex equation determines the choice of either a transcription-dependent or -independent pathway for wild-type p53-mediated apoptosis.

According to the current state of knowledge, some wild-type p53 activities are dependent on the transcriptional activity of the protein, whereas others are independent of this activity. While growth arrest has been shown to be dependent on the transactivation of specific downstream genes (El-Deiry *et al*., 1993), p53-dependent apoptosis may involve either transcription-dependent or -independent mechanisms. Interestingly, we have observed that in DNA repair, particularly in the base excision repair pathway, p53 acts through a transcription-independent mechanism (Offer *et al*., 2001).

Mutant p53 has been shown to act either by a transdominant mechanism involving inactivation of the wild-type p53 form or a by gain of function, independent of wild-type p53 (Sigal and Rotter, 2000). Protection from chemotherapy can be the result of wild-type p53 loss, but we and others have previously demonstrated that it can also result from mutant p53 gain of function (reviewed in Hickman and Dive, 1999). We have observed that p53 135 (Ala to Val), expressed in M1/2 p53 non-producer cells, provides protection from p53-independent apoptosis mediated by γ-irradiation and several chemotherapeutic agents (Li *et al*., 1998). Likewise, drug resistance following mutant p53 expression has been reported in the H1299 cell line (Blandino *et al*., 1999).

In this report we demonstrate that various human p53 mutant proteins can interfere with several drug-induced apoptotic pathways. Expression of the p53 mutants 143 (Val to Ala), 248 (Arg to Trp) or 273 (Arg to His) resulted in resistance to cisplatin and etoposide, drugs commonly used in chemotherapy, and also to α-amanitin, which may serve as a potential agent for cancer treatment (Koumenis and Giaccia, 1997). These effects of mutant p53 were observed in the absence of wild-type p53 and therefore represent a gain of function activity of the mutant p53 protein. Interestingly, mutant p53 expression did not interfere with doxorubicin-, DRB- or actinomycin D-induced apoptosis, suggesting that mutant p53 protein forms may affect differently the outcome of various drugs. The fact that mutant p53 proteins did not confer resistance to all drugs may have important implications for the choice of drugs in patients with p53 mutations.

Our present study shows that, as in the case of wild-type p53, core mutant p53 acts as a transcription factor. However, it is expected that the two will engage different downstream pathways. We observed that alterations in the N-terminal domain of core mutant p53 abrogated the apoptosis-blocking activity. This conclusion is further substantiated by studies showing that mutant p53 can transactivate genes involved in malignancy. Among the specific target genes of mutant p53 that were identified, several are candidates for mediating the anti-apoptotic effect. p53 mutants can transactivate the MDR-1 gene promoter (Dittmer *et al*., 1993; Lin *et al*., 1995), and insulin-like growth factor II (Lee *et al*., 2000), as well as genes that are involved in proliferation (Deb *et al*., 1992; Margulies and Sehgal, 1993; Ludes-Meyers *et al*., 1996).

The c-myc oncoprotein has also been identified as a specific target gene of mutant p53 (Frazier *et al*., 1998). In agreement with these observations, we found that in M1/2 cells c-myc RNA and protein expression are elevated by mutant p53 protein expression, and that this upregulation is dependent on an intact mutant p53 transactivation domain. The elevated expression of *c-myc* in the mutant p53 expressing clone may be directly linked to the resistant phenotype of these clones. This idea is supported by studies showing that activation of *c-myc* contributes to chemoresistance to cisplatin and other drugs (Niimi *et al*., 1991; Sklar and Prochownik, 1991; Kinashi *et al*., 1998). Furthermore, decreasing the expression of *c-myc*, by using antisense oligonucleotides, has been shown to enhance the efficacy of cisplatin treatment both *in vitro* and *in vivo* (Mizutani *et al*., 1994; Van Waardenburg *et al*., 1996; 1997; Citro *et al*., 1998; Leonetti *et al*., 1999). However, it should be borne in mind that others have reported that overexpression of c-myc increases the sensitivity to drugs by activating apoptosis (Lotem and Sachs, 1993, 1995;

Dong *et al*., 1997). If the latter is the case, mutant p53 expressing cells may tolerate higher levels of c-myc than the empty vector clone, and therefore do not undergo c-myc-induced apoptosis upon c-myc expression. Such tolerance to c-myc overexpression has been shown (Zindy *et al*., 1998) for p53-null murine embryonic fibroblasts, compared with their normal counterparts. In this scenario, c-myc elevation in mutant p53 expressing cells may play a role in gain of function activities of mutant p53 other than chemoresistance, such as immortalization and tumorigenicity.

Since the p53 (22-23) was also shown to be defective in the repression of promoters (Roemer and Mueller-Lantzsch, 1996), it may well be that mutant p53-mediated chemoresistance is acquired by the repression of apoptotic genes.

Our present results show that integrity of the N-terminus is critical for mutant p53 anti-apoptotic function. Furthermore, we recently found that modification of the extreme C-terminus of the murine mutant p53 135 (Ala to Val) by alternative splicing resulted in the loss of this gain of function activity (Sigal and Rotter, 2000). These results are consistent with a transactivation-based mechanism for mutant p53 gain of function, since truncation of the extreme C-terminus of the p53 281 (Asp to Gly) mutant rendered it transcriptionally inactive (Frazier *et al*., 1998).

It should be borne in mind that the N-terminus of p53 is also the site that interacts with mdm2. Therefore, it could not be entirely ruled out that the abolishment of the apoptosis-blocking activity of mutant p53, by insertion of the 22Gln and 23Ser mutations, could at least be partly due to the disruption of this pathway. Indeed, it has recently been demonstrated that while various p53 mutants, including p53 281 (Asp to Gly), may stabilize the mdm2 oncoprotein, p53 22,23,281 is defective in this activity (Peng *et al*., 2001). In addition, it has been reported recently (Buschmann *et al*., 2000) that mutant p53 may interact with mdm2 or JNK, known components in the control of wild-type p53 stability (Woods and Vousden, 2001). Interestingly, we observed that treatment of cells with cisplatin, doxorubicin and actinomycin D induced the accumulation of mutant p53. These observations further support the idea that, in addition to the existence of the mdm2–wild-type p53 feedback loop, mdm2 may also affect the mutant p53 protein.

Taken together, we propose that the protective activity of mutant p53 relies upon an intact N-terminus (as shown in this report) as well as C-terminus (Sigal *et al*., 2001). This hypothesis is supported by the molecular epidemiology of p53, in which mutations in primary human tumors were found to be clustered at the core domain of p53, leaving both the N- and the C-terminus unaffected (Hussain and Harris, 2000). This suggests that, like the wild-type p53, activity of mutant p53 requires the extreme parts of the molecule, which contain various functional domains. Our present findings support the notion that mutant p53 protein is transcriptionally active and that in malignant transformation it acts via a gain of function mechanism. This conclusion provokes the need for a specific search of target genes that are activated in this mutant p53 gain of function pathway.

Materials and methods

Plasmids and cell lines

The cDNAs encoding the various p53 mutants were cloned into the pBabe-puro retroviral vector (Morgenstern and Land, 1990). Retroviral stocks were generated by using the highly efficient 293GP packaging cell line (Yee *et al*., 1994). The 293GP cells were transfected with the cDNA constructs by the calcium phosphate method. Stable clones expressing the p53 mutations were generated by infection of the M1/2 parental p53 non-producer cell line as described previously (Peled *et al*., 1996). For each p53 mutant, several clones expressing different levels of the mutated protein were selected. Clones were grown continuously in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and CM. Each set of experiments was performed with the same batch of CM.

Drugs

Cisplatin (Abic) and α-amanitin (Sigma) were prepared as 1 mg/ml stock solutions in water. Actinomycin D and doxorubicin (Sigma) were prepared, respectively, as 500 μg/ml and 2 mg/ml stock solutions in water. DRB (Sigma) was prepared as a 0.5 M stock solution in DMSO. Etoposide (Sigma) was dissolved in DMSO to a concentration of 50 mM.

Western blot analysis

Cell extracts for western blot analysis were lysed in TLB buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS). The samples were normalized according to quantitation by Bradford assay (Bio-Rad). Protein sample buffer (140 mM Tris pH 6.8, 22.4% glycerol, 6% SDS, 10% β-mercaptoethanol, 0.02% bromophenol blue) was added, and the samples were boiled for 10 min and loaded onto 10% SDS–polyacrylamide gel. The proteins were transferred to nitrocellulose membranes using a semi-dry transfer cell (Bio-Rad). The p53-specific monoclonal antibody Pab-1801 was used to detect p53. c-myc protein was detected by an antibody kindly provided by H.Kahana. The protein–antibody complexes were detected by using a horseradish peroxidase-conjugated secondary antibody by the super-signal enhanced chemiluminescence system (Pierce).

FACS analysis of apoptosis

The AO DNA denaturability assay was performed as described previously (Li *et al*., 1998). Briefly, cells were fixed in 80% ethanol–20% Hanks balanced salt solution (HBSS) and stored at –20°C. Cells were washed once with HBSS, resuspended in HBSS containing 0.25 mg/ml RNase, and incubated at 37°C for 1 h and 15 min.

Cells were then harvested and resuspended in HBSS. Cell suspensions (200 μl) were added to 0.5 ml of 0.1 M HCl for a 40 s incubation. Acid denaturation was quenched by the addition of 2 ml of AO (Molecular Probes) staining solution pH 2.6, containing 90% v/v 0.1 M sodium citrate, 10% v/v 0.2 M Na_2HPO_4 and 6 μg/ml AO (Darzynkiewicz *et al*., 1994). The PI (Sigma) exclusion assay was performed as for the AO assay, except that following the incubation with RNase, 20 μg/ml PI was added to the cell suspensions (500 μl). The cells were analyzed by the FACScan flow cytometer (Beckton-Dickinson), using the CellQuest (Beckton-Dickinson) software.

RT–PCR analysis

Total RNA was extracted from 2×10^6 cells using the Tri-Reagent kit (Sigma) according to the manufacturer's instructions. For the generation of cDNA, 3 μg of RNA were incubated with 0.1 μg of oligo dT at 70°C for 10 min. Reverse transcription was performed using Superscript II following the manufacturer's instructions (Gibco-BRL). The following primers were used for PCR amplification under the following conditions: HPRT, 23 cycles at 50°C (5′ primer, CAGAGGACTAGAACACCT; 3′ primer, GCTGGTGAAAAGGACCTCT); waf1, 30 cycles at 58°C (5′ primer, GATTGCGATGCGCTCATGG; 3′ primer, CCTCCTGACCCA-CAGCAGAAG); and c-myc, 22 cycles at 58°C (5′ primer, GGA-AACTTTGCCCATTGCAG; 3′ primer, ACGTAGCGACCGCAA-CATAG).

Acknowledgements

We would like to thank Professor H.Kahana for the generous gift of the anti-c-myc antibody. This work was supported in part by grants from the Israel–USA Binational Science Foundation (BSF) the DIP (Deutsch–Israelische Projektkooperation) and the Kadoori Foundation. V.R. is the incumbent of the Norman and Helen Asher Professorial Chair in Cancer Research at the Weizmann Institute.

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Received January 25, 2001; revised May 10, 2001; accepted June 14, 2001