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Studies of p53-Mediated mdm2 and gadd45 Gene Activation in Nuclear Chromatin

by

GU XIAO

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

Studies of p53-Mediated mdm2 and gadd45 Gene Activation in Nuclear Chromatin

by

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Advisor: Professor Jill Bargonetti

The tumor suppressor p53 is a transcription factor with the sequence-specific DNA binding activity. A variety of its target genes have been identified when p53 was overexpressed. However, none of the sequence of the binding site completely fits the consensus sequence, and whether p53 is able to bind to these binding sites *in vivo* still unknown. In addition, protein products of the p53-target genes have diverse functions. In this study, we have investigated p53-mediated mdm2 and gadd45 gene activation in two systems. One is that it utilizes murine cells containing a temperature sensitive p53-Val135. At 32°C, p53-Val135 assumes a wild-type conformation, while at 37°C this p53 is conformationally mutant. The other system is by an inducible promoter that regulates p53 levels to analyze p53-mediated gene activation both in the presence and absence of DNA damage. Gadd45 protein induces cell growth arrest and protects genome integrity while Mdm2 protein promotes p53 degradation and blocks its transcriptional activity. Additionally, the transcriptional activity of p53 was considered to be part due to the increasing protein levels. Here, we provide evidence that (1) p53 binds specifically to the murine mdm2 P2 promoter in nuclear chromatin, and this binding correlates with mdm2 endogenous mRNA

induction; (2) the mdm2 P2 promoter is maintained in a nucleosome free state and activation of the mdm2 gene does not require chromatin remodeling; (3) the p53 target genes p21, gadd45, and mdm2 are differentially activated in the presence and absence of DNA damage. Both induced p53 and DNA damage signaling are necessary to activate the gadd45 gene; (4) the p53-mediated gadd45 induction by camptothecin did not require a further increase in nuclear p53 levels, or DNA binding activity, or phosphorylation of p53 at serine 15 and acetylation of p53 at lysine 382; (5) constitutive DNase I hypersensitivity was detected by indirect Southern blot analysis at both the gadd45 promoter and its putative p53-RE, but after camptothecin treatment increased DNase I sensitivity was only detected at the gadd45 promoter region. We conclude that both mdm2 and gadd45 regulatory regions are preset for rapid activation by p53. Up-regulation of mdm2 is independent of change of chromatin structure. Induction of gadd45 gene expression by p53 and DNA damage signaling alters the configuration of chromatin at the gadd45 promoter region. Finally, the stability of p53 and the transcriptional activity of p53 are regulated at different levels.

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TABLE OF CONTENTS

LIST OF FIGURES

Page

Chapter I

Introduction

Loss of cell cycle control and increased genomic instability are central features in the development of cancer (reviewed in Agarwal et al., 1998). Activation of proto-oncogenes concomitant with inactivation of tumor suppressor genes leads to abnormal proliferation and tumor formation. p53 is one of the most important tumor suppressor proteins. Mutations which disrupt the functions of p53 are among the most common found in human cancers (reviewed in Prives and Hall 1999). Consistent with its role as a tumor suppressor, p53 works as a multifunctional protein in regulating cell growth, including cell cycle arrest, apoptosis, control of genome integrity, and DNA replication and repair.

1.1 p53: Guardian of the Genome

p53 was first identified as a protein that co-immunoprecipitated with Simian Virus 40 (SV40) large T antigen in SV40-transformed cells (Linzer et al., 1979). The knowledge that p53 was a tumor suppressor and not a oncoprotein was based on the fact that mutant p53 can transform cells in culture while wild-type p53 can suppress the transformation by viral or cellular oncogenes, such as *myc* and *ras* or E1A and *ras* (Finlay et al., 1989; Eliyahu et al., 1989). In 1989, p53 gene mutations were found in both alleles of colon cancer cells as well as other human tumors (Baker et al., 1989; Nigro et al., 1989). Moreover, using retroviral vectors to introduce wild-type p53 into human osteogenic sarcoma cells demonstrated p53 dependent reduction in tumorigenicity (Chen et al., 1990). Further studies indicated that from more than 5,000 human tumor samples, representing 43 different tumor types, 50-60% of all cancers have a mutated p53 gene (Hollstein et al., 1994; Momand and Zambetti 1997). In addition, members of Li-Fraumeni cancer-prone families were shown to carry germ-line p53 mutations (reviewed by Ko and Prives, 1996). While p53 knockout mice are developmentally

normal they are susceptible to spontaneous tumors, predominantly lymphomas (Donehower et al., 1992). Mice heterozygous for the inactivated p53 allele also show an increased incidence of spontaneous malignancies as compared to p53+/+ mice. The heterozygous mice develop predominantly osteosarcomas and soft tissue sarcomas and the wild-type p53 allele has been lost in the tumors in the most cases (reviewed by Ko and Prives 1996).

a. Cell Cycle Checkpoints

It has been reported that human fibroblast cells TR9-7 revealed a marked reduction in S phase and an increase in both G1 and G2/M after induction of wild-type p53 (Agarwal et al., 1995).

G1-S transition. The cyclin (type D and type E)-cyclin dependent kinase (Cdk4/6 and Cdk2)-Retinoblastoma protein (Rb)-E2F pathway is well documented for the regulation of the G1-S phase transition (reviewed in Sherr and Roberts, 1999). The Rb protein is a target of Cdks. The phosphorylation of Rb leads to the release of E2F, which then proceeds to activate target genes (reviewed in Cox and Lane 1995). The E2F protein can regulate a number of genes required to initiate or propagate the S phase of the cell cycle, including cyclin E, cyclin A, dihydrofolate reductase, and Proliferating Cell Nuclear Antigen (PCNA) (reviewed in Levine 1997). When hypophosphorylated, Rb binds and sequesters the transcription factor E2F. The best known p53-mediated G1 arrest is through p21/waf1, which is a p53 transcriptional target and binds to the cyclin-Cdk complex (including cyclin D1-Cdk4/6 and cyclin E-Cdk2). Although at first glance p21 appeared to be the critical target of p53, p21 knockout mice develop normally, do not

develop tumors and are only partially deficient in their ability to arrest cells in G1 phase upon DNA damage (Deng et al., 1995).

G2-M transition. It has been shown that overexpression of wild-type p53 can prevent cells entry into mitosis (Agarwal et al., 1995). 14-3-3σ is highly induced in a p53 dependent manner after DNA damage and induction of 14-3-3σ causes inhibition of the G2/M progression by inhibiting Cdc25C (Hermeking et al., 1997).

Spindle checkpoint. It was found that cells with wild-type p53 treated with the micotubule assembly inhibitor nocodazole arrested with 4N DNA content. However p53 null cells treated with nocodazole resulted in cells with DNA contents of 8N or 16 N (Cross et al., 1995). In addition, mouse fibroblasts from p53 null mice produce abnormal numbers of centrosomes after a few doublings in cell culture and initiate spindles with three or four poles (reviewed in Levine 1997). These observations suggest that p53 is involved in a checkpoint that blocks the re-replication of DNA.

b. Control of Apoptosis

There is evidence that the apoptotic activity of p53 is of central importance to its tumor suppressive activity (reviewed in Bates and Vousden 1999). It seems that cell cycle arrest can function to inhibit the growth of normal cells, but cells that have attained oncogenic activation are less susceptible. The ability of p53 to induce apoptosis appears to correlate with its ability to suppress transformation (reviewed in Bates and Vousden 1999). In addition, it has been reported that levels of p53 can sometimes dictate cellular response. Lower levels of p53 result in cell cycle arrest whereas higher levels result in apoptosis, however under some conditions DNA damage can heighten the apoptotic

response to p53 without altering the protein level of p53 (Chen et al., 1996b). Apoptosis is physiological cell death and it involves a genetically determined cellular program that is essential for normal development of multicellular organisms and maintenance of tissue homeostasis (Raff 1992). Cells undergoing apoptosis display shrinkage, loss of cell-cell contact, chromatin condensation, internucleosomal degradation of DNA and finally the loss of membrane integrity (reviewed in Bates and Vousden 1999). Cells obtained from p53 null mice have been shown to be resistant to the induction of apoptosis by radiation and chemotherapy (reviewed in Gallagher and Brown 1999). An effect for p53 in the apoptotic response to DNA damage was further proved by the fact that the introduction of exogenous p53 into some tumor cell lines was able to activate programmed cell death (reviewed in Bates and Vousden 1999). Wild-type p53 mediated apoptosis results from several stimulus, including DNA damage, withdrawal of growth factors, and overexpression of Myc, adenovirus E1A, human papilloma virus E7 or in the absence of Rb (reviewed in Ko and Prives 1996). Activation of Bcl-2 associated X protein (Bax) and Insulin-like Growth Factor-Binding Protein -3 (IGF-BP3) by wild-type p53 has been shown to play a role in apoptosis (Miyashita and Reed, 1995; Buckbinder et al., 1995). Bax has been identified as a protein that forms a complex with Bcl-2. Bcl-2 functions as a repressor of cell death and Bax acts as an accelerator of apoptosis. Following a death stimulus, cytosolic and monomeric Bax protein translocates to the mitochondria where it becomes an integral membrane protein and induces dimerization with Bcl-2 protein (reviewed in Gross et al., 1999). The dimerization results in altered mitochondrial membrane potential, production of reactive oxygen species, and release of cytochrome *c* , which activates a downstream caspase program. Therefore, the ratio between Bcl-2 and Bax determines the susceptibility of cells to a death signal (reviewed in Gross et al., 1999). In addition, IGF-BP3 binds to IGF and prevents its interaction with its receptor, in

this way it blocks the IGF mitotic signaling pathway and enhances apoptosis or reduces the mitogenic response of cells (Buckbinder et al., 1995).

Additionally, some studies have indicated that p53 may also have a transcription-independent function in apoptosis. For example, inhibition of transcription by actinomycin D or translation by cycloheximide does not always inhibit p53-dependent apoptosis (Caelles et al., 1994). In addition, apoptosis is also mediated by overexpression one of the mutant p53 proteins (residues Leu22 and Trp23), which is defective as a transcription factor (Chen et al., 1996b).

c. Control of Genome Integrity

It has been reported that cells with wild-type p53 do not undergo gene amplification readily (Livingston et al., 1992). However embryo fibroblast cells from p53-null mice are permissive for gene amplification, and primary human cells from Li-Fraumeni patients became permissive as soon as they have lost their single copy of wild-type p53 (reviewed in Agarwal et al., 1998). Additionally, fibroblasts from p53-deficient mice demonstrate chromosomal abnormalities that appear in early passage in homozygous null fibroblasts and at later passages in heterozygous fibroblasts (Harvey et al., 1993). Transformation of wild-type p53 cells with either SV40 large T antigen or activated *ras* plus E1A abolishes p53-dependent cell cycle control and allows these cells to become permissive for gene amplification (Perry et al., 1992). The mechanism how p53 inhibits gene amplification is still unclear, but p53 might recognize the recombination intermediates and kill these cells (reviewed in Levine 1997).

d. DNA Replication

It has been shown that purified p53 can inhibit nuclear DNA replication in a transcription-free system (Cox et al., 1995a). In addition, p53 interacts with the single-strand binding protein RPA which is essential for the early stages of DNA replication (Dutta, et al., 1993). It has been reported that complementary DNA strands are separated during the replication processes, and RPA binds to single stranded DNA to stabilize this intermediates and remove secondary structures (reviewed in de Laat et al., 1999). Moreover, p53 has been found to promote re-annealing of separated DNA strands and act as an anti-helicase (Oberossler, et al., 1993; Brain, et al., 1994). Finally, two p53 responsive gene products, p21 and GADD45, can bind to the C-terminal domain of PCNA and block its role as a DNA polymerase processivity factor in DNA replication (Waga et al., 1994; Smith et al., 1994).

e. DNA Repair

It has been found that p53 can recognize and bind to irradiated DNA and insertion/deletion mismatched DNA (Lee et al., 1995; Reed et al., 1995). In addition, p53 interacts with TFIIH, a basal transcription factor consisting of two DNA-dependent ATPase and helicases that are involved in DNA repair: ERCC2 and ERCC3, as well as cyclin H-Cdk7-p36 complex (Leveillard et al., 1996). TFIIH contains a zinc finger motif and putative DNA binding activity. It has been shown that TFIIH is required to catalyze melting of the DNA double helix at the lesion and facilitates repair complex assembly (reviewed in de Laat et al., 1999). p53 also exhibits a 3' to 5' exonuclease activity which suggests p53 might be directly involved in DNA repair processing (Mummenbrauuer, et al., 1996).

p53 can block cell growth using both transcriptional and non-transcriptional level mechanisms to prevent inappropriate proliferation. Analysis of the protein structure provides crucial insight into understanding how p53 functions and may dysfunction.

1.2 Structure of p53: Domains and Functions

p53 is a gene of approximately 20 kb which is located on the short arm of chromosome 17. It contains 11 exons and codes for a protein of 393 amino acids (reviewed by Elledge and Lee 1995). The p53 protein can be divided into three domains: the amino terminus, containing the transcriptional activation domain (residues 1-43); the central region containing the sequence-specific DNA binding domain (residues 100-300); and the carboxyl terminus, containing both oligomerization and nuclear localization sequences (residues 300-393) (reviewed in Ko and Prives 1996) (Fig. 1.1).

a. N-terminus: Transcriptional Activation Domain

The acidic N-terminal region has been shown to have a potent transcription activating sequence by analyzing it as a fusion protein (Fields and Jang 1990). The fusion protein, composed of a heterologous DNA binding domain linked to a 72 amino acids from the N-terminus of p53, can activate transcription of a test gene. It was also found that a mutation at residues Leu-22 and Trp-23 of p53 inactives p53 transcriptional activity but stills retains the ability to specifically bind to DNA *in vivo* (Lin et al., 1994). Using affinity chromatography, protein blotting, and immunoprecipation technique, many proteins have been found to interact directly with the N-terminus of p53, these include transcription machinery molecules such as TATA box binding protein (TBP) (Seto et al.,

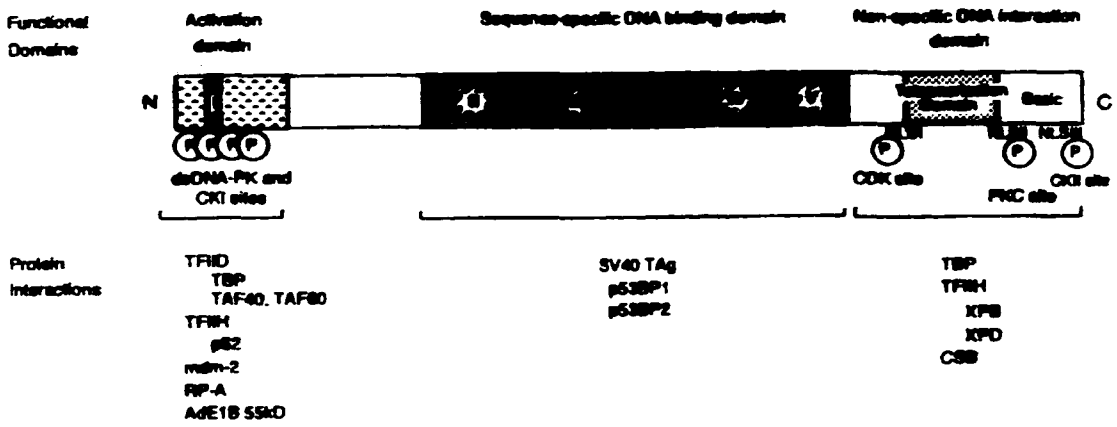


Figure 1.1: Schematic representation of structural organization of p53. Hatched boxes with roman numerals indicate the five regions of the gene that conserved across species. Nuclear localization sequences (NLS) and phosphorylation sites (circled P's) are shown below. Proteins that are known to interact with particular region of p53 are shown at the bottom (Ko and Prives 1996).

1992; Liu et al., 1993; Truant et al., 1993) and several TBP-associated factors (TAFs), such as TAF31 and TAF 70 which are subunits of THIID (Lu and Levine, 1995; Thut et al., 1995). The association of p53 with these general transcription factors suggests that transcriptional activation by p53 might require a direct interaction between p53 and these components of TFIID. In addition, some viral and cellular oncoproteins such as MDM2 and adenovirus 2 E1B 55kD protein can inhibit the trans-activation function of wild-type p53 by forming a complex with p53 at its N-terminus (Chen et al., 1993; Oliner et al., 1993; Kao et al., 1990; Momand et al. 1992). Their inhibition correlates with their oncogenic transforming ability.

b. Central-core: Sequence-specific DNA Binding Domain

Sequencing analysis of tumor-derived p53 genes in nearly 2000 cancer patients demonstrated more than 90% of missense mutations are clustered within the central region and contain high frequency in a number of "hot spots" (reviewed by Prives 1994 and see Fig. 1.1). This observation indicated the essential role of p53 as a tumor suppressor is associated with the central region. The central core region of p53 has been reported as the sequence-specific DNA binding domain (Bargonetti et al., 1993; Pavletich et al., 1993). A stable protease-resistant fragment (aa 100-290) of p53 identified by antibodies, amino-terminal sequencing and cdc2 kinase treatment, has been localized in the central region. This fragment can bind specially to p53 DNA-binding sequences (Bargonetti et al., 1993). The crystal structure of this domain, determined by X-ray diffraction, reveals that this domain folds into four-stranded and five-stranded antiparallel β sheets that in turn are scaffold by two α-helical loops which interact directly with the DNA (Cho et al., 1994). The residues K120, S241, R273, A276, and R283 make contact with the phosphate backbone in the major groove of the DNA helix, while K120, C277,

and R280 interact via hydrogen bonds to the DNA bases. R248 makes multiple hydrogen bond contacts in the minor groove (Cho et al., 1994). Based the above observation, mutations in the central region can be divided into 2 classes: Contact mutants (including R248 and R273) result in directly defective contacts with the DNA; Conformation mutants (including R175, R249, R282 and G245) disrupt the structural basis of the β sheet and the loop-sheet helix motif that act as a scaffold in this domain (reviewed in Levine 1997) (Fig. 1.2).

The central region of p53 is also targeted by the SV40 large T antigen viral oncoprotein which functions in oncogenic cell transformation (Ruppert and Stillman 1993). SV40 large T antigen is a replication origin binding protein required for the initiation of viral DNA synthesis. p53 blocks SV40 large T antigen replication function by preventing T antigen from binding to the viral replication origin (Gannon and Lane 1987; Wang et al., 1989; Friedman et al., 1990; Bargonetti et al. 1991). p53-T antigen complexes have also lost the sequence-specific DNA binding ability of wild-type p53 (Bargonetti et al., 1991). In addition, human papilloma virus E6 protein binds to p53 at the central region to inhibit p53 function either by blocking the sequence-specific DNA binding activity of p53 or by labeling newly synthesized p53 as a target for ubiquitin-mediated degradation (reviewed by Gallagher and Brown 1999).

Consensus binding site for p53

Wild-type p53 was identified as a sequence-specific DNA-binding protein in 1991 (Kern et al., 1991; Bargonetti et al., 1991). While Bargonetti et al. identified a p53 binding site in the SV40 viral genome, Kern et al. used immunoprecipitation technique to

Figure 1.2: Crystal structure of a p53 tumor suppressor-DNA complex. Ribbon drawing of the p53 core domain-DNA complex showing the six most frequently mutated residues of p53 (Cho et al., 1994).

screen a specific binding site from cloned human genomic DNA. A human DNA fragment was found specifically bound to affinity-purified, baculovirus-produced wild-type p53 but not to mutant form (His-273). After subcloning, they found that 33 base pairs were sufficient for p53 binding and that the 3 copies of TGCCT within the 33 base pairs were important for p53 binding (Kern et al., 1991).

Since TGCCT repeats are very common in human genome and these 33 base pairs have very little homology with SV40 origin which is one of the known p53 binding site (Bargonetti et al., 1991), El-Deiry et al. set up another experiment to identify the p53 consensus binding sequence (El-Deiry et al., 1992). Human genomic DNA was cut randomly into 200-400 bp by sonication, and then these fragments were ligated to specific catch linkers. After incubating with wild-type p53 *in vitro* and immunoprecipitated with anti-p53 antibodies and protein A sepharose, the bound DNA was amplified by PCR using catch linkers as primers. The amplified DNA was then cloned and tested for p53 binding, methylation and DNase I protection. A common feature that these binding sites had was 2 copies of a 10 bp motif **5'-PuPuPuC(A/T)(T/A)GPyPyPy-3'**, separated by 0-13 bp. In addition, it was observed that the oncogenic mutant forms of p53 from central region of p53 can not bind to the consensus sequence (El-Deiry et al., 1992). Stereospecific alignment proposed that spacing distance between two copies is an important determinant of p53 affinity. The binding is optimal when the two copies are contiguous or separated by insertions so that the two halves of the binding sites are orientated on the same helical face (reviewed in Hupp 1999). Using a yeast-based method, it was found that up to 300 binding sites for p53 protein might exist in the human genome (Tokino et al., 1994). Since 1992 a lot of cellular genes have been identified which are regulated by wild-type p53 utilizing a form of the consensus binding site, however none of the sequences completely fits the

consensus sequence. Two p53 consensus binding sites have been described in the p21/waf1 promoter located at -1.3 kb and -2.2 kb upstream from the first exon and they are thought to participate in the induction of p21/waf1 gene (El-Deiry et al., 1995). The p53 binding site of the gadd45 gene is located in the third intron and contains 2 copies of the 10 bp motif without any nucleotides in between (Kastan et al., 1992). The Bax gene can be activated by p53 at the promoter region by a site which is 70 bp 5' of the TATA box and contains 1 perfect and 3 imperfect p53 consensus binding sites (Miyashita and Reed 1995). The mdm2 gene contains a p53-dependent promoter (P2) in addition to a p53-independent promoter (P1). The mdm2 P2 promoter is localized in the first intron of the gene and consists of two adjacent imperfect p53 consensus binding sites, and a TATA box located 10 bp downstream of the p53 responsive elements (Juven et al., 1993; Barak et al., 1994; Zauberman et al., 1995). All these pieces of evidence suggest that the DNA sequence variability and genomic location might be significant, perhaps controlling which gene is expressed at distinct cellular times.

c. C-terminus: Oligomerization, Apoptotic, Transcriptional Regulatory Domain

The carboxyl terminal portion of p53 is largely unaffected by mutational change in human tumors, however, it contains several functionally significant regions and it is the most complex region of p53. A major nuclear location signal (NLS) has been identified between amino acid 316 and 325 (Dang and Lee 1989). In addition, a segment from amino acid residues 315 to 360 not only enhances transformation with other oncogenes (Wang et al., 1994a) but also functions in facilitating tetramerization (Pavletich et al., 1993; Sturzbecher et al., 1992) and binding DNA nonspecifically (Wang et al., 1993). It has been determined that immunopurified full-length p53 is predominantly a tetramer and that the C-terminus of p53 by itself can form a tetramer

(Friedman et al., 1993; Wang et al., 1993). Human and murine p53 can form stable heterotetramers, indicating that the oligomerization region is functionally conserved (reviewed in Prives 1994). The structure of tetramerization domain (aa 319-360) was solved by using multidimensional heteronuclear magnetic resonance spectroscopy (Clore et al., 1994). This domain contains a turn (aa 324-326), a β strand (aa 326-334), a second turn (aa 335-336) and a α helix. In order to form a tetramer, each monomer interacts with the other one to form a dimer in a way helices and β strands are antiparallel. Two dimers then form a four-helix bundle. Several different salt bridges stabilize the interactions between the helices (reviewed in Prives 1994).

The C-terminal domain is involved in the apoptotic function of p53. Deletion of the C-terminal 30 amino acids of human p53 has been shown to impair the ability of p53 to induce apoptosis efficiently, while induction of G1 arrest is unimpaired (Chen et al., 1996). p53 can bind directly to two helicases XPB and XPD, which function in DNA repair, through the same region, and inhibits the activity of these two proteins to induce apoptosis (Wang et al., 1996b). In addition, the hepatitis B virus X protein binds to the p53 C-terminus and inhibits p53-mediated apoptosis (Wang et al., 1994b).

The C-terminal region is regarded as a negative regulatory domain, since it has been found that the specific DNA binding ability of p53 can be activated by either deletion of the C-terminus, phosphorylation at residue S378 by protein kinase C or at residue S392 by casein kinase II, or by adding a monoclonal antibody (PAb421) which binds to the C-terminus (reviewed in Levine 1997). In addition, more piece of evidence have been reported to support this hypothesis: (1) acetylation of the p53 C-terminal domain was associated with increased DNA-binding activity of p53 (Gu and Roeder 1997b), (2) microinjection of PAb421 into cells activates the p53 transcriptional activity

without DNA damage (Hupp et al., 1995). (3) small peptides derived from the C-terminus activated the sequence-specific DNA binding function of p53 (Hupp et al., 1995). However, as neither the crystal structure of the full-length unphosphorylated p53 tetramer nor that of the phosphorylated p53 tetramer is known, it is still not clear how the C-terminus negatively regulates p53 DNA binding activity. According to Hupp's model, the C-terminal amino acid side chains of p53 interacts with the central region of p53. This interaction blocks the central region of p53 and inactivates the DNA binding ability of p53. When modification or deletion disrupts this interaction, the central region changes to an active form (Hupp et al., 1995). These observation have led to the suggestion that there may exist in cells at least two forms of p53: a latent (inefficient DNA binding) species and an active (efficient DNA-binding) one. Conversion from the former to the latter may be orchestrated by distinct cellular signals, which are important for cell cycle control.

It is believed that p53 is the central component of a complex network of signaling pathways in response to DNA damage. Therefore it is important to understand how the function of wild-type p53 as a tumor suppressor can be regulated by upstream signals.

1.3 Post-translational Modifications of p53: Phosphorylation and Acetylation

In normal cells, p53 is an unstable nuclear protein with a half-life of 20-30 minutes (reviewed in Cox and Lane 1995). However, upon stress from DNA damage, hypoxia, nucleotide depletion or activated oncogenes, p53 is stabilized and post-translationally modified (reviewed in Freedman and Levine 1999). Phosphorylation has been postulated to be an important regulatory mechanism of p53 function. There are

several potential phosphorylation sites where post-translational modification of p53 can occur (Fig. 1.3).

Phosphorylation sites at the N-terminus of human p53 are serine 6, serine 9, serine 15, threonine 18, serine 20, serine 33 and serine 37 (reviewed in Meek 1998 and Prives 1998). Serine 15, serine 33 and serine 37 of human p53 have been found to be targets of various kinases both *in vitro* and *in vivo*. For example, members of the phosphoinositide-3 kinase-related kinase (PIK) superfamily can phosphorylate p53 at serine 15 and serine 37 (reviewed in Milczarek et al., 1997). The PIK superfamily can be divided into three structurally and functionally distinct subfamilies: the TOR family which is mainly involved in transducing signals required for G1 to S phase progression, the ataxia telangiectasia mutated (ATM) / ATM-Rad-3-related protein (ATR) family, and double-stranded DNA-dependent protein kinase (DNA-PK) which respond to DNA damage (Tibbetts et al., 1999). *In vitro* studies have demonstrated that ATM phosphorylates p53 at a single residue, serine 15 (Banin et al., 1998; Canman et al., 1998). Interestingly, ATM kinase activity is increased by ionizing radiation (IR) but not UV in normal AT cells (Canman et al., 1998). This observation suggests different forms of DNA damage may activate different upstream kinases signaling to p53. Phosphorylation of serine 15 in AT^- cells is delayed after IR (Siliciano et al., 1997). In addition, ATR/ATM and DNA-PK can phosphorylate p53 at both serine 15 and serine 37 *in vitro* (Canman et al., 1998; Tibbetts et al., 1999; Lees-Miller et al., 1992). Furthermore, it has been shown that phosphorylation of serine 15 *in vivo* is induced by IR, camptothecin (CPT) and UV irradiation (Shieh et al., 1997; Siliciano et al., 1997). It has been reported that triple mutation of serine 6, serine 15 and serine 33 reduces p53

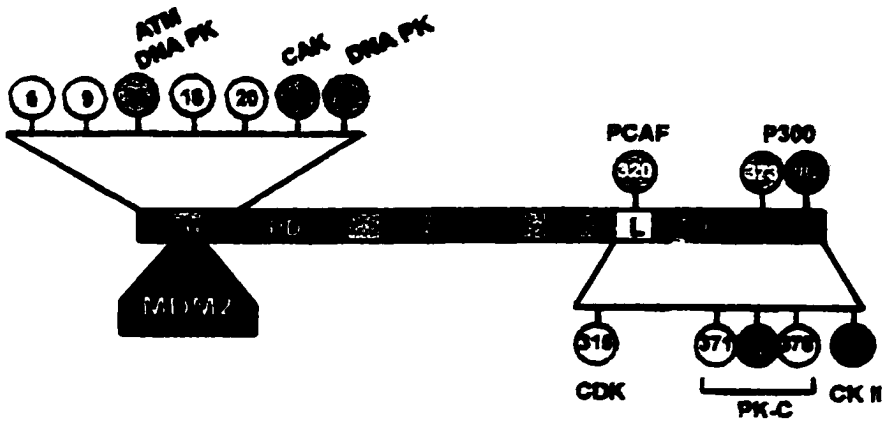


Figure 1.3: Schematic representation of stress-induced post-translational modifications of p53. p53 protein can be characterized to Activation domain (AD), aa 1-60; Growth suppression proline rich region (PD), aa 63-97; DNA-binding domain (DBD), aa 100-300; linker region (L), aa 305-323; tetramerization domain (TD), aa 323-356; and basic C-terminal regulatory region (BD), aa 363-393. The AD region is the MDM2 binding region. Several potential phosphorylation sites are located in N-terminus (Ser6, 9, and 20, and Thr18). Sites known to be phosphorylated in vitro by ATM, DNA-PK or the cyclin-activating kinase complex, CAK, as indicated. C-terminal phosphorylation site for CDK, PK-C and CKII protein kinase are shown. PCAF and p300 acetylate lysine 320 and 382 respectively *in vitro* (Prives 1998).

ability to suppress transformation of rat embryo fibroblasts transfected with E1A and oncogenic *ras* (Mayr et al., 1995).

DNA damage induced phosphorylation of p53 at the N-terminus is considered to act in two separate pathways. The first pathway works at the level of reducing the binding of MDM2 to p53 protein (Shieh et al., 1997), which inhibits the ability of MDM2 to promote the degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997). The second is involved in increasing the ability of p53 to recruit CREB-Binding Protein (CBP/p300), followed by increasing the overall level of acetylation of the C-terminus of p53 (Sakaguchi et al., 1998; Lambert et al., 1998). It has also been suggested that DNA damage activates p53 as a transcription factor through signaling for an N-terminal phosphorylation and C-terminal acetylation cascade (Sakaguchi et al., 1998).

In addition, *in vitro* phosphorylation of the p53 C-terminal region by protein kinase C (PKC), casine kinase II or CDK7-cycH-p36 complex can differentially stimulate sequence-specific DNA binding ability to various p53 DNA binding sites (Hupp et al., 1992; Takenaka et al., 1995; Lu et al., 1997 and Wang and Prives 1995). The serine 315 lies within a consensus recognition site for the cell cycle regulatory $p34^{cdc2}$ kinase (McVey et al., 1989) and can be phosphorylated by the cdc2 kinase *in vitro* (Bischoff et al., 1990). The serine at codon 392 is phosphorylated *in vitro* by casein kinase II (Meek et al., 1990). In addition, IR induced dephosphorylation at serine 376 (a PKC site), leads to both p53 association with 14-3-3 proteins and activation of sequence-specific DNA binding (Waterman et al., 1998).

Furthermore, acetylation at Lysine 382, Lysine 373 and Lysine 320 was found after IR and UV radiation (Sakaguchi et al., 1998; Liu et al., 1999). Recombinant histone

acetyltransferases (HATs) CBP/p300 and CBP-associated factor (PCAF) acetylate p53 at lysine 382 and lysine 320 respectively (Gu et al., 1997b; Sakaguchi et al., 1998). CBP/p300 and PCAF are transcriptional coactivators and p300/CBP is associated with various sequence-specific activators such as bHLH factors, NF*k*B p65, c-Jun/ v-Jun, c-Myb/v-Myb, c-Fos, MyoD, CREB, AP-1, STATs, and nuclear hormone receptor (reviewed in Janknecht and Hunter, 1996). Interestingly, CBP interacts with p53 at its N-terminus and enhances p53-mediated transactivation (Gu et al., 1997a; Lill et al., 1997). The association of p53 with these two HATs may provide an answer for the question how p53 transactivates its target genes at the chromatin level.

1.4 mdm2: Keeping p53 under Control

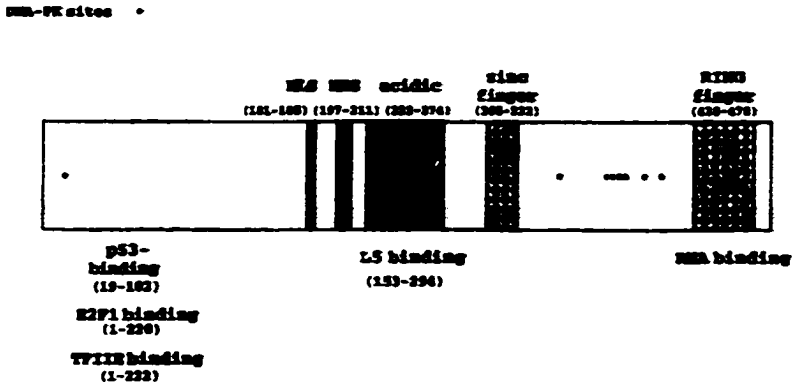
p53 functions as a sequence-specific transcription factor and mdm2 is the product of a p53 inducuble gene, leading to a negative feedback loop between p53 and mdm2 (reviewed in Prives 1998; Wu et al., 1993). The mdm2 gene was originally cloned from a spontaneously transformed 3T3 cell line containing amplified DNA sequences in the form of double minutes which are small, self-replicating, extra chromosomal nuclear bodies (Cahilly-Snyder et al., 1987). In these cells, mdm2 is amplified approximately 50 folds. Afterwards the mdm2 gene was mapped to mouse chromosome 10, region C1-C3 and human chromosome 12q13-14. mdm2 gene was found amplified in over a third of 47 human sarcomas containing wild-type p53 alleles (Oliner et al., 1992). The mdm2 gene is considered as an oncogene because overexpression of mdm2 induces tumorigenicity when tested in nude mice by subcutaneous injection (Fakharzadeh et al., 1991). Moreover, co-transfection of mdm2 with an activated *ras* gene results in the transformation of rat embryo fibroblasts (Finlay 1993). Furthermore, it was reported that overexpression of mdm2 decreased the

susceptibility of human glioblastoma cells to cisplatin mediated apoptosis, while expression of anti-sense mdm2 increased their susceptibility (Kondo et al., 1995). Overexpression of MDM2 results from either gene amplification (in soft tissue sarcomas and breast) or enhanced transcription and translation (in testicular germ cell tumours and transitional carcinoma of the bladder) (Arriola, et al., 1999). The oncogenic transforming ability of MDM2 can be attributed to its interactions with proteins involved in regulation of cell cycle progression and apoptosis including p53.

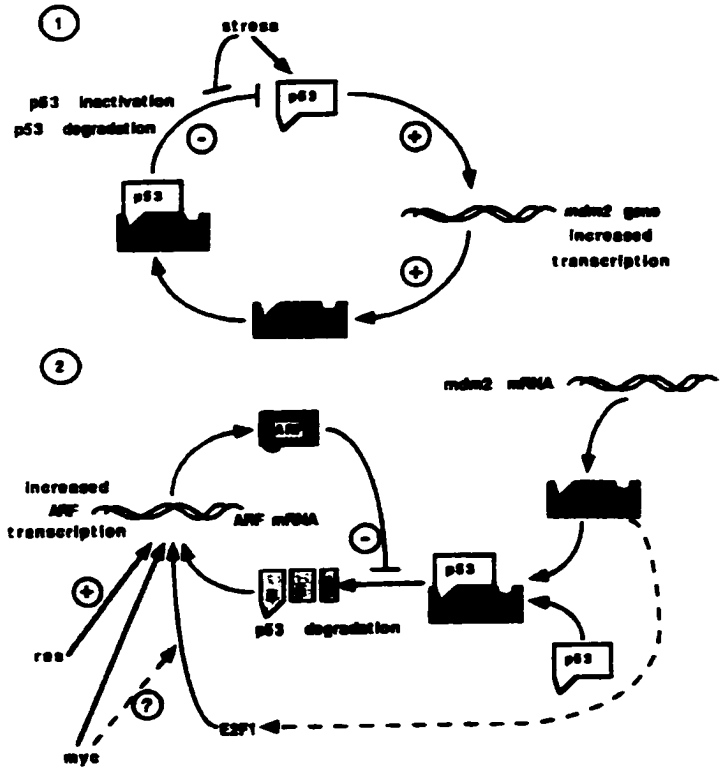
The mdm2 gene is approximately 25 kb which contains 12 exons (Montes de Oca Luna et al., 1996). The mouse MDM2 protein is 489 amino acids and can be divided into 4 conserved domains (Fakharzadeh et al., 1991) (Fig. 1.4A). Region I is about 90 amino acids and lacks any obvious sequence domain, region II is a highly acidic region which is often found in transactivators, region III contains a potential zinc finger and region IV contains a ring finger (reviewed in Piette et al., 1997). In 1991, Momand et al. found that the MDM2 protein co-immunoprecipitates both wild-type and mutant p53 as well as inhibiting the transactivation function of p53 in cotransfection assays (Momand et al., 1992). The region of MDM2 that can bind to p53 was mapped at region I (aa 14-130) by co-immunoprecipitation experiments, *in vitro* translation analysis and functional assays (Chen et al., 1993; Oliner et al., 1993; Lin et al., 1994). The co-crystallization experiment demonstrated that the residues TFSDLW (aa 18-23) of p53 are essential for binding of MDM2 (Picksley et al., 1994). The three dimensional structure of the 109 amino acids at the N-terminal domain of *xenopus laevis* MDM2, bound to the 15-residue transactivation domain peptide of p53, revealing that MDM2 has a deep hydrophobic cleft on which the p53 peptide binds as an amphipathic α helix (Kussie et al., 1996). Residues F19, W23 and L26 of p53 insert deeply into the MDM2 cleft which contains 14 conserved hydrophobic and aromatic amino acids that make multiple van der Waals

Figure 1.4: Schematic diagram of the primary structure of MDM2 and functional inter-relationship between MDM2 and p53. (A) MDM2 protein contains a putative nuclear localization signal (aa 181-185), two zinc fingers (aa 305-322 and aa 438-478), and an acidic domain (aa 223-274). The binding sites for p53, E2F, and TFIIE are indicated. (B) In loop 1, activation of p53 induces mdm2 gene transcription. The production of MDM2 protein will bind to p53 and inactivates it and targets it for degradation. Stress signals may allow p53 activation and accumulation through interfering with the inhibitory effects of MDM2 on p53. In loop 2, MDM2 protein can down-regulate of p53 which relieves the inhibitory effect on ARF transcription and consequently in inhibition of MDM2 function through ARF-MDM2 protein interactions (Juven-Gershonand Oren 1999).

A



B



contacts to p53. It should be noted that the residue L22 and W23 of p53 were required for transcription activity of p53 and binding to TAFII31 and TAFII70 (Lin et al., 1994; Lu and Levine, 1995; Thut et al., 1995). Through this interaction, MDM2 inhibits the transcriptional activation of p53 both in transfection experiments and in an *in vitro* transcription assay (Momand et al., 1992; Oliner et al., 1993; Thut et al., 1997).

In addition to preventing p53 transcription activity by direct contact to p53, MDM2 also promotes p53 degradation by a proteasome mediated pathway (Haupt et al., 1997; Kubbutat et al., 1997). Several phosphorylation sites of p53 have been identified located in the N-terminal region, some are involved in regulating the stability of p53 (reviewed in Milczarek, 1997). It was reported that MDM2 can function as an E3 ubiquitin ligase, forming a trimeric complex with p53 and conjugating ubiquitin to the p53 protein, a prerequisite for targeting and degradation through the proteasome (Honda et al., 1997). Additionally, nuclear-cytoplasmic shuttling has been shown to be necessary for the degradation of p53 by MDM2 (Roth et al., 1998; Freedman et al., 1998). Wild-type p53 must be localized in the nucleus to transactivate growth inhibitory genes to promote tumor suppression. However, it has been found that greater than 95% of primary human neuroblastomas contain over-expression wild-type p53 that is located in the cytoplasm (reviewed in Momand and Zambetti 1997). Therefore, MDM2 and p53 may interact in the nucleus and MDM2 then shuttles p53 to the cytoplasm for subsequent degradation by cytoplasmic proteasomes (Freedman and Levine, 1999). A conserved, leucine-rich sequence in MDM2 resembles a Nuclear Export Signal (NES) sequence and mutations of the NES in mdm2 have been shown to impede degradation of p53 (Roth et al., 1998).

It has been reported that abrogation of mdm2 mediated p53 degradation is via two pathways. First, DNA-damage induced phosphorylation of p53 at its N-terminus can block the interaction between MDM2 and p53 (Shieh, et al., 1997). Second, the tumor suppressor $p19^{ARF}$ can bind to MDM2 and inhibit the degradation of p53. This ARF mediated stabilization of p53 occurs in the absence of DNA damage and the p53 DNA damage-responsive pathway is intact in $ARF^{-/-}$ cells (Pomerantz, et al., 1998; Zhang, et al., 1998; de Stanchina et al., 1998). Activation of p53 by the oncoprotein /ARF upstream pathway does not involve phosphorylation of p53 on serine 15 (de Stanchina et al., 1998; reviewed in Prives 1998). It has been demonstrated that transcription of ARF is induced by cellular c-myc, adenovirus E1A and oncogenic activated ras. In addition, p53 can down-regulate ARF expression while ARF is able to stabilize p53 (Stott, et al., 1998). All these observations suggest that an additional negative autoregulatory loop exists to safeguard cells hyperproliferative, oncogenic signals (Fig. 1.4B).

On the other hand, some oncogenic effects of MDM2 are independent of p53. It has been shown that overexpression of mdm2 stimulates the activity of the E2F transcription factor and reverses an arrest in the cell cycle mediated by the RB protein (Martin et al., 1995; Xiao et al., 1995). This effect of MDM2 does not depend on the presence of p53. Additionally, in mice lacking p53 the mammary tumors arose from overexpressed mdm2 and the mice developed polyploidy and enlarged cells with giant nuclei (Lundgren et al., 1997). The tumor capacity of mdm2 occurs by multiple mechanisms in addition to inactivating p53 (Sigalas et al., 1996; reviewed in Momand and Zambetti 1997).

The relationship between p53 and MDM2 extends beyond the protein-protein interaction. The mdm2 gene expression is induced by wild-type p53 (Barak et al., 1993; Wu et al., 1993; Otto and Deppert 1993). A p53 responsive element was screened for by cloning different segments of the mdm2 gene linked to a minimal promoter adjacent to the reporter gene (CAT), then cotransfected with the wt-p53 plasmid into a p53-null cell line and a temperature-sensitive p53 line (Wu et al., 1993). A 85 bp fragment from the first intron was found to be sufficient to active a test gene in a p53-dependent manner (Wu et al., 1993). Juven et al. fused a genomic mdm2 DNA segment with a promoterless CAT gene and then cotransfected it with wt-p53 into p53-deficient Saos-2. In this way, they found that the p53 putative binding sites are adjacent to an internal p53-inducible promoter near the 3' end of intron 1 of the mdm2 gene (Juven et al., 1993). Furthermore, p53-DNA immunoprecipitation assays showed that both purified wt-p53 and cell extracts containing wt-p53 could bind to the putative binding sites (Wu et al., 1993; Juven et al. 1993). The mdm2 gene is regulated by two promoters, one p53 independent (P1) and the other p53 dependent (P2) (Barak, et al., 1994; Zauberman et al., 1995). The P1 promoter regulates the level of transcripts initiating from exon 1 while P2 promoter controls transcription from exon 2.

Homologous deletion of mdm2 is lethal at a very early stage of embryogenesis (day 5 of gestation) but is completely rescued by the additional deletion of the p53 gene. This result demonstrates that the inhibition of p53 by MDM2 is essential for life at an early developmental stage (Montes de Oca Luna et al., 1995; Jones et al., 1995).

Multiple mRNA splice forms of mdm2 are expressed and different MDM2 proteins are found (Sigalas et al., 1996). Although the biochemical function of these different mdm2 splice forms has not yet been determined, their existence suggests that

MDM2 may perform multiple functions. Several MDM2 proteins are detectable in cells including human tumors and transformed murine cell lines (Olson et al., 1993; Landers, et al., 1994). Multiple MDM2 proteins can be translated from a single mRNA in a rabbit reticulocyte lysate system, indicating that internal initiation may be a mechanism whereby multiple MDM2 proteins are expressed in cells (Barak et al., 1994).

1.5 Gadd45: Growth Arrest and DNA Damage

Five gadd genes (gadd153, gadd45, gadd34, gadd33 and gadd7) were isolated by subtractive hybridization on the basis of rapid induction after UV irradiation in Chinese hamster ovary (CHO) cells (Fornace et al., 1988). The gadd45 gene was the only one found to be strongly induced by IR (Papathanasiou et al., 1991). In addition to being rapidly induced by the DNA damaging agents, etoposide, methyl methanesulfonate, cis-Pt (II)-diamminedichloride and H_2O_2, the gadd45 gene was also coordinately induced in growth arrests cells (Fornace et al., 1989; Kastan et al., 1992; Zhan et al., 1998; Hollander et al., 1993). Stress induction of the gadd45 gene has been detected in every mammalian cell line examined to date (Fornace et al., 1992). The gadd45 gene shown 89% nucleotide homology between the human and hamster gene in the coding region which translates to 96% at the putative amino acid level (Hollander et al., 1993). This considerable conservation between organisms that are separated by 70 million years of evolution suggests that the GADD45 protein is an essential component of mammalian cells (Hollander et al., 1993).

The human gadd45 gene contains 4 exons and is located on chromosome 1p31.1-31.2 (Hollander, et al., 1993). GADD45 is a small acidic protein of 21 kDa, found in low abundance in the nucleus (Carrier et al., 1994). It has been shown that over

expression of GADD45 protein reduces by more than 75% the formation of cellular colonies in three different human cell lines (Zhan et al., 1994). However the specific function of GADD45 in involving in growth arrest and DNA repair is not well understood. Unlike p21, GADD45 is not detectable in Cdc2, Cdk2, or cyclin D, E, or A immune complexes, and it does not inhibit cyclin E-Cdk2-dependent activity when added to *in vitro* kinase assays (Smith et al., 1994). It was found that GADD45 interacts directly with the essential replication factor DNA Polymerase δ and with the ε auxiliary factor PCNA as well as with the cyclin dependent kinase inhibitor p21. Gadd45 blocks DNA replication and possibly co-ordinarily enhances nucleotide excision repair of damaged DNA (Smith et al., 1994; Hall et al., 1995). Recently, it was reported that GADD45 can recognize damaged chromatin and modify chromatin accessibility to cellular protein because GADD45 can facilitate topoisomerase I relaxing and cleavage activity in the presence of core histones (Carrier et al., 1999). In addition, microinjection of an expression vector into primary human fibroblasts has shown that overexpression of gadd45 arrests the cells at the G2/M boundary (Wang, et al., 1999).

Gadd45 is a p53 responsive gene (Kastan et al., 1992, Zhan et al., 1996, Gujuluva, et al., 1994). Gadd45 induction by radiation is deficient in a large variety of tumor cells lacking p53 function (Kastan et al., 1992). In addition, medium starvation stress responses were appreciably reduced for gadd45 in mouse fibroblasts from p53-null mice and in human lines where p53 function was blocked with dominant-negative mutant p53 (Zhan et al., 1996). Reduced induction of gadd45 mRNA after UV irradiation is evidenced in human papillomavirus-immortalized keratinocytes and an oral cancer cells compared to normal human keratinocytes (Gujuluva, et al., 1994). A conserved 20-bp sequence in the third intron of human and hamster gadd45 gene corresponds to the p53 consensus binding sequence was obtained (Kastan et al., 1992).

Nuclear extracts from γ-irradiated ML-1 cells contain a factor that bound to this sequence and that can be supershifted by 421 antibody (Kastan et al., 1992).

Investigation of regulation of gadd45 gene expression has been carried out predominately using transient transfection reporter assay. Two cis-elements were studied separately. One is the gadd45 promoter region and the other one is the putative p53 RE. Sequence analysis of the gadd45 promoter revealed no conservation of the consensus, the AP-1 recognition site, or other potential DNA damage-responsive sequences, such as SREs or NFκB elements. Instead, a G-C rich region contains one WT1 consensus sequence and three overlapping Egr-1 sites (Amundson, et al., 1998, Marhin et al., 1997). It has been documented that p53 might form a complex with WT1 and bind to the gadd45 promoter in response to DNA damage (UV & MMS) (Zhan et al., 1998). Deletion of various portions of the gadd45 promoter in promoter-driven CAT construct analysis demonstrated that the G-C rich region is necessary for p53 dependent activation of the gadd45 promoter. The co-transfection study demonstrated that p53 alone can not activate gadd45 promoter without WT1. In contrast, the combination of p53 and WT1 resulted in a consistent large increase in expression of the CAT reporter (Zhan et al., 1998). Interestingly, the Myc-mediated repression of gadd45 in response to UV was this G-C rich region dependent (Amundson et al., 1998). In addition, it has been shown when the promoter region of the hamster gadd45 gene was inserted into a eukaryotic expression vector, that construct was inducible by the alkylating agent MMS and UV radiation in both hamster and human cell lines. This construct however is not inducible by IR in either hamster or human lines (Hollander et al., 1993). Kastan et al. found that a vector containing one copy of the p53 RE linking to a basal promoter adjacent to the CAT gene could be activated by wild-type, but not mutant p53. The specificity of IR inducible gadd45 indicates that multiple mechanisms can affect gadd45 expression.

Regulation of gene expression is mediated in part by the interaction of specific transcription factors with their respective recognition sequences situated within the DNA (reviewed in Saluz and Jost 1993). In particular, the cellular transcription machinery is designed to function with physiological template chromatin not naked DNA. Biochemical and genetic evidence indicate that chromatin structure functions not only to constrain the genome within the boundaries of the cell nucleus but also plays an active role in gene transcription (reviewed in Struhl 1996 and Wu 1997).

1.6 Chromatin: Implications for Transcription

The basic chromatin unit is a nucleosome core, which consists of an octamer of one tetramer of histones H3 and H4 and 2 dimers of histone H2A and H2B: $(H2A\text{-}H2B)_2$-$[(H3)_2\ (H4)_2]$ around which 146bp of DNA is looped in one and three quarter left-handed superhelical turns. In addition, histone H1, H5 and some other nuclear proteins join each nucleosome at linker regions (reviewed in Beato and Eisfeld 1997; Studitsky et al., 1995). Histones were identified as a group of small molecules that associated non-covalently with the DNA. In addition, histones are highly basic and only the central domain adopts a structured conformation while the N- and C- terminal tails are flexible (reviewed in van Holde et al., 1995). Genetic and biochemical evidence indicates that the nucelosome can act as a general repressor in transcription. *In vitro* experiments have shown that promoters are inactivated when reconstituted into nucleosomes (Grunstein 1990), and *in vivo* reduction in histone H4 synthesis in yeast resulted in expression of a variety of genes that should be repressed (Han and Grunstein 1988). Importantly, *in vitro* transcription experiments have indicated that TBP can not bind to the TATA box if the TATA box is located in a nucleosome core (Imbalzano et

al., 1994). However, in addition to acting as a barrier of transcription, nucleosomes can behave as scaffolding structures to bring in close proximity regulatory factors bound at distant sites to activate transcription (reviewed in Woodcock and Horowitz 1995).

Histones undergo several forms of post-translational modification, including methylation, acetylation, phosphoryaltion, polyADP ribosylation, and ubiquitination (reviewed in van Holde et al., 1995). In 1964, Allfrey et al. provided evidence for the importance of histones as transcriptional repressor and histone acetylation as a modulator of the repression (reviewed in van Holde 1997). The core histones, particularly H3 and H4 can be acetylated at their lysine rich N-terminal tails. Acetylation greatly reduces the affinity of the histone tail for DNA because the acetylated N-terminal tail extends outwardly from globular core of the histone octamer (reviewed in Wade et al., 1997; Kadonaga 1998). Using an antibody directly against acetylated lysine can selectively precipitate nucleosomes containing active gene sequences (reviewed in Struhl 1998). Several transcription coactivators have been found to function as histone acetyltransferases, including GCN5p in yeast, P/CAF, p300/CBP in human, and TAF230/250 in human and Drosophila (reviewed in Wade et al., 1997). GCN5p and TAF250 preferentially acetylates free histone H3 over H4 and has little or no activity on nucleosomal histones (reviewed by Wu 1997). This may involve specific gene activation through selectively interaction between transcription factor and basal transcriptional machinery (reviewed in Wolffe and Pruss 1996).

In addition to histone acetyltransferases, a variety of complexes have been identified that may assist transcription factors to reconfigure chromatin. For example, in yeast the RNA polymerase II holoenzyme contains stoichiometric amounts of Swi/Snf, a multiprotein complex with the capability to disrupt nucleosomes (Wilson et al., 1996). *In*

vitro experiments demonstrated that purified Swi/Snf was able to induce perturbation of histone-DNA interactions in a mononucleosome as well as facilitating binding of GAL4-AH (a sequence-specific DNA-binding protein) to a mononucleosome. The Swi/Snf complex is a DNA-stimulated ATPase, and it disrupts nucleosomal arrays in an ATP-dependent manner *in vitro* . Disruption of nucleosomal arrays can facilitate binding of activator proteins or TBP to their target sites on nucleosomal templates (reviewed in Struhl 1996).

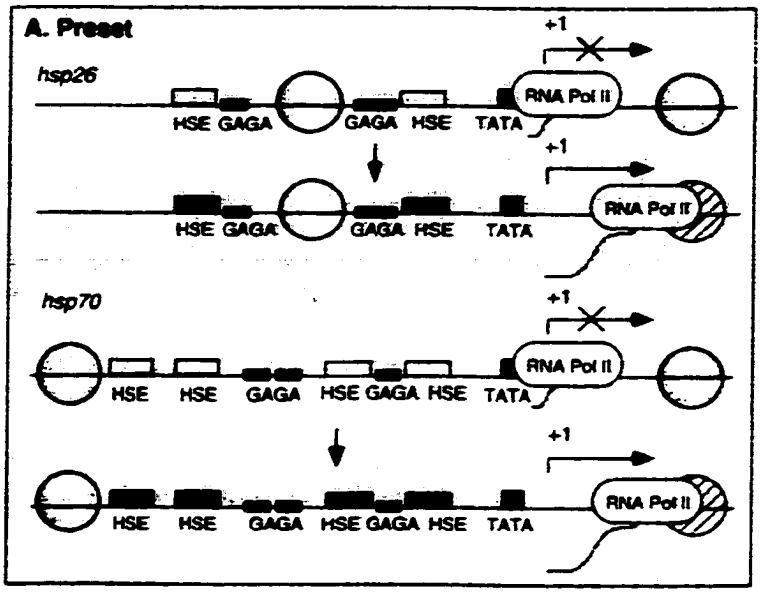
Repressive nucleosomes may prevent either the association or function of the basal transcriptional machinery on a particular promoter (reviewed in Wade et al., 1997), but differences in intrinsic nucleosomal positioning of individual genes have to be taken into considered. To understand the mechanism of transcription factor access to chromatin, two models have been proposed based on genomic footprinting data and chromatin reconstitution experiments (reviewed in Beato and Eisfeld 1997). First, the transcription factor can bind to its target sites within the nucleosome, remodeling the chromatin configuration thus leading to the promoter region being more accessible to the basal transcription machinery complex. The activity of this kind of activator may involve histone acetylation. Second, the transcription factor is unable to bind to regular chromatin. In contrast, it can bind to an altered nucleosome or a nucleosome free region of DNA which is marked by constitutive nuclease hypersensitivity. The activator may function to recruit the pre-initiation complex to its promoter and stabilize the binding of the factors to the sequence. This may result in an increased transcription initiation rate or the release of a paused RNA polymerase II from its downstream nucleosomes. Two examples described below will illustrate the details of these two models (Fig. 1.5).

Remodeling model. The steroid hormone receptors belong to the class of remodeling initiators which can bind to regularly organized chromatin. It has been reported that mammalian Mouse Mammary Tumor Virus (MMTV) LTR promoter region has 6 positioned nucleosomes. There is no indication of proteins other than histones bound to DNA before hormone induction (Truss et al., 1995). The Glucocorticoid Responsive Elements (GREs) and Nuclear Factor 1 (NF1) sites are all located within a positioned nucleosome. The NF1 can not bind to their sites in regular chromatin. Upon hormone treatment, the Glucocorticoid Receptor (GR) has the ability to bind to the GRE and initiate promoter remodeling (Truss et al., 1995). The remodeling can be accomplished by hyperacetylation at lysine residues of the amino-terminal tails of the histones, thereby neutralizing the positive charge of histone tails and decreasing their affinity for DNA (reviewed in Struhl 1998). As a consequence, NF1 obtains the access to chromatin template while the nucleosome remains in place. In this case, GR works as an initiator to remodel chromatin and NF1 binds to an altered nucleosome.

Preset model. This model has been well documented using the Drosophila heat shock genes hsp26 and hsp 70. Before and after heat shock, there are two constitutively DNase I hypersensitive sites which correspond to the Heat Shock Factor (HSF) and binding sites for GAGA factor. GAGA protein is a constitutively expressed transcription factor that binds to GA/CT-rich site present in many Drosophila genes (Tsukiyama, et al., 1994). The positioned nucleosomes are flanked by these 2 nucleosome-free regions. UV crosslinking experiments indicate the RNA polymearse II holoenzyme transcriptionally engages with the TATA box but pause at +25bp downstream of start site before heat shock. Upon heat shock, HSF binds to the HSEs and RNA polymearse II holoenzyme is facilitated to continue transcription past the pause site. No major changes in chromatin structure occur. The GAGA factor has been found binds to its

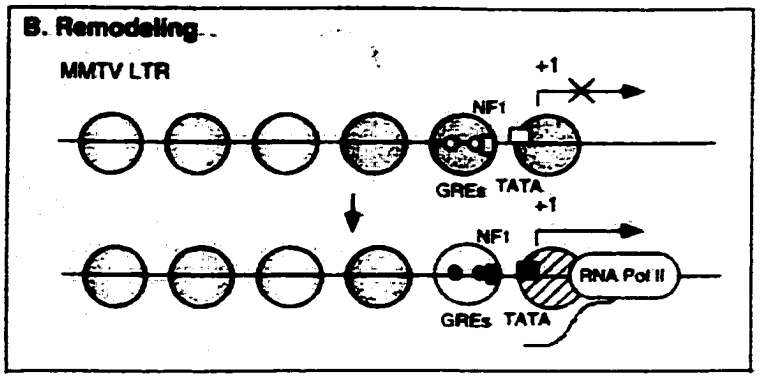
Figure 1.5: Examples of preset and remodeling genes. (A) Diagram of the Drosophila hsp26 and hsp70 preset promoters. (B) Diagram of the mammalian MMTV LTR remodeling promoters. Shaded large circles represent stable nucleosomes, cross-hatched large circles represent nucleosomes downstream of the transcription start of active genes and unshaded large circles represent perturbed nucleosomes associated with remodeling promoters. Cis-acting regulatory elements are shown as small boxes and circles; those bound by trans-activators are filled (Wallrath et al., 1994).

Drosophila



HSE: heat shock element
HSF: heat shock factor

mammalian



GRE: glucocorticoid receptor element
MMTV: mouse mammary tumor virus
NF1:nuclear factor one

binding sites constitutively (reviewed in Wallrath et al., 1994). The formation of nucleosome free region is thought to be controlled by two factors. First, *in situ* deletion or mutation of HSE sequences indicates that these sites can affect nucleosome positioning (Gross et al., 1993). Second, *in vitro* nucleosome assembly assays show binding of GAGA factor on existing nucleosomes leading to nucleosome disruption and a rearrangement of adjacent nucleosomes in an ATP-dependent manner (Tsukiyama et al., 1994). Interestingly, GAGA factor does not contain the canonical ATP binding motif which suggests GAGA may act in conjunction with another protein that needs to hydrolyze ATP for nucleosome disruption (Tsukiyama et al., 1994). Tsukiyama et al. continued this study and purified a factor called Nucleosome Remodeling Factor (NURF) which contains ATPase activity and is capable of perturbing the structure of a reconstituted mononucleosome. NURF facilitates binding of the GAGA to its cognate sites, leading to an enhanced reconfiguration of nucleosome structure (Tsukiyama et al., 1995). The activity of NURF is only stimulated by nucleosomes and not by free DNA (Tsukiyama et al., 1995). The transcription factors that are found constitutively bound to nucleosome-free DNA likely bind to their cognate sites during DNA replication on mitosis and set the stage for a rapid response of the corresponding genes (reviewed in Beato and Eisfeld 1997).

1.7 The Goals of my Work

As mentioned above, the sequence-specific DNA binding ability of p53 has been shown to be essential for its transcriptional activity. Much progress regarding p53 binding to its putative response elements (REs) has been made using electrophoretic mobility shift assays (EMSAs), co-immunoprecipitation, *in vitro* footprints and transient transfection assays, but little is known about its ability to bind to specific target sites *in*

vivo. *In vivo*, the chromatin structure and gene position effects must be taken into account.

Two model systems have been used to study transcriptional regulation by p53. One is the stabilization of p53 through DNA damage and the other is the overexpression of p53 either by an inducible promoter or temperature sensitive alleles. Increased p53levels are found in both systems. DNA damage has been shown to trigger p53-mediated transcription of the gadd45 gene but it has not been determined whether this is the result of an increase in p53 level or the post-translational modification of p53. p21, gadd45 and mdm2 gene constructs have been shown to be activated from cells overexpressing wild-type p53 (El-Deiry et al., 1993; Juven et al., 1993; Kastan et al., 1992). The protein products of the p53-target genes have an intriguingly diverse number of functions. p21 and GADD45 are involved in growth arrest while MDM2 positively regulates cell growth. Does p53 turn on all its target genes at the same time? Do post-translational modifications play a role in controlling the regulation of different targets? Are the chromatin structures of the p53 target genes the same? In addition, overexpression of large quantity of one protein in transfection assays may not predict physiological cooperative effect on endogenous target genes. It is also possible that since the process of transfection itself can cause a DNA damage response and trigger p53 signaling pathways (Renzing and Lane 1995). To further our understanding of the transactivaton of endogenous p53 target genes correlated with the DNA binding activity at nuclear chromatin both in the presence and absence of DNA damage, my research has investigated the following:

1. Ligation mediated PCR *in vivo* footprinting was carried out to investigate the intra-nuclear binding of p53 to the mdm2 P2 promoter in a murine temperature-sensitive

p53Val135 cell line. In addition, the nucleotide sequence protected from DNase I digestion on naked DNA of mdm2 P2 promoter by purified wild-type p53 was compared to the p53 dependent DNase I protection in nuclei. 2. Chromatin accessibility of the mdm2 gene and the reconfiguration ability of p53 were analyzed by nuclease hypersensitivity. Nuclease hypersensitive sites in the mdm2 P1 and P2 promoter region were searched for by indirect end labeling Southern blot analysis. 3. The p53 mediated transcription profile of the gadd45, p21 and mdm2 genes in the presence and absence of DNA damage were studied using a tetracycline-regulated wild-type p53 expression cell line in both presence and absence of DNA damage. 4. Whether p53 nuclear-protein level, DNA binding activity, post-translational modifications, and chromatin configuration are mechanistic factor for p53-mediated gene activation was investigated and the results follow in chapter III and IV.

Chapter II

Materials and Methods

2.1 Cells and Viruses

The 10-1 cell line is a mouse fibroblast cell line lacking endogenous p53 protein due to a deletion of p53 gene (Harvey and Levine 1991). 10-1 cells were co-transfected with the temperature-sensitive mutant p53-Val135 plasmid (pLTRp53cGval135) (Michalovtiz et al., 1990) and a Neomycin resistant plasmid to create a stable p53 expressing cell line called 3-4 (Chen et al., 1995). The MDAH041 line is a human fibroblast cell line lacking functional p53 protein due to a frameshift mutation of one p53 allele at codon 184 and loss of the normal p53 allele (Agarwal et al., 1995). The TR9-7 line is an isogenic line derived from MDAH041 that contains tetracycline regulated wild-type p53 (Agarwal et al., 1995). These cell lines were generously provided by G. Stark and M. Agarwal (The Cleveland Clinic Foundation, OH). The 184A1 line is an immortalized human mammary epithelial cell line that contains wild type p53 (Lehman et al., 1993). This cell line was obtained from American Type Culture Collection.
Spodoptera frugiperda cells (Sf21 cells) and recombinant baculoviruses expressing wild-type human p53 were as described (O'Reilly and Miller, 1988).

10-1 and 3-4 cells were grown on 150 mm plates at 37°C in Dulbecco's Modified Eagle Medium (GIBCO) containing 10% heat-inactivated fetal bovine serum (GIBCO) until 80% confluence. Sub confluent plates were shifted to either 32°C for 4 hours or 32°C for 24 hours. 184A1 cells were grown on 150 mm plates at 37°C in 1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle Medium (GIBCO) containing 0.1 ug/ml cholera enterotoxin, 10 ug/ml insulin, 0.5 ug/ml hydrocortisol, 20 ng/ml epidermal growth factor and 5% horse serum (GIBCO). TR9-7 cells were grown on 150 mm plates at 37°C in Dulbecco's Modified Eagle Medium (GIBCO) containing 10% heat-inactivated fetal bovine serum (GIBCO) and 2ug/ml tetracycline until 70% confluence.

Sub confluent plates were grown either in media containing tetracycline (Control) for 24 hours, withdrawal of tetracycline (-Tet) for 24 hours, or after 24 hrs withdrawal of tetracycline with overlapping 0.1 mM Camptothecin treatment for the last 4hrs (-Tet & CPT). Sf21 cells were grown at 27°C in TC-100 medium (GIBCO) containing 10% heat-inactivated fetal calf serum.

2.2 DNase I Treatment of Nuclei

The cells were washed twice with 4°C 1x PBS, scraped off the plate and spun down at 2,500 rpm (4°C) for 10 minutes. The pellet was resuspended in 2 ml of RSB (10 mM Tris-HCl PH 7.4; 10 mM NaCl; 3 mM $MgCl_2$ PH 7.4; 0.5% NP-40) with 1 mM PMSF. The cells were homogenized 15-20 strokes and checked by trypan blue exclusion. Nuclei were isolated by centrifugation at 4,000 rpm at 4°C for 10 minutes and washed once with RSB. 2.0 $x10^6$ nuclei in 250 ul of RSB were treated with increasing amounts of DNase I (Worthington; 2,932 units/mg) for 10 minutes at either 37°C or 32°C. The reactions were stopped by adding 250 ul of DNase I stop buffer (2M NH_4OA_c; 100 mM EDTA; 0.2% SDS) followed by proteinase K digestion (final concentration of 400 ug/ml) overnight at 37°C. The DNA was extracted once with phenol and three times with chloroform followed by ethanol precipitation.

2.3 DNase I Sensitivity Assay

Equal amounts (20 ug) of DNase I treated genomic DNA was digested with either EcoRI (10-1 and 3-4 cells) or Xba I (TR9-7 and 184A1 cells) to completion. The digested DNA was electrophoresed on a 0.8% agarose gel overnight at 23V and transferred to positively charged nylon membrane (Schlelcher and Schuell) by electric

transfer at 0.1 Amp overnight at 4°C in 1x TBE buffer. The blots were hybridized murine mdm2 1.1kb probe (10-1 3-4 cells) which was obtained as a EcoRI-XhoI fragment from a genomic subclone (Juven et al., 1993) or the blots were hybridized with an Xba I- Msc I gadd45 probe (TR9-7 and 184A1 cells) which contained upstream of gadd45 p53 RE. All probes were labeled with [^{32}-P] by random prime labeling (Boehringer Mannheim). The specific activity of all probes was at least 10^8 cpm/ug.

2.4 Ligation-mediated PCR for *in vivo* Footprinting

The ligation-mediated PCR footprinting technique carried out was an adaptation of that first described by Mueller and Wold (Mueller and Wold, 1989) and later revised by (McPherson, 1993). The reactions were carried out in a volume of 15ul containing 1 ug of genomic DNA from nuclei treated with 0.1 ug of DNaseI, 1X Sequenase buffer 1(25 mM Tris-HCl PH 7.5; 80 mM N_aCl; 0.5mM M_gCl_2) and 1 ul of 0.3 pmol/ul oligo#1. The mixture was denatured at 95°C for 5 minutes and annealed at 50°C for 30 minutes then chilled on ice. 9 ul of Sequenase buffer 2 (40 mM Tris-HCl PH 7.5; 5 mM M_gCl_2; 20 mM DTT; 0.1 mM dNTPs) and 0.5 ul Sequenase DNA Polymerase, version 2.0 (USB, 13 U/ul) were then added and the mixture was incubate at 37°C for 10 minutes followed by inactive of the enzyme at 68°C for 10 minutes. The ligation was carried out by adding 20 ul of Ligase buffer 1 (80 mM Tris-HCl PH 7.5; 180 ug/ml BSA; 30 mM M_gCl_2), 20 ul of Ligase buffer 2 (12 mM ATP; 70 mM DTT), 5 ul Linker solution (20 pmol/ul of LMP-T1 and LMP-B1 in 250 mM Tris-HCl PH 7.5; 5 mM M_gCl_2, LMP-B1: 5-GAATTCAGATC-3'; LMP-T1: 3'-CTTAAGTCTAGAGGGCCCAGTGGCG-5') and 3 ul of T4 DNA Ligase (GIBCO, 1 Weiss unit/ul) to the mixture followed by incubation at 20°C overnight. The DNA was ethanol precipitated and resuspended in 14 ul of dH_2O. Additional components were then added for PCR reactions as follows: 16

ul of 2.5 mM dNTP, 2 ul of 5 pmol/ul oligo#2, 2 ul of 5 pmol/ul LMP-T1, 1 ul of 4 mg/ml BSA, 4 ul of 10xTaq buffer (650 mM Tris-HCl PH 8.8; 100 mM β-mercaptoethanol; 165 mM $(NH_4)_2SO_4$; 15 mM $MgCl_2$) and 0.5 ul of Taq DNA polymerase (Boehringer Mannheim 5 U/ul). The samples were cycled 20 times by denature at 94°C for 1 minute, annealing at 65°C for 2 minutes and extension at 74°C for 3 minutes. The PCR product was visualized using polynucleotide kinase (New England Biolabs) [^{32}P] radiolabeled for primer extension. 1.5 ul of [^{32}P] oligo#3 (1 pmol/ul) was added to 15 ul of PCR reaction product along with 0.5 ul of 10xTaq buffer, 3 ul of 2.5 mM dNTPs and 0.5 ul Taq DNA polymerase. 7 PCR cycles of denaturation at 94°C for 1 minute, annealing at 68°C for 2 minutes and extension at 74°C for 3 minutes were carried out and then 30 ul stop mixture (24 ul TE; 1 ul of 5mg/ml tRNA; 5 ul of 3M N_aOAc) was added, followed by chloroform extraction and ethanol precipitation. The pellet was resuspended in 12 ul of sequencing loading buffer and the sample was electrophoresed on a 6% denature polyacrylamide gel. Sequencing reactions were carried out using oligo #3 and Sequenase DNA Polymerase, version 2.0 (USB, 13 U/ul).

For murine mdm2 p53 REs, the following primers were used for LM-PCR.

oligo#1 (5'-TCGAGGTAGAAATACCAACC-3');

oligo#2 (5'-CGAAGCTGGAATCTGTGAGG-3');

oligo#3 (5'-GGAATCTGTGAGGTGCTTGCAGCA-3').

For human mdm2 p53 REs, the following primers were used for LM-PCR.

Oligo#1: 5'-ACAGCACCATCAGTAGGTAC -3';

Oligo#2: 5'- AAGCTACAAGCAAGTCGGTG -3';

Oligo#3: 5'- AAGTCGGTGCTTACCTGGATCAGCAG -3'.

For human gadd45 p53 RE, the following primers were used for LM-PCR.

Oligo#1: 5'- CCCTGAAAACATAACTTCCC -3';

Oligo#2: 5'- GAAGCTGACTCCTTAATGAGGG -3';

Oligo#3: 5'- TGACTCCTTAATGAGGGGTGAGCCAG-3'.

2.5 Preparation of Nuclear Protein Extracts

Two methods have been carried out to get nuclear protein extracts. Method 1 was used for 10-1 and 3-4 cells and Method 2 was used for o41 and TR9-7 cells.

Method 1:

Nuclear lysates were prepared from cells maintained as described above. The cells were washed two times with 4°C 1x PBS. Cytoplasmic Lysis Buffer was prepared with 8.8 ml of Lysis Buffer Stock (20mM Hepes pH 7.5, 20% Glycerol, 10mM NaCl, 1.5mM $MgCl^2$, 0.2 mM EDTA pH 8.0, 0.1% Triton X-100, 1 mM DTT, 1mM PMSF, 50 ug/ml aprotinin, 50uM leupeptin) and 6.2 ml of ddH_2O. Two milliliters of Cytoplasmic Lysis Buffer was added to each washed 150 mm plate. The cells were scraped off the plate and spun at 2300 rpm at 4° C for 7 min. The supernatant was removed and the pellet was resuspended in 1 ml of Nuclear Extraction Buffer (prepared with 8.8 ml of Lysis Buffer Stock, 0.5 M NaCl, and 4.7 ml of ddH_2O). The cells were rocked with the Nuclear Extraction Buffer for 1 hr at 4° C and then centrifuged at 14000 rpm for 5 min. to extract the nuclear proteins. The supernatant was removed and the pellet was discarded. Protein concentrations of the extracts were determined via Bradford Microassay (BIORAD).

Method 2:

The TR9-7 cells were washed with ice-cold 1xPBS twice and were scraped off. The cells were pelleted by centrifugation at 3000 rpm for 10 min. The pellet was resuspended in 5

packed cell volumes of hypotonic buffer (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 0.2 mM PMSF; 0.5 mM EDTA) and quickly pelleted by spinning as above for 5 minutes. After swelling for 10 minutes in 3 pack cell volume of hypotonic buffer, they were homogenized in a glass homogenizer with 10 stokes using a type B pestle and spun at 3800 rpm for 15 minutes. The pellet containing the nuclei was resuspended in 1/2 packed nuclei volume of low salt buffer (20 mM HEPES, pH 7.9; 25% glycerol; 1.5 mM $MgCl_2$; 20 mM KCl; 0.02 mM EDTA, 0.2 mM PMSF; 0.05 mM DTT) followed by drop-wise addition of 1/2 pack nuclei volume of high salt buffer (20 mM HEPES, pH 7.9; 25% glycerol; 1.5 mM $MgCl_2$; 1.2M KCl; 0.02 mM EDTA, 0.2 mM PMSF; 0.05 mM DTT) and rocking at 4°C for 30 minutes. The nuclear protein was obtained by centrifugation for 30 minutes at 13000 rpm at 4°C and stored at -80°C. To prevent rapid deacetylation, trichostatin A (TSA), an inhibitor of histone deacetylases, was added in the nuclear extraction buffer at a final concentration of 5 uM (Taunton et al., 1996; Sakaguchi et al., 1998). Wortmannin is a fungal metabolite that has been shown to act as a selective inhibitor of phosphoinositide 3-kinase family members (reviewed in Ui et al., 1995). TR9-7 cells were incubated in 5uM of wortmannin for 4 hours before cells were lysed.

2.6 Western Blot Analysis

Samples were electrophoresed on a 10% SDS-PAGE and electrotransferred to nitrocellulose. The blots were probed with different specific antibodies and visualized by incubation with goat anti-mouse or goat anti-rabbit second antibody followed by ECL solutions (Amersham).

2.7 Purification of p53 Proteins

Sf21 cells (2.5 x 10^7 / 150 mm dish) were infected with recombinant viruses and harvested 48 hr post infection. Extracts of infected cells were prepared as described (Bargonetti et al., 1992). p53 proteins were purified from cell lysates by immunoafinity procedures. A monoclonal antibody column with p53-specific antibody PAb 421 cross-linked to protein A-Sepharose was used to purify p53 proteins. The proteins were eluted with ethylene glycol (Bargonetti et al., 1992) and dialyzed into a buffer containing 10mM HEPES (pH 7.5), 5mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 50% glycerol.

2.8 Cytoplasmic RNA Extraction and mRNA Purification

The cells were washed two times with 4°C 1x PBS and were scraped off. The cells were pelleted by centrifugation at 2500 rpm for 10 min on Beckman centrifuge. The pellet was resuspended in in 200 ul of RNA extraction buffer (0.14M NaCl; 1.5mM $MgCl_2$; 10mM Tris.Cl ph 8.6; 0.5% NP-40; 1mM DTT; 100 units/ml RNasin) and vortex for 15 seconds. The tubes were standed on ice for 5 minutes. The cytoplasmic part was separated by centrifuged at 12,000g for 90 seconds at 4°C and remained in supernatant. The proteins were digested by adding 200 ul of proteinase digestion buffer (0.2 M Tris.Cl ph 8.0; 25 mM EDTA ph 8.0; 0.3M NaCl; 2% SDS; 50 ug/ml proteinase K) and incubate at 37°C for 30 minutes. The RNA was extracted with phenol and chloroform followed by ethanol precipitation. The cytoplasmic RNA was resuspended in 200 ul of TE and store at -80°C.

The mRNA was purified from oligo(dT)-cellulose described as Maniatis. The cellulose was first resuspended in 0.1 N NaOH and packed to a column. The column then was washed with 3 volumes of DEPC-dH_2O and 1x column-loading buffer (20 mM Tris.Cl

ph7.6; 0.5M NaCl; 1 mM EDTA ph 8.0; 0.1% sodium lauryl sarcosinate) until the PH is less than 8.0. The RNA was heated at 65°C for 5 minutes and then added equal amount of 2x column-loading buffer. The RNA was loaded on the column and the elution was collected by centrifugation at 2,500 rpm. Repeat this step until OD_{260} absorbency is close to 0. The poly(A) RNA from the column was eluted by washing the column with elution buffer (10 mM Tris.Cl ph 7.6; 1mM EDTA ph 8.0; 0.05% SDS). The mRNA was precipitated with ethanol and stored at -80°C.

The mRNA was run in 1% agarose gel (GIBCO) and transferred to positive charged nylon membrane (Schleicher and Schuell) by electric transfer at 0.1 Amp overnight at 4°C in 1xTAE. The membrane was baked at 80°C for 2 hours and UV crosslinked.

2.9 EMSA

The SCS synthetic oligonucleotide used in this study, obtained form Operon, contained consensus p53 binding sites. The sequence of this oligonucleotide was the following: 5' TCG AGC CGG GCA TGT CCG GGC ATG TCC GGG CAT GTC - 3'
The murine mdm2 p5REs synthetic oligonucleotide used in this study, obtained from Operon, contained two p53 response elements. The sequence of this oligonucleotide was the following: 5' - GAT CCC TGG TCA AGT TGG GAC ACG TCC GGC GTC GGC TGT CGG AGG AGC TAA GTC CTG ACA TGT CTC CG - 3'
The human gadd45 putative p53 RE site synthetic oligonucleotide used in this study, obtained form Operon, contained p53 binding sites. The sequence of this oligonucleotide was the following: 5' AAT TCT CGA GGA ACA TGT CTA AGC ATG CTG CTC GAG 3'

Labeling of the oligonucleotides was performed with the large fragment of DNA polymerase and [^{32}P] dCTP. Reaction mixtures for EMSA experiments (30 ul) were carried out with 0.1 pmoles oligonucleotide, 500 ng dIdC and 1 ug BSA, in 2 mM Spermidine, 0.9 mM DTT, 2mM MgCl2, 0.1mM EDTA pH 8.0, 25mM KCl, 20mM HEPES pH 7.5 and 10% Glycerol. In addition to 2ug of 041 or TR9-7 nuclear protein extract, 0.5 ug of PAb 1801 was added to each reaction. All samples were incubated at room temperature for 20 min. The protein-DNA complexes were resolved on a 4% acrylamide gel, which was pre-run at 100V at 4°C for 20 minutes prior to loading. The samples were then electrophoresed at 200V for 3 hours.

2.10 Plasmid DNase I Footprinting

Reaction mixtures for plasmid footprinting (50 ul) were carried out with 25 ng of Xho I-Apa I digested genomic murine mdm2 subclone and 1 ug BSA, in 2 mM Spermidine, 0.9 mM DTT, 2 mM MgCl2, 0.1 mM EDTA pH 8.0, 25 mM KCl, 20 mM HEPES pH 7.5 and 10% Glycerol. Increasing amounts of p53 were added as indicated in the figure legend and the reaction mixture was incubated on ice for 30 minutes followed by digestion with 2 ng of DNase I (Worthington; 2,932 units/mg) on ice for 1 minute followed by the addition of 50 ul of DNase I stop buffer (2 M ammonium acetate, 100 mM EDTA, o.2% SDS, 100 ug/ml sheared salmon sperm DNA).The DNAs were phenol and chloroform extracted followed by ethanol precipitation. The pellet was resuspended in 35 ul of dH_2O followed by primer extension of [^{32}P] labeled oligo#3 (5'-GGAATCTGTGAGGTGCTTGCAGCA-3'). 1 ul (1pmol/ul) [^{32}P] oligo#3 and 4 ul of 50 mM N_aOH were added to the DNase I digested samples and heated at 80°C for 3 minutes and chilled in ice. 10 ul of primer extension buffer (500 mM Tris-HCl, PH 7.2; 100 mM $MgSO_4$; 2 mM DTT) was added and the sample was incubated at 65°C for 3

minutes. 10 ul of 2.5 mM dNTPs and 40 ul of dH_2O were then added and primer extension was carried out at 37°C for 10 minutes with Klenow DNA polymerase (Boehringer Mannheim). Reactions were stopped by adding 25 ul of stop mix (4 M N_aOAc; 20 mM EDTA) followed with ethanol precipitation. The pellet was resuspended in 12 ul of sequencing loading buffer and electrophoresed on a 6% denaturing polyacrylamide gel.

2.11 Real-time PCR with Molecular Becon

Reverse transcription (RT)

For each sample, 5 ug of cytoplasmic RNA were incubated at 65°C for 10 min with 250 umol oligo$(dT)_{15}$ primer (Bohringer) in a total volume of 10 ul. After cooling on ice, 10 ul of RT mix was added: 2X AMV buffer, 15U AMV (Amersham), 2.5 mM dNTP, 20U RNase inhibitor RNasin (Promega). Samples were incubated for 1 hour at 37°C. The reaction was stopped by heating at 94°C for 2 min. Samples were stored at –80°C.

PCR with molecular beacons

PCR primer pairs were designed to anneal to their target at the same temperature (55°C) and to amplify DNA fragments of approximately 100 bp. Molecular beacons were designed with a DNA folding program (http:www.ibc.wustl.edu/~zuker/dna/form1.cgi) to have an hairspin stem that dissociate at a temperature 10°C higher than the detection temperature. The Molecular beacons were synthesized as previously described (Tyagi and Kramer, 1996).

2 ul of RT products were used in the PCR reaction carried out under the following conditions: 1X TaqMan Buffer (Perkin-Elmer), 2.5 mM $MgCl_2$, 250 uM dNTP, 15 pmol of each primers, 2.5 U AmpliTaq Gold polymerase (Perkin-Elmer) and 125 ng of

the appropriate molecular beacon. Forty cycles of amplification (94°C denaturation for 30s, 55°C annealing for 1 min and 72°C elongation for 30s) were carried out in sealed tubes in an Applied Biosystems 7700 Prism spectrofluorometric thermal cycler (Perkin-Elmer). Fluorescence was measured during the annealing step and plotted automatically for each sample.

The primer pairs used for PCR reaction were following:
Gadd45, Forward primer: 5'-CCATGCAGGAAGGAAAACTATG-3'; Antisense primer: 5'-CCCAAACTATGGCTGCACACT-3'.
Waf1, Forward primer: 5'-ACCTTCCAGCTCCTGTAACATACT-3'; Antisense primer: 5'-GTCTAGGTGGAGAAACGGGAA-3'.
GAPDH, Forward primer: 5'-AGAGCACAAGAGGAAGAGAGAGACC-3'; Antisense primer: 5'-AACTGTGAGGAGGGGAGATTCAG-3'.

The sequences of the molecular beacons were following:
Gadd45, 5'-CGCTGCAGAATGGTTGAGTTACATTAAAATAAACCGCAGCG-3';
Waf1, 5'-CGCTGCAGGACACATGGGGAGCCGAGCAGCG-3';
GAPDH, 5'-GGACGCGGTGGGGGACTGAGTGTGGCGTCC-3'.

Chapter III

p53 Binds to a Constitutively Nucleosome Free Region of the mdm2 Gene

3.1 Introduction

Temperature sensitive p53 provides us a useful system to study p53 since normal or untransformed cells contain very low levels of p53 and overexpressed p53 will result in cellular growth arrest. One murine mutant p53, p53-Val135 (an alanine to valine change at residue 135) was found to transform primary rat embryo fibroblasts in cooperation with the *ras* oncogene in 1989 (Hinds et al., 1989). In 1990 Michalovitz et al. demonstrated that the p53-Val135 was temperature sensitive for transforming activity. At 32.5°C, it was unable to cooperate with *ras* to transform cells in transformation assays. In addition, dependent on the reactivity against conformation-dependent antibodies (PAb 246 which binds wild-type but not mutant p53; PAb 240 which binds mutant but not mutant p53), p53-Val135 is conformationally wild-type ($PAb246^+$, $PAb240^-$) at 32.5°C whereas at 39.5°C p53-Val135 is conformationally mutant ($PAb246^-$, $PAb240^+$) (Martinez et al., 1991). Moreover, immunostaining demonstrates that at 32.5°C, the p53 enters the nucleus and at 39.5°C the mutant p53 is localized in the cytoplasm of the cell (Gannon et al., 1991; Martinez et al., 1991). It was believed that at the non-permissive temperature (37°C) the mutant protein assumed a thermodynamically less stable folded structure, thereby losing any resemblance to wild-type p53, with concomitant abrogation of p53-mediated activities (reviewed in Gallagher and Brown 1999). In addition, the dominant-negative function of p53-Val135 was carried out in a transgenic mice system by introducing p53-Val135 into wild-type or p53 deficient mice. Expression of this mutant p53 increased tumor incidence in mice carrying one or both wild-type alleles of p53 but not in mice that were homozygous null for p53 (Harvey, et al., 1995).

It has been shown the endogenous mdm-2 mRNA is enhanced in rodent fibroblasts carrying overexpressed temperature-sensitive p53 at 32.5°C (Barak et al., 1993). The region of the mdm2 gene with which p53 interacts was originally identified by co-immunoprecipitation of a specific DNA element as well as by demonstrating a minimal sequence able to confer p53 specific transactivation of a reporter construct (Wu et al., 1993; Juven et al., 1993). However the nucleotides within this region that are specifically bound by p53 and thereby protected from DNase I digestion have not been identified. A better understanding of the precise functioning of p53 sequence-specific DNA binding *in vivo* has therefore been the focus of this project.

Studies on sequence-specific transcription factor binding to its binding sites in cells are carried out by using *in vivo* footprinting techniques. Several DNA modifying agents such as DNase I (deoxyribonuclease I), dimethyl sulfate, exonuclease III, micrococcal nuclease and KMnO4 have been used to provide the information on how and when proteins occupy regulatory regions in chromatin. In 1989, Mueller et. al. developed ligation-mediated PCR *in vivo* footprinting which has a great advantage in sensitivity to allow single-copy genomic cleavages to be detected quantitatively at the nucleotide level. We chose DNase I because of its relatively low DNA sequence specificity. DNase I cuts every bond along the sugar phosphate chain with only a mild preference for TpX, ApX, XpT, and XpA, where X is any base (reviewed in Saluz and Jost 1993). DNase I attacks and cleaves DNA by binding to the minor groove of the double helix and creates nicks on both strands. The LM-PCR *in vivo* footprinting was carried out by using DNA samples digested with DNase I *in vivo*. First, gene-specific oligonucleotide#1 was annealed to denatured genomic DNA and extended with Sequenase DNA Polymerase to make blunted ends. Next, a common linker oligo was ligated to the blunt ends of genomic DNA fragments and the DNA fragments were

amplified by PCR utilizing gene-specific oligonucleotide#2 and linker top as primer pairs. At the end, the amplified DNA was visualized by elongation of radiolabeled gene-specific oligonucleotide#3. The three oligonucleotides were selected by using Mac vector computer software. They were based on the genomic sequence and had increasing melting temperature for each one to ensure the specificity (Fig. 3.1).

It has become increasingly apparent that modulation of chromatin structure plays an important role in the regulation of transcription in eucaryotes (reviewed in Kadonaga et al., 1998). It is believed that nuclease-hypersensitive sites are associated with the regulatory elements. The sensitivity to nuclease of these regions is usually one or two orders of magnitude above those of the bulk chromatin (Boyes and Felsenfeld 1996). The most commonly used method to map nuclease hypersensitive sites is DNase I digestion of nuclei followed by indirect end-labeling of the resulting purified double-stranded DNA. Within a given cell type, DNase I hypersensitive sites fall into two major categories: constitutive and inducible. Constitutive sites are often present in promoter regions of genes "poised" for transcriptional induction; their presence precedes transcriptional activation and is independent of gene expression. Inducible sites are most often associated with concomitant transcriptional activation of the linked gene (Gross and Garrard 1988).

We have investigated the intranuclear interaction of p53 with the p53 dependent promoter region of the mdm2 gene by comparing the genomic DNase I protection of this region in two different cell lines, a p53-null murine fibroblast cell line (10-1) and a temperature-sensitive p53-Val135 overexpressing line (3-4). The 3-4 cell line was derived from 10-1 by co-transfecting with the temperature-sensitive mutant p53-Val135 plasmid (pLTRp53cGval135) and a Neomycin resistant plasmid.

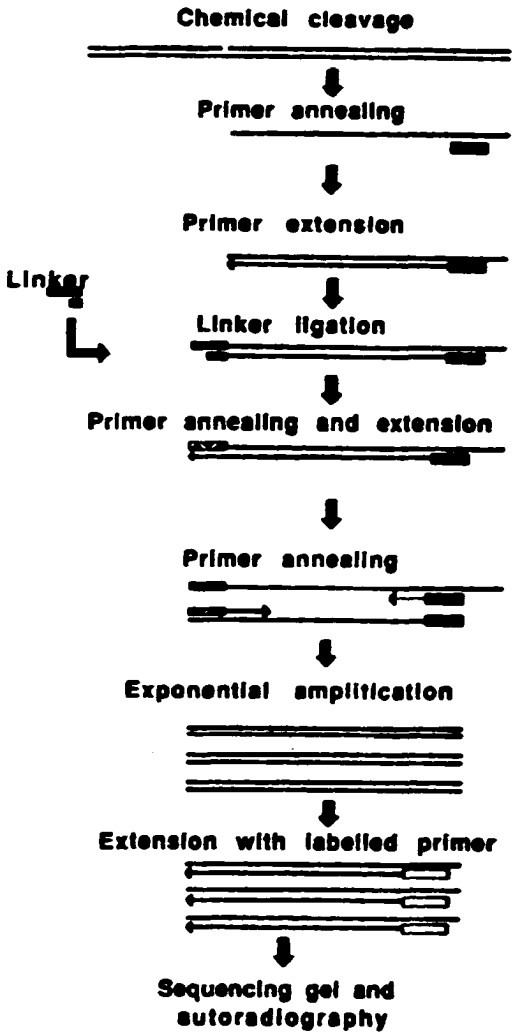


Figure 3.1: Schematic outline of the procedure of ligation-mediated PCR *in vivo* footprinting. The LM-PCR *in vivo* footprinting was carried out by using DNA samples digested with DNase I *in vivo* . First, gene-specific oligonucleotide#1 was annealed to denatured genomic DNA and extended with DNA Polymerase. Next, a common linker oligo was ligated to the blunt ends and the DNA fragments were amplified by PCR utilizing gene-specific oligonucleotide#2 and linker top as primer pairs. At the end, the amplified DNA were visualized by elongation of radiolabeled oligonucleotide#3. In this example only one cleaved fragment is indicated. The different primers are represented as boxes (Saluz and Jost 1993).

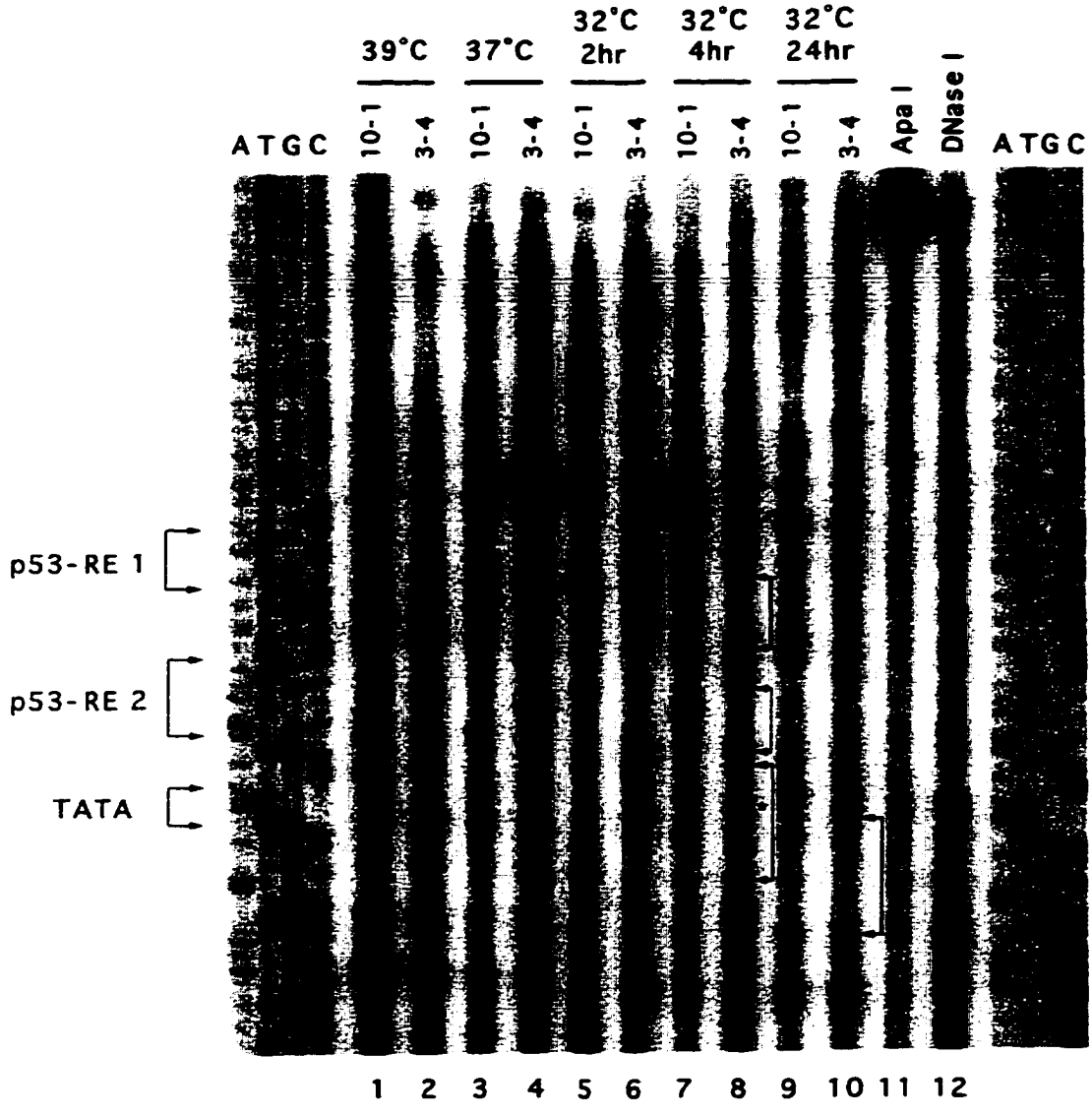
3.2 Results

3.2.1 Simultaneous intra-nuclear protection of the p53-REs and the TATA box

We used ligation mediated PCR genomic footprinting analysis to define the nucleotide sequences (within putative p53 binding site region of the mdm2 gene) that were protected in a p53 dependent manner. Comparing 10-1 and 3-4 cell lines at 32°C and 37°C, striking protection of the p53-REs was observed in nuclei of 3-4 cells maintained at 32°C for 4 hours (Fig. 3.2A, lane 8; the protected regions are shown by brackets on the right while the putative p53-RE are shown on the left). While no changes were observed between the protection patterns at p53 RE-1 or p53 RE-2 in 3-4 cells maintained at 39 °C or 37°C. Footprints in cells maintained at 32°C for 2 hours showed some differences with possible changes occurring at the p53-REs and the TATA box (Fig. 3.2A, lane 6). After the 3-4 cells had been shifted to 32°C for 4 hours the two putative p53 binding sites were protected over their downstream 3' halves. While some changes occur in the 10-1 genomic protection analysis at the different shift time points the pattern at 39°C and 37°C was similar to that seen in 3-4 (Fig. 3.2A, lanes 1-4). While the 32°C 4 hours shift point demonstrated dramatic contrasts (Fig. 3.2A, lanes 7 & 8). Interestingly the p53-REs DNase I protected sequence in 3-4 nuclear chromatin did not contain the total 20 bp consensus sequence. Additionally, a change in protection at the TATA box region was evident, with a hypersensitive site emerging in the middle and prolonged protection occurring over the 3' region (Fig.3.2A, lane 8 indicated by bracket with *). The DNase I hypersensitive site which emerged (marked *) was also visible in the naked DNA sample indicating that this region may be protected by a factor at the other time points. It has been shown that p53 and TFIID are able to bind cooperatively to DNA (Chen, 1993). The simultaneous change in protection at the TATA box and p53

Figure 3.2: Ligation-mediated PCR *in vivo* footprinting demonstrates p53 mediated protection of the p53 REs. (A) The published p53-REs and adjacent TATA box were identified by sequencing genomic mdm-2 in a plasmid clone (lanes indicated as ATGC) and are indicated on the left. DNA in 2 x10^6 isolated nuclei from 10-1 and 3-4 cells was digested with 0.1ug of DNase I and purified. Samples are from cells maintained at 39°C (lanes 1 and 2), 37°C (lanes 3 and 4), 32°C for 2 hours (lanes 5 and 6), 32°C for 4 hours (lanes 7 and 8) and 32°C for 24 hours (lanes 9 and 10). Purified DNA from undigested nuclei is shown in lane 11 and naked mouse genomic DNA digested with DNase I is shown in lane 12. Ligation mediated PCR was carried out followed by primer extension with [^{32}P] labeled oligonucleotide #3 which hybridized approximately 350 bp downstream from the P2 promoter. Samples were electrophoresed on a 6% urea sequencing gel. This experiment was repeated 3 times.(B) Sequence representation of the mdm2 P2 promoter region showing areas of predicted protection overlapping with areas protected during *in vivo* footprinting. The **large bold** letters indicate protection as seen during *in vivo footprinting*. The <u>underlined</u> regions depict areas that were the predicted *cis* acting elements. The asterisk represents the location of hypercutting found in both the 3-4 32°C 4 hour sample (lane 8) and in the DNase I digested naked genomic DNA sample (lane 12). Oligonucleotides used for ligation mediated PCR are numbered and shown in gray. The overlap between oligonucleotides #2 and #3 is denoted by larger letters and a dashed underline.

A



B

ApaI

(1bp)--gggcc|cgctccgggtcgcgctggctcgttgctgggtccaggaggtgacaggtgcctggtc

ccggactcgccgggatgcggcttccgggacgggtgggactggtctgggccgagttgactc

agctcttcctgtggggct**ggtcaagttgggacacgtccggcgt**cggctgtcggagg

p53 RE-1

agctaagtcctgacatgtctcc**agctggggttat*ttaaacgctgccccgt**

p53 RE-2 **TATA box**

ttccgcagccgtctgctgggcgagcgggagaccgaccggacacccctgggggaccctctcggatcaccgcgctt

ctcctgcgggcctccaggtaagggacagctcgccgacgtcgttttgcatttgagagctatgaaagacgt

NsiI

tttctgttccttcgtaaat|gcatgtatctatttgtccctttcgtagatgttttataatttcaagttttt

atcgtgtgtgttgttgttttttttctacttgtaggccaatgtgcaataccaacatgtctgtgtctaccgagggt

*gctgcaagca**gctcacagattcc**agcttcg*gaacaagagactct*ggttggtatttctacctcga*-(585bp)

oligo #3 <==========|-oligo #2 <----------oligo #1

REs suggests that binding of p53 facilitates recruitment of TFIID to the promoter region. The 3-4 samples shifted to 32°C for 24 hours had reduced protection of the two p53-REs with concomitant extended protection visible over the 3' adjacent TATA box region (Fig. 3.2A, lane 10). The pattern of genomic protection in 10-1 and 3-4 cells was similar (although not identical) at 39°C and 37°C (Fig. 3.2A, lanes 1-4). Changes in the 10-1 protection pattern were observed at 32°C but these changes did not correlate with the putative p53-REs. Figure 3.2B shows the mdm2 P2 promoter sequence with the areas of predicted protection compared with the actual p53 dependent chromatin protection pattern. Northern blotting indicated that the protection at p53-REs and TATA box correlated with an increase in transcription of the mdm2 gene of 16-fold in the 3-4 cells at 32°C for 4 hours shift normalized to the housekeeping gene GAPDH (Fig. 3.3).

3.2.2 p53 mediated DNase I protection of the mdm2 p53-REs is different on naked DNA

We examined the *in vitro* DNase I footprinting ability of purified p53 on the mdm2 P2 promoter region in order to determine if the pattern of protection at the p53-REs was the same as that demonstrated *in vivo* (Fig. 3.4). Surprisingly, the *in vitro* protection pattern demonstrated with purified p53 on naked DNA differed considerably from the genomic protection patterns that were observed. Protection of the 5' half of p53-RE1 was observed on naked DNA with p53 however no clear protection of p53-RE2 was identified (Fig. 3.4, lanes 1& 2). The footprinting pattern with purified p53 demonstrated a pattern of protection in which sequences extending from the specific site were alternately protected and hypersensitive to DNase I cutting; this is consistent with our previous observation with purified p53 at other p53 binding sites (Bargonetti et al., 1992). The auxilary protection due to may be a result of p53 protection multimers

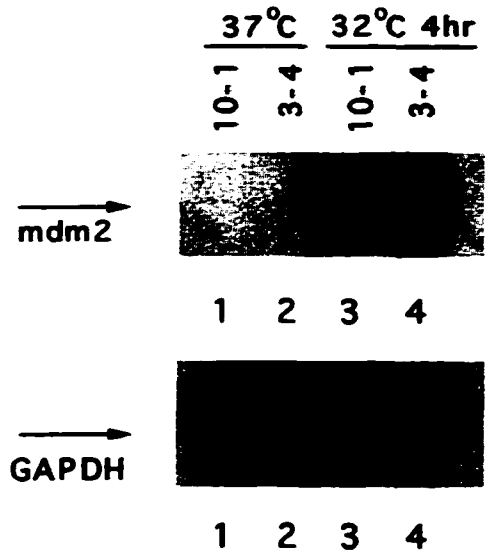


Figure 3.3: Endogenous mRNA levels of mdm2 are enhanced by the presence of wild-type p53 activity. Northern blot analysis of mdm2 polyA RNA in 10-1 cells (lanes 1-3) and in 3-4 cells with the temperature sensitive p53 Val135 (3-4) (lanes 4-6). Cells were grown at 37°C and then shifted to 32°C for either 4 hours (lanes 2 and 5) or 24 hours (lanes 3 and 6). The blot was hybridized first with Nsi I- Apa I mdm2 probe then stripped and reprobed with labeled GAPDH fragment. Data were also quantitated from multiple exposures on a PhosphorImager. This experiment was repeated 3 times.

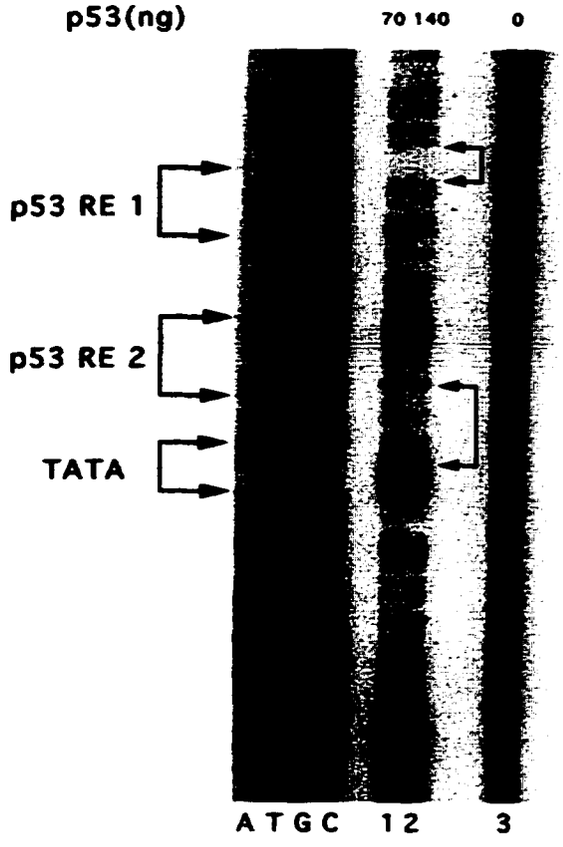


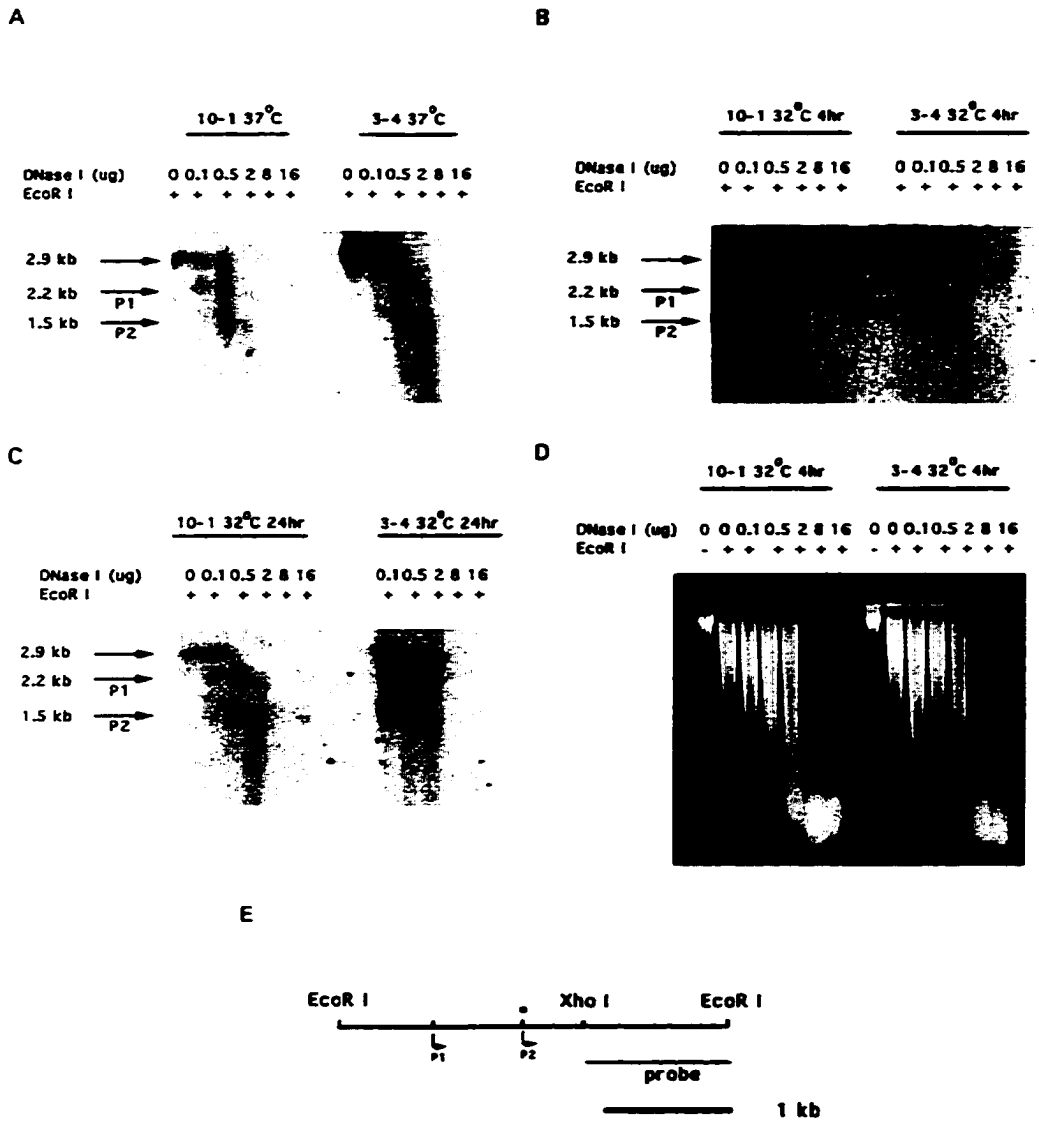
Figure 3.4: Protection of the p53-REs of mdm2 *in vitro* differs from that observed *in vivo*. *In vitro* footprinting of a naked mdm2 DNA fragment with purified p53. The published p53-REs and adjacent TATA box were identified by sequencing genomic mdm-2 in a plasmid clone (lanes indicated as ATGC) and are indicated on the left. Increasing amounts of purified p53 were added to a complete Apa I-XhoI digest of the naked mdm2 genomic plasmid clone (lanes 1 & 2, as indicated). Footprints were visualized by primer extension using [^{32}P] labeled oligonucleotide #3 which hybridized 350 bp downstream from the TATA box. Lane 3 contains no p53 protein. This experiment was repeated 2 times.

wrapping around the DNA (Stenger et al., 1994). The difference between the genomic footprinting pattern and the *in vitro* protection pattern suggests that the chromatin structure of the mdm2 P2 region organizes the DNA sequences into an optimal p53 DNA binding site or either p53 post-translational modification or association of p53 with other factors facilitates p53 binding to the mdm2 gene.

3.2.3 The mdm2 P1 and P2 promoters are nucleosome free

When genes controlled by specific promoters are transcriptionally active, or are primed to be activated, their promoter regions in chromatin are marked by increasing sensitivity to DNase I caused by an induced disruption or constitutive binding nucleosome (Gross and Garrard, 1988). We took advantage of this fact and screened for the appearance of p53 dependent DNase I hypersensitive sites at the mdm2 P2 promoter. Indirect end-labeling analysis of the genome from isolated nuclei of 10-1 or 3-4 cells maintained at 37°C and 32°C for 4 hours or 32°C for 24 hours were subsequently treated with DNase I (Fig. 3.5A-C). We observed the same DNase I hypersensitive sites in the two cell lines at the different temperature shift points. One hypersensitive site was observed at 2.2 kb from the EcoR I cutting site which corresponded to the mdm2 P1 promoter region (Fig. 3.5, indicated by the P1 arrow) while another was observed 1.5 kb from the EcoR I cutting site and corresponded to be the location of the P2 promoter region (Fig. 3.5, indicated by the P2 arrow). The overall DNase I sensitivity of these sites did not change at these regions in the presence p53. The constitutive hypersensitive site present at the mdm2 P2 promoter indicated that this region had an altered chromatin structures in the absence of p53 and that this structure was not influenced by the binding of p53 which was demonstrated in the genomic footprint. To further analyze the nucleosome arrangement, micrococcal nuclease sensitivity of the p53 dependent mdm2

Figure 3.5: The mdm2 P1 and P2 promoters are constitutively nucleosome free. DNA in 2 x 10^6 isolated nuclei was digested with increasing amounts of DNase I (0 ug, 0.1 ug, 0.5 ug, 2 ug, 8 ug or 16 ug as indicated above each lane). Nuclei were isolated from 10-1 and 3-4 cells maintained at 37°C (A), 32°C for 4 hours (B) or 32°C for 24 hours (C). Purified DNA was restricted with EcoR I and electrophoresed on 0.8 % agarose gel, probed with a [^{32}P] labeled EcoR I-Xho I genomic mdm2 probe fragment (shown in D). The arrows indicate the hypersensitive sites in the mdm2 gene. (D) The ethidium stained agarose gel of (B). (E) Physical map of the upstream region of the murine mdm2 gene (Barak et al. 1994). The * delineates the location of the p53 REs. This experiment was repeated 2 times.



P2 was assayed. Although the agarose gel showed the nucleosome fragmentation ladder, probing with the P2 promoter specific probe gave a barely detectable smearing signal without the typical DNA digestion ladder from either the 10-1 or 3-4 genome (Fig. 3.6). This result confirmed that the P2 promoter region was constitutively nucleosome-free because it was digested by micrococal nuclease. Interestingly, a reproducible difference in the intensely of the mdm2 signal found in the 3-4 and 10-1 cell lines of 4-7 fold existed although the same amount of genomic DNA was loaded on the gels. This suggests that in the presence of ts p53 Val-135 the mdm2 gene has been amplificatied. We compared this intensity further by probing the same blot for Gadd45 (a p53 responsive gene that has not been shown to be amplified). In this case no difference was observed in the level of the signal obtained from the 10-1 vs. the 3-4 genome (data not shown).

3.2.4 The p53 protein level peaks at the same time as the genomic footprint

Previous reports on the properties of ts p53-Val135 have shown increased levels of nuclear p53 protein when cells are shifted to 32°C (Martinez, 1991). In order to access if our genomic footprinting peak corresponded to an increase in nuclear p53 we analyzed the protein's level at the different temperature shift time points (Fig. 3.7). An increase of 2 fold was seen for the p53 level was observed when the cells had been maintained at 32°C for 4 hours (Fig. 3.7, lane 8) and a decrease in the level of p53 was observed after 24 hours at 32°C (Fig. 3.7, lane 10). Therefore it appeared that an increased level of p53 protein present in the nucleus could be involved in controlling the ability of p53 to protect the mdm2 promoter consensus site sequences within chromatin.

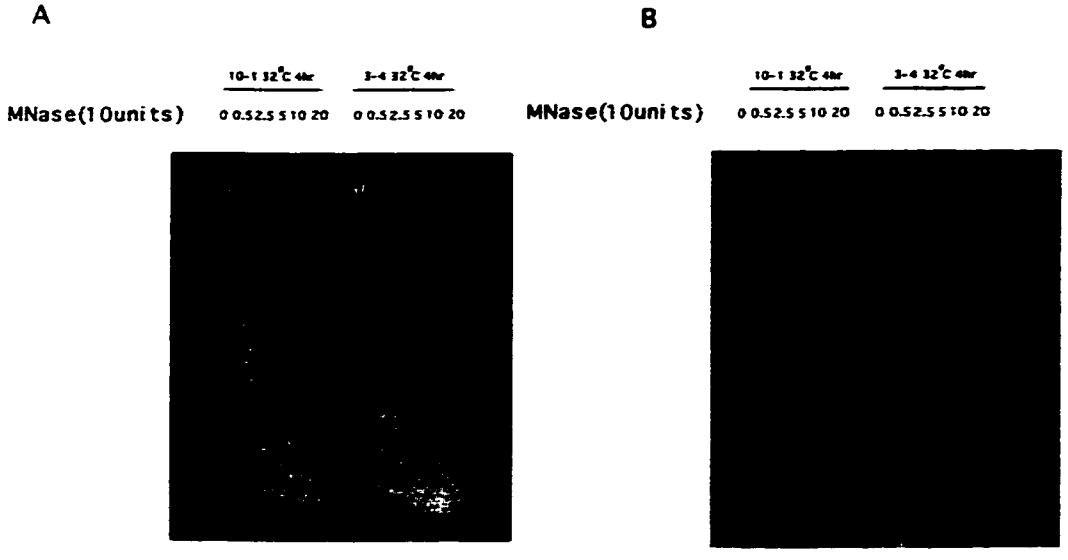


Figure 3.6: The nucleosomal organization over the mdm2 P2 promoter was assayed by micrococcal nuclease digestion. DNA in 2 x 10^6 isolated nuclei was digested with increasing amounts of micrococcal nuclease (0 u, 5 u, 25 u, 50 u, 100 u or 200 u as indicated above each lane). The ethidium stained agarose gel is shown (A). (B) Purified DNA was electrophoresed on 1.5 % agarose gel, probed with a [^{32}P] labeled Apa I-Nsi I genomic mdm2 probe fragment . This experiment was repeated 2 times.

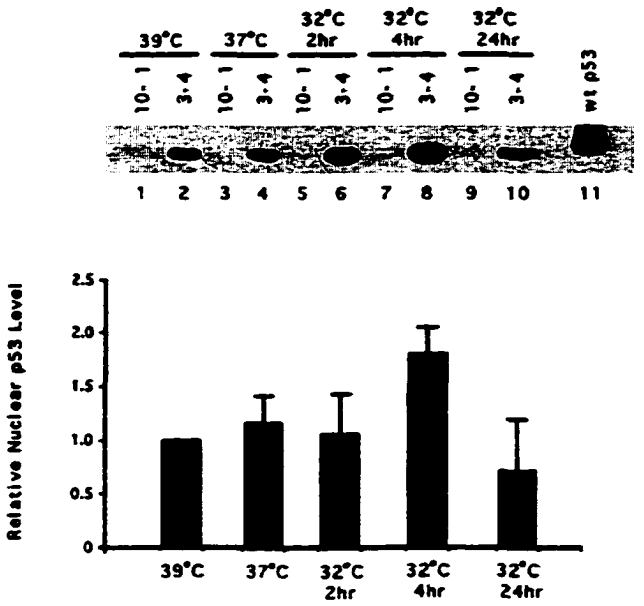


Figure 3.7: p53 protein levels in 10-1 and 3-4 nuclear extract. Nuclear extracts were prepared as described in the Materials and Methods. 100 ug of total protein was resolved by electrophoresis on a 10% SDS-PAGE. The p53 in samples was visualized by Western blotting with a mixture of p53 specific monoclonal antibodies PAb 421, PAb 240 and PAb 1801 and detection with ECL reagent (Amesham). The relative levels of p53 are represented as a histogram of normalized pixel values determined by laser densitometer analysis and quantitation by Image QuanNT software (version 4.1) setting the level detected at 39°C as 1. The standard deviation for all other samples was calculated from three experiments. This experiment was repeated 3 times.

3.2.5 EMSA with nuclear extracts containing p53-Val135 suggests p53 may recruit other factors to the mdm2 site

It has been demonstrated that p53 can bind differentially to p53-REs that contain variations on the consensus binding sequence and that phosphorylation of p53, in addition to site-specific binding affinity, can be regulators of this differential binding (Funk, 1992; Halazonetis et al., 1993; Hecker et al., 1996; Takenaka et al., 1995; Wang, 1995). Using a standard electrophoretic mobility shift assay (EMSA) and 2ug of 10-1 or 3-4 nuclear extracts, we examined the p53 dependent gel shift of an oligonucleotide containing the mdm2 P2 p53-REs. With nuclear extracts from cells maintained at 37°C, no p53 dependent gel shift species was observed either in the absence or presence of the p53 specific antibody, PAb421 (Fig. 3.8, compare lanes 1& 2 to lanes 7&8). The DNA binding ability of p53 is activated in the presence of PAb421 and therefore induction of a PAb421 induced supershift is a sensitive indication of the amount of p53 in the sample that is able to bind to DNA. Nuclear extracts from 3-4 cells maintained at 32°C for 4 hours demonstrated a strong p53 dependent gel shift of the mdm2 oligonucleotide (Fig. 3.8A, compare lanes 3 &4 to lanes 9 &10 as indicated by the arrow). Nuclear extracts from cells maintained at 32°C for 24 hours gave a reduction in the p53 dependent gel shift species (Fig. 3.8A, compare lanes 10 &12). The mdm2 oligonucleotide with nuclear extracts bound migrated slower than the purified p53 PAb 421 gel shift complex (Fig. 3.8A, compare lanes 10 & 14) suggesting that additional proteins were bound.

A competition gel shift assay was carried out to further support the specificity of p53 binding to the mdm2 oligonucleotide. The PAb421 induced supershift from the 3-4 nuclear extracts at 32°C for 4 hours was competed by both the non-radioactive mdm2 oligonucleatide as well as the oligonucleotide containing the p53 super consensus site

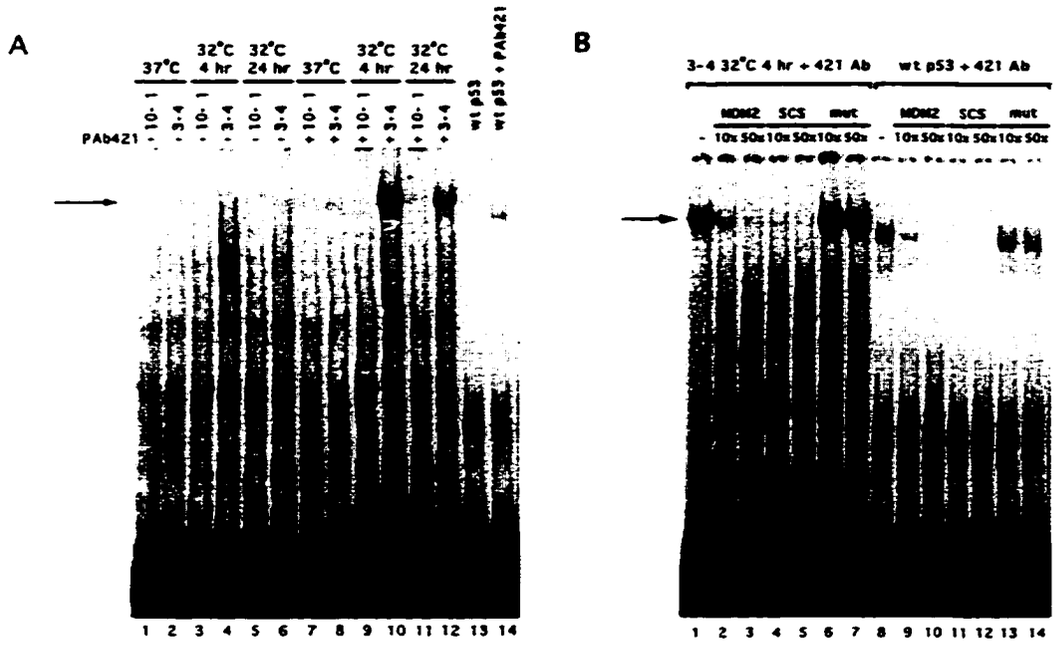


Figure 3.8: Protection of the mdm2 P2 promoter *in vivo* corresponds the enhance binding of p53 to an oligo contain the mdm2 P2 promoter region. (A) EMSA of a mdm2 P2 containing oligonucleotide with 10-1 and 3-4 nuclear extracts in the presence of PAb 421. (B) EMSA of mdm2 P2 containing oligonucleotide with 3-4 nuclear extracts and purified wild-type p53 in the presence of PAb 421 competed with either cold mdm2 oligo, cold super consensus p53 binding sequence, or mutant RGC oligo (10 times or 50 times). The arrows indicate the p53 dependent gel shift species present in nuclear extract. This experiment was repeated 3 times.

sequence (SCS, lanes 4, 5, 11 and 12). Non-radioactive mdm2 oligo or super consensus p53 binding sequence (10 times or 50 times) competed the p53 dependent gel shift species in 3-4 cells maintaining at 32°C for 4 hours (Fig. 3.8B, lanes 2, 3, 4 and 5). However, the cold mutant RGC oligo (10 times or 50 times) could not compete the p53 dependent gel shift species.

3.3 Discussion

3.3.1 The mdm2 locus is primed for transcriptional activation

The mapping of the DNase I hypersensitive sites located in the mdm2 gene identified that both P1 and P2 promoters were localized in altered chromatin regions. Chromatin structure has been shown to regulate gene expression however gene expression has also been shown to remodel chromatin structure. The mdm2 gene appears to be a member of the class of genes which exist in a preferentially DNase I sensitive state suggesting that the mdm2 gene is primed for both transcriptional activation and repression (Gross and Garrard, 1988). Within the mdm2 DNase I sensitive region we have identified two DNase I hypersensitive sites. One hypersensitive site is located 2.2 kb from the EcoRI cutting site and corresponds to the mdm2 basal promoter P1. The other hypersensitive site at 1.5 kb which is located at the p53 dependent P2 promoter. These two DNase I sensitive sites occurred in both 10-1 and 3-4 cells maintained at 37°C and 32°C therefore neither site was dependent upon the presence of p53. Hypersensitive sites in chromatin reflect the absence of a canonical nucleosome. Our results suggest that activation of mdm2 transcription does not require nucleosome disruption at the p53 dependent promoter.

A model can be proposed that p53 can bind to TFIID and TFIIH recruiting the complex to the P2 TATA box and may involve stabilization of the complex to the DNA binding sites in such a way as to alter the transcription initiation rate. The 32°C 24 hours shift sample demonstrated that although the promoter may be occupied by transcription factors, stimulation of transcription does not require that the promoter always be occupied.

It has been reported that chain elongation by RNA polymerase II is unimpeded by the presence of one or two histone octamers in their path *in vitro*. When longer arrays of octamers are present, pol II is still able to elongate RNA *in vitro*, but transcription is partially inhibited by pausing along the template, an effect that may well be alleviated *in vivo* by histone modification or the presence of additional factors. Since we did not find other DNase I hypersensitive sites downstream of the TATA box in either the 32°C 4 hours or 24 hours samples, we would like to suggest that drastic disruption of the nucleosome does not occur during elongation. Our results suggest chromatin structure may be important for recognition by p53. This altered chromatin structure may not be present at all p53 REs. However most of the genes activated by p53 negatively regulate cell growth while mdm2, in contrast, positively regulates cell growth. All p53 target genes contain versions of the consensus sequence, none of the sequences are identical nor are their locations similar. It can therefore be proposed that chromatin structure is one of the mediators of p53 recognition binding sites.

3.3.2 p53 protects sequences of the mdm2 P2 promoter *in vivo*

Using ligation mediated PCR *in vivo* footprinting, we have shown that protection of the p53 REs in the mdm2 gene occurred after a 4 hour temperature shift of the 3-4 cells. At this time point transcription of the mdm2 gene increased approximately 16 fold. Therefore, it was not surprising that the *in vivo* footprints also demonstrated protection of the TATA box region. It is clear that the pattern of protection at the TATA box region showed dramatic differences in the 3-4 cells maintained at 32°C for 4 hours when compared to the 3-4 cells maintained at 39°C or 37°C as well as when compared to 10-1 cells. A hypercutting site resulted (marked by *) which was also present when the naked DNA was treated with DNase I demonstrating that this area is protected at most other times (perhaps by nucleosome contact) and that in cells actively undergoing p53 transcriptional activation a nucleosome contact is disrupted. It is also evident that p53 is not constitutively bound to the p53-REs in the mdm2 P2 promoter while mediating transactivation because after the cells have been maintained at 32°C for 24 hours protection of the p53 REs is no longer observed.

3.3.3 Regulation of p53 DNA Binding

p53 consensus DNA consists of two functional half-sites (el-Deiry et al., 1992; Wang et al., 1995). These half sites can be separated by 0 to 13 bp but p53 binds separated half sites best when they are centered on the same face of the DNA helix (Wang et al., 1995). The mdm2 P2 promoter in both humans and mice contains two consensus p53-REs, each has two contiguous half sites (Zauberman et al., 1995). Interestingly, deletion of one of the mdm2 p53-REs virtually abolishes p53's ability to transactivate a reporter construct (Zauberman et al., 1995). It has been suggested that the

mdm2 P2 promoter region may contain two relatively weak p53 binding sites as a means of preventing premature activation (Zauberman et al., 1995). DNA binding by p53 oligomers and p53 mediated DNA looping are linked with transcriptional activation (Stenger et al., 1994). DNA looping at the mdm2 P2 promoter may be facilitated (and necessary) in the presence of the two p53-REs. Additionally, as suggested by the position of the p53-mdm2 oligonucleotide gel shift species, other proteins may associate with the looped p53-DNA complex. In addition to chromatin configuration facilitating p53 binding, another explanation for the difficulty in obtaining an *in vitro* footprint of the mdm2 p53 REs may be that p53 needs to associate with other proteins in order to efficiently bind to this region. TFIID and p53 bind cooperatively to DNA (Chen et al., 1993) and the mdm2 promoter may be an example of one instance where p53 will not bind efficiently unless TFIID is present. We are investigating this possibility.

The fact that the EMSA experiments with nuclear extracts from 3-4 cells maintained at 32°C for 24 hours showed some p53 dependent DNA binding while the dynamic p53-RE protection was no longer evident at 24 hours suggests that p53 may be tethered to the complex via association with other DNA bound proteins. This type of p53 binding could be confined to specific promoters that must be both up and down regulated as is suggested by the p53-mdm2 autoregulatory loop. Activation of mdm2 transcription by p53 increases MDM2 protein which in turn gives the cells a growth advantage by inactivating p53 function (Chen et al., 1996a; Chen et al., 1994). However in the case of the system used for these experiments the mdm2 protein must be maintained low enough for the cells to undergo a cell cycle arrest. Therefore regulatory mechanisms must exist to down regulate mdm2 expression while maintaining p53 activity because p53 activity is necessary to keep the 3-4 cells at 32°C growth arrested. If these cells are shifted back to 37°C they resume growth.

3.3.4 Hypersensitive sites formation

From the results of DNase I sensitivity assay, it is suggested that p53 is not involved in formation of nuclease-hypersensitive sites, but rather that the chromatin structure at the mdm2 promoter region in the inactive state is preset. The gene locus might be in a precise spatial alignment of DNA sequences, leading p53 to recruit TBP to this region at the permissive temperature. Alternatively TBP might be bound and p53 bending might then activate the TBP. The mechanisms leading to the formation, maintenance, and propagation of hypersensitive sites are not well understood. Nucleosome-free regions can be created by the DNA sequence itself or boundary constraints imposed by proteins that block nucleosome formation (Gross et al., 1993). For example, it has been shown that polypurine-polypyrimidine sequences which are often found in promoter regions and are known to "repel" histones *in vitro* (Kunkel and Martinson 1981). In addition, the evidence that deletion or substitution of HSE1 in yeast results in generating two sequence-positioned nucleosome in heat shock promoter directly demonstrates this sequence is necessary for formation of nucleosome-free regions (Gross et al., 1993). Furthermore, introduction of Drosophila hsp70 gene constructs into yeast has revealed that the HSTF-binding site is important for generating hypersensitivity on immediately adjacent 5'-sequences, even when these flanking sequences are of foreign origin (Gross 1988). It has been suggested that a hypersensitive site must be formed *de novo* during or after each replication cycle, since replication through a pre-formed transcription complex destroyed that complex, both *in vivo* and *in vitro*. One model is that the hypersensitive site is re-established during or immediately after replication, when a competition between nucleosome assembly and factor binding could be won by one or the other process (Boyes and Felsenfeld 1996). It has been

proposed that limited amounts of transcription factors would be titrated by the early replicating sequences and thereby provide a mechanism for specific chromatin structures, such as hypersensitive sites (Gross and Garrard 1988).

Chapter IV

A DNA damage signal is required for p53 to activate gadd45

4.1 Introduction

Given the importance of p53 DNA binding and the observation of additional proteins associating with p53, it is likely that the activity of protein is subject to multilevel regulation to correctly control p53 function at the correct time. In 3-4 cells, we detected the induction of the mdm2 gene concomitant with an increase in the p53 nuclear protein level. The assumption was made that the p53 protein level plays a role in controlling mdm2 gene activation. How p53 responds to DNA damage is not yet fully understood. DNA damage results in an increase in the p53 level and it is assumed that this p53 increase is at least in part responsible for the activation of the downstream genes. It has been difficult to dissect what causes DNA damage induced activation of p53 because DNA damage generally causes both an increase in the level of p53 as well as causing a signal transduction cascades which result in a number of p53 post-translational modifications (reviewed in Agarwal et al., 1998).

In our study, we utilized the tetracycline-regulated wild-type p53 expressing cell line TR9-7 to analyze p53-mediated transactivation in the presence and absence of DNA damage. The TR9-7 line is derived from the MDAH041 cell line. The MDAH041 line is a human fibroblast cell line from a patient of Li-Fraumeni syndrome which lacks functional p53 protein due to a frameshift mutation of one p53 allele at codon 184 and the normal p53 allele has been lost. However, MDAH041 cells behave very much like normal cells when functional p53 is restored under a normal p53 promoter, suggesting that the downstream functions are intact (Agarwal et al., 1995).

The TR9-7 cell line was constructed by Agarwal et. al. in the following way: the MDAH041 cells have been cotransfected with 2 plasmids (Fig. 4.1), one which contains

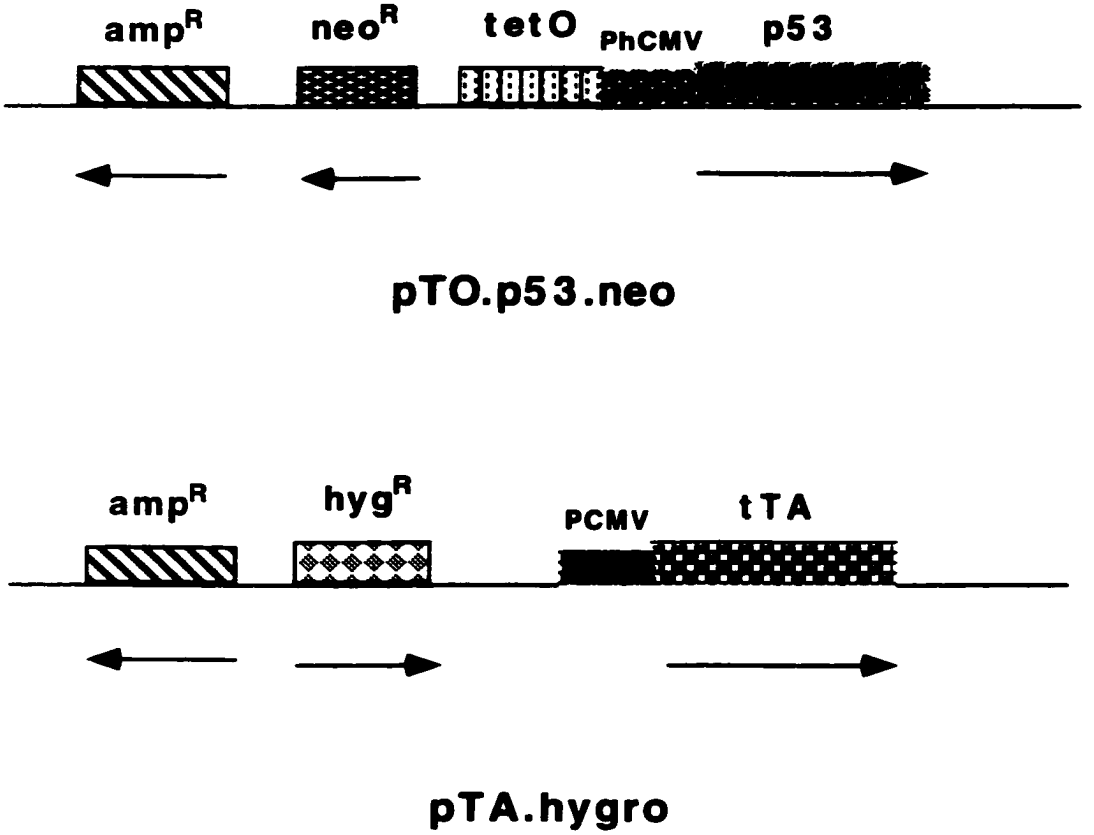


Figure 4.1: Schematic representation of a tetracycline-regulated system for expression of wild-type p53. pTO.p53.neo contains the wild-type p53 sequence under control of a derivative of the human cytomegalovirus promoter without the enhancer region. This minimal promoter is fused to a heptad of tetracycline operator. pTA.hygro contains the tetracycline-regulated transactivator (tTAs), driven by the entire cytomegalovirus promoter, including the enhancer region. amp^R, ampicillin resistance gene: neo^R, neomycin resistance gene; hyg^R, hygromycin resistance gene (Agarwal et al., 1995).

the wild-type p53 coding sequence under the control of human cytomegalovirus promoter without enhancer region. This minimal promoter is fused to a heptad of tetracycline operators (tetO). The other plasmid contains the tetracycline-regulated transactivator (tTA), driven by the entire cytomegalovirus promoter, including the enhancer region. The tetracycline-regulated transactivator was generated by fusing the tet repressor with activating domain of virion protein 16 of herpes simplex virus (Gossen and Bujard 1992). The transactivatior tTA produced in HeLa cells binds specifically to tetO sequences *in vitro* but this association is prevented by tetracycline. When bound to tetOs placed upstream of minimal promoters, tTA efficiently activates transcription from such promoters *in vivo* in a tetracycline-dependent manner (Gossen and Bujard 1992). In the presence of tetracycline tTA does not bind to its operator, and p53 can not be induced. After withdrawal of tetracycline, increased p53 can be detected in 2 hours and its expression continues to increase with time, reaching a peak at 20 hours (Agarwal et al., 1995). TR9-7 cells were used to study the p53 dependent cell cycle checkpoint. p21 was found to be activated by p53 after withdrawal of tetracycline. In addition, cell cycle analysis after induction of p53 revealed a marked reduction in S phase and an increase in both G1 and G2/M and this growth arrest is reversible (Agarwal et al., 1995). Because the induced p53 in this cell line is able to transactivate p21/Waf1 it has been assumed that this p53 also activates other p53 inducible genes, including gadd45 (Kastan et al., 1992). At the same time the activation of gadd45 by DNA damage has been attributed to in part to an increase in p53 levels (Agarwal et al., 1995).

Camptothecin is a topoisomerase I targeted DNA damaging agent. Topoisomerase I is involved in DNA transcription, DNA replication, and folding of the chromatin fiber to a compact form. It reduces torsional stress on DNA (Wang 1991). Topoisomerase I changes DNA topology by transiently breaking one strand of DNA,

passing a segment of DNA through this break, and finally resealing the break. It functions in relaxation of either negatively or positively supercoiled domains (Stryer 1988). Camptothecin functions by stabilizing the formation of transient complex between the enzyme and DNA and it inhibits the religation step of topoisomerase catalysis, thereby causing the accumulation of normally short-lived cleavage intermediate (Gobert et al., 1996). It has been shown wild-type p53 induction by topoisomerase-targeted drug requires replicative DNA synthesis and DNA strand breakage (Nelson et al., 1994).

A hypothesis exists that the specific transcriptional activity of p53 may be increased after DNA damage. Therefore it is important for a study to be done which would examine the activation of p53 target genes by constant p53 levels in both in the presence and absence of DNA damage in order to determine if combinatorial signal transduction pathways could modify the regulation of any p53 target genes. Such a study is presented here and shows that differences in the ability of p53 to activate gadd45 in the presence or absence of DNA damage was not dependent on a change in the nuclear protein level of the tetracycline-regulated over-expressed wild-type p53.

4.2 Results

4.2.1 Differential activation of p53 responsive genes, waf1, mdm2 and gadd45

The tetracycline-regulated wild-type p53 expressing cell line TR9-7 and its isogenic p53 minus part MDAH041 were used to analyze p53-mediated transactivation of waf1/p21, mdm2 and gadd45 in the presence and absence of DNA damage (Agarwal et al., 1995). It has been shown that increased p53 DNA binding activity was detected when fibroblasts were treated with 100 uM camptothecin for 4 hours by a time course

assay (Tishler et al., 1993). The RNA levels of the p53 target genes waf1, gadd45 and mdm2 in TR9-7 and MDAH041 cells under different conditions were examined by Northern blot analysis (Fig. 4.2). Barely detectable expression of the p53 target genes was observed in RNA samples derived from MDAH041 cells prior to drug treatment (Fig. 4.2, lane 1). No change was observed for waf1/p21 after camptothecin treatment in the absence of p53 while a slight increase was observed for both mdm2 and gadd45 (Fig. 4.2, lane 2). The activation resulting from the induced p53 or the induced p53 in the presence of camptothecin was examined using the inducible cell line TR9-7. Induction of p53 by the withdrawal of tetracycline caused a 5 fold increase for both waf1/and mdm2 RNAs above that seen in the controls (Fig. 4.2A & 1C, compare lanes 3 & 4). The waf1/p21 gene was activated to the same extent with or without the addition of camptothecin (Fig. 4.2A, lanes 4 & 5). Interestingly, in the TR9-7 cells, while no activation of gadd45 RNA was detected after tetracycline withdrawal, a 8.8 fold activation was observed when the induced cells were incubated with camptothecin (Fig. 4.2B, compare lanes 3, 4 & 5). In addition, the mdm2 gene was activated in a p53-dependent manner when tetracycline was removed (Fig. 4.2C, lane 4), but the mdm2 RNA level decreased when the cells were treated with camptothecin (Figure 4.2D, lane 5). A similar result showing inhibition of mdm2 in the presence of etoposide was recently reported (Arriola et. al., 1999).

4.2.2 p53 nuclear protein level does not correlate with transcription activity

Increased levels of nuclear p53 protein often result when cells are treated with DNA damaging agents (reviewed in Freedman et al., 1999). We examined the p53protein level in the presence and absence of drug in order to see if the differential

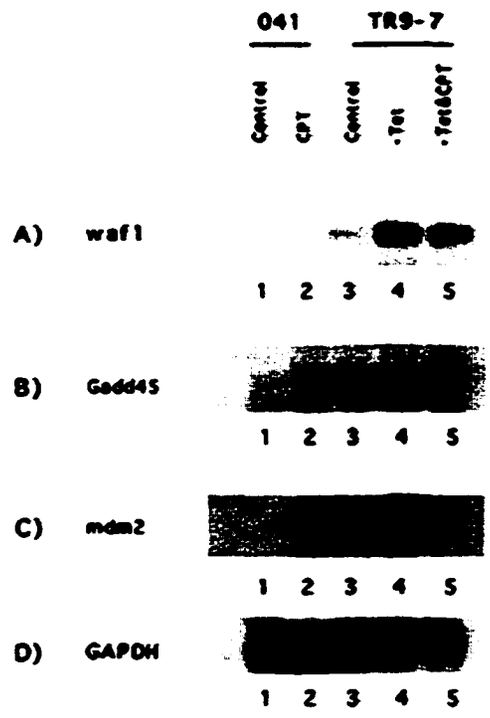


Figure 4.2: Differential activation of wt-p53 target genes occurs after camptothecin induced DNA damage. The RNA extraction was made from cells grown in media containing tetracycline (Control), after 24 hrs withdrawal of tetracycline (-Tet), or after 24 hrs withdrawal of tetracycline with overlapping 0.1 mM camptothecin treatment for the last 4hrs (-Tet & CPT). Northern blot analysis of the wt p53 responsive genes was carried out by separating 25 ug of cytoplasmic RNA in 1% formaldehyde-agarose gel and transferring to a nylon membrane. The blot was hybridized with full-length cDNA probes for waf1, gadd45, mdm2, and GAPDH as indicated. The results were reproducibly obtained in multiple blots using both mRNA and total cytoplasmic RNA. The signals were analyzed using a Molecular Dynamics PhosphoImager with Image Quant software. This experiment was repeated 3 times.

activation of the p53 responsive gene gadd45 corresponded to an increase in nuclear p53 (Fig. 4.3). The protein level was detected by Western blotting with the p53 specific monoclonal antibody PAb 240. Nuclear p53 protein was induced after the withdrawal of tetracycline for 24 hours and the protein level did not change after camptothecin treatment (Fig 4.3, lanes 4&5). Densitometer analysis of the Western blot clearly demonstrated that there was no significant change in the p53 level after camptothecin treatment. Therefore, it appeared that an increased level of p53 protein in the nucleus was not required for the p53 dependent camptothecin mediated induction of gadd45.

4.2.3 p53 is phosphorylated at serine 15 and acetylated at lysine 382 in response to camptothecin treatment and these modifications can be inhibited by wortmannin *in vivo*

Post-translational modification of p53 has been suggested as one of mechanisms that regulates p53 activity. Gadd45 can be activated by an ATM- and p53 -dependent mechanism mediated by the p53-RE in intron 3 (Kastan et al., 1992). *In vitro* studies have demonstrated that ATM phosphorylates p53 at a single residue, serine 15 (Banin et al., 1998; Canman et al., 1998). We analyzed the DNA damage induced phosphorylation status of p53 using the p53 specific anti-phosphoserine-15 antibody. The phospho-ser-15 antibody was raised against a chemically synthesized, KLH (Keyhole limpet hemocyanin) -conjugated phosphopeptide SVEPPLS(PO3)QETFSDC (amino acids 9-21). The affinity-purified αp53-P-ser-15 was then passed through a column conjugated with unphosphorylated peptide SVEPPLSQETFSD to deplete antibodies that recognize unphosphorylated p53 (Shieh et al., 1997). Specificity of the antibody was confirmed by ELISA with phosphorylated and unphosphorylated peptides representing the respective site. Similarly we analyzed the DNA damage induced

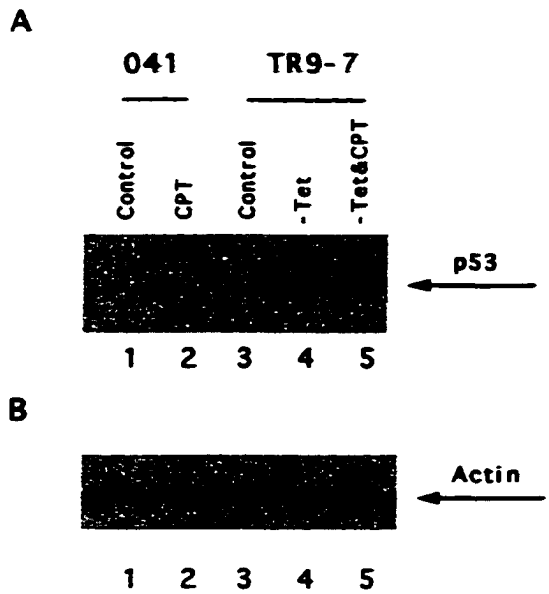
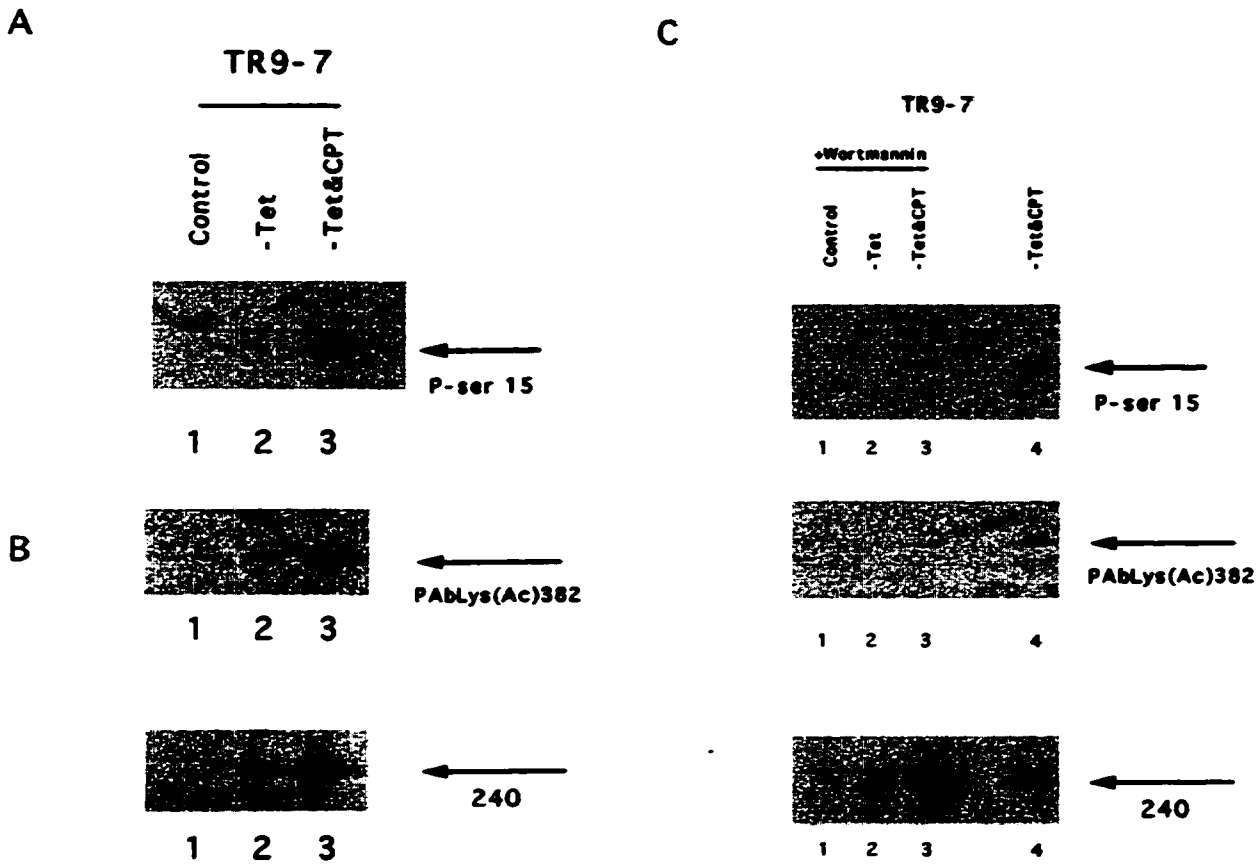


Figure 4.3: p53 protein levels in 041 and TR9-7 nuclear extract. (A) 100 ug of nuclear protein was resolved by electrophoresis on a 10% SDS-PAGE. The p53 in samples was visualized by Western blotting with the p53 specific monoclonal antibody PAb 240 and detected with ECL reagent. (B) The blot was reprobed with anti-Actin antibody as a control of loading. This experiment was repeated 3 times.

acetylation of p53 using a p53 specific anti-acetylated lysine 382 antibody. Western blot results demonstrated that p53 was phosphorylated at serine 15 only when camptothecin was added (Fig. 4.4A, lane 3). Likewise, acetylation at lysine 382 was induced after treatment with camptothecin (Fig. 4.4B). These results correlate differential post-translational modification at these amino acid residues with the differential activation of the gadd45 gene.

An inhibitor that could block phosphorylation of p53 was added to examine if phosphorylation of p53 at serine 15 was required for activation of gadd45. Previous *in vitro* studies have shown that wortmannin can inhibit phosphorylation of p53 at serine 15 by blocking both DNA-PK and ATM kinase activity (Shieh et al., 1997; Banin et al., 1998). Additionally, it has been reported that wortmannin inhibits actinomycin D induced activation of a p53 CAT reporter construct. This construct contained two copies of the p53 DNA-binding site from the murine creatine phosphokinase gene linked to a basal promoter and a CAT reporter gene (Price and Youmell, 1996). Wortmannin is a fungal metabolite that has been shown to act as a selective inhibitor of phosphoinositide 3-kinase family members. In particular, wortmannin can enter intact cells, making whole-cell studies of signaling pathways possible (reviewed in Ui et al., 1995). PI 3-kinase is a heterodimer of an 85 kD regulatory subunit (p85α, β) and a 110 kD catalytic subunit (p110α, β). The p85 subunit possesses two SH2 domains and one SH3 domain. Wortmannin binds irreversibly to the p110 catalytic subunit and inhibits its kinase activity (Yano et al., 1993). Wortmannin is a specific inhibitor of PI 3-kinase, but does not affect protein kinase C, cAMP or cGMP-dependent kinase, c-src, phospholipase C, or calmodulin-dependent protein kinase (Powis et al., 1994). The post-translational modification of p53 derived from cells grown in media containing wortmannin for the last 4 hours of growth prior to protein extraction was analyzed (Fig. 4.4C, lanes 1, 2 &3).

Figure 4.4: Wortmannin inhibits camptothecin-activated phosphorylation of p53 at serine 15 and acetylation at lysine 382 *in vivo*. (A) 100 ug of nuclear protein (same samples as Figure 2) was resolved by electrophoresis on a 10% SDS-PAGE. The phosphorylated serine 15 in samples was visualized by Western blotting with a specific antibody anti-phosphorylated-serine 15 and detected with ECL reagent. (B) The acetylated lysine 382 in samples was detected by Western blotting with a specific antibody anti-acetylated lysine 382. The nuclear protein extraction buffer contained 5 uM TSA. The blot was also probed with PAb240 to detect total p53. (C) Nuclear extract was made from the TR9-7 cells grown in media containing 5 uM wortmannin for the last 4 hours. 100 ug of nuclear extract was resolved by electrophoresis on a 10% SDS-PAGE and transferred to nitrocellous paper. The blot was probed with anti-phosphorylated serine15, anti-acetylated lysine 382 and PAb 240 as indicated. This experiment was repeated 2 times.



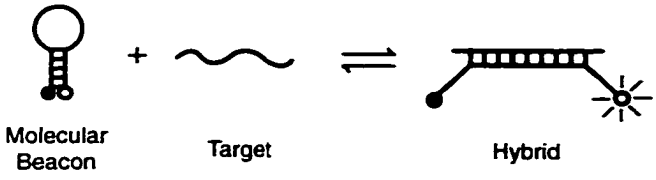
Wortmannin reduced both *in vivo* phosphorylation of serine 15 and acetylation of lysine 382 (Fig. 4.4C, compare lane 3 to 4). Wortmannin did not alter the induction or level of p53 in the TR9-7 cells (Fig. 4.4C, with antibody PAb240). It has been shown *in vitro* that acetylation of p53 at lysine 382 is dependent on N-terminal phosphorylation (Sakaguchi et al., 1998). Our results suggest that this is also the case *in vivo* and that camptothecin can induce this cascade.

4.2.4 Phosphorylation at serine 15 and acetylation at lysine 382 are not necessary to control p53 mediated activation of gadd45 expression

We investigated if there was any change in p53 mediated gadd45 gene expression when wortmannin was present since wortmannin reduced camptothecin induced phosphorylation of p53 at serine 15 and acetylation at lysine 382 (Fig. 4.5). Cytoplasmic RNA was extracted from TR9-7 cells with or without wortmannin for the last 4 hours of growth. Real-time PCR with a novel fluorescent molecular beacon designed for the gadd45 gene was used to investigate the levels of gadd45 mRNA in each sample because this method is more rapid and specific than Northern blot analysis (Tyagi and Kramer 1996; Manganelli et al., 1999) (Fig. 4.5). We also analyzed the camptothecin-induced activation of gadd45 using real-time RT-PCR. The mRNA level of gadd45 in the TR9-7 cells was observed not to increase when p53 was induced by of tetracycline. A 7 fold increase was detected however, when cells with induced p53 were treated with camptothecin (Fig. 4.5C). The addition of wortmannin did not inhibit the p53-mediated transcriptional activation of gadd45 (Fig. 4.5C). This result suggests that camptothecin induced DNA damage does not require the phosphorylation of p53 at serine 15 to effect the activation of gadd45. CPT induced damage activates a yet to be determined component of the p53-dependent signal transduction cascade that does not

Figure 4.5: Wortmannin does not block DNA damage induction of the gadd45 gene. Analysis of gadd45 mRNA expression was analyzed by real-time RT-PCR using specific sequence molecular beacons. (A) Molecular beacons are hairpin-shaped oligonucleotide probes that consist of a central part complementary to the target mRNA, flanked by two 6 bp inverted repeats that can form a stable stem. The 5' end of the beacon is coupled to a fluorophore, while the 3' end is coupled to a quencher. In the absence of the target, the stem is closed and the fluorophore is quenched, whereas in the presence of the target, an opened conformation allows the fluorophore to fluoresce. (B) The PCR was carried out in a spectrofluorometric thermal cycler that monitored the fluorescence in each reaction tube at the annealing stage of each thermal cycle. The 4 reactions shown in the left panel correspond to PCR done with increasing amounts of GAPDH-plasmid DNA (10^3 to 10^6 copies). The cycle at which the fluorescence signal becomes detectable above the background gives the threshold cycle (TC). An inverse relationship between the threshold cycle and the logarithm of the initial number of template was observed (right panel). (C) The reverse transcription reactions were carried out with 5ug of cytoplasmic RNA and 1/10 of the RT products were used in the PCR reactions specific for Gadd45 and GAPDH. The initial number of targets in each sample was calculated according to the TC. The results were normalized using the control samples and the GAPDH values to give relative units of mRNA induction. This experiment was repeated 3 times.

A.



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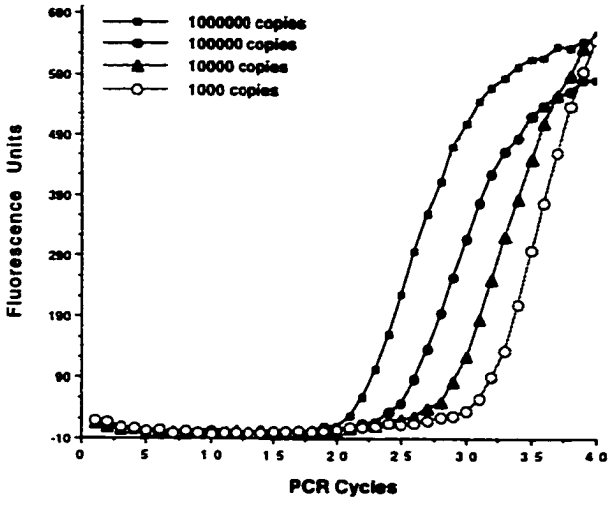

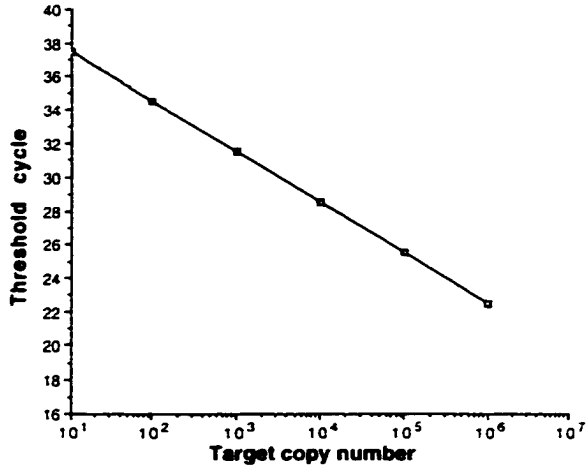

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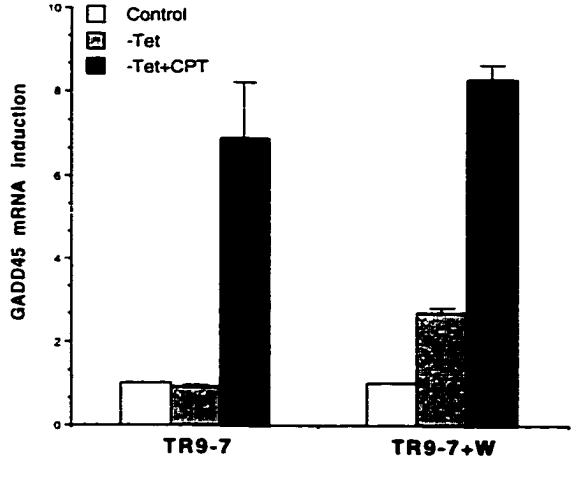
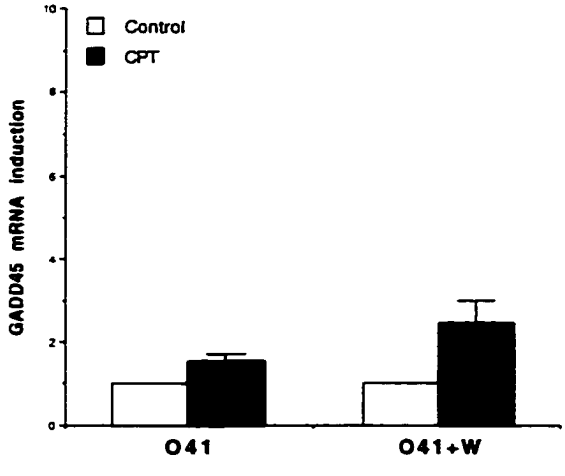


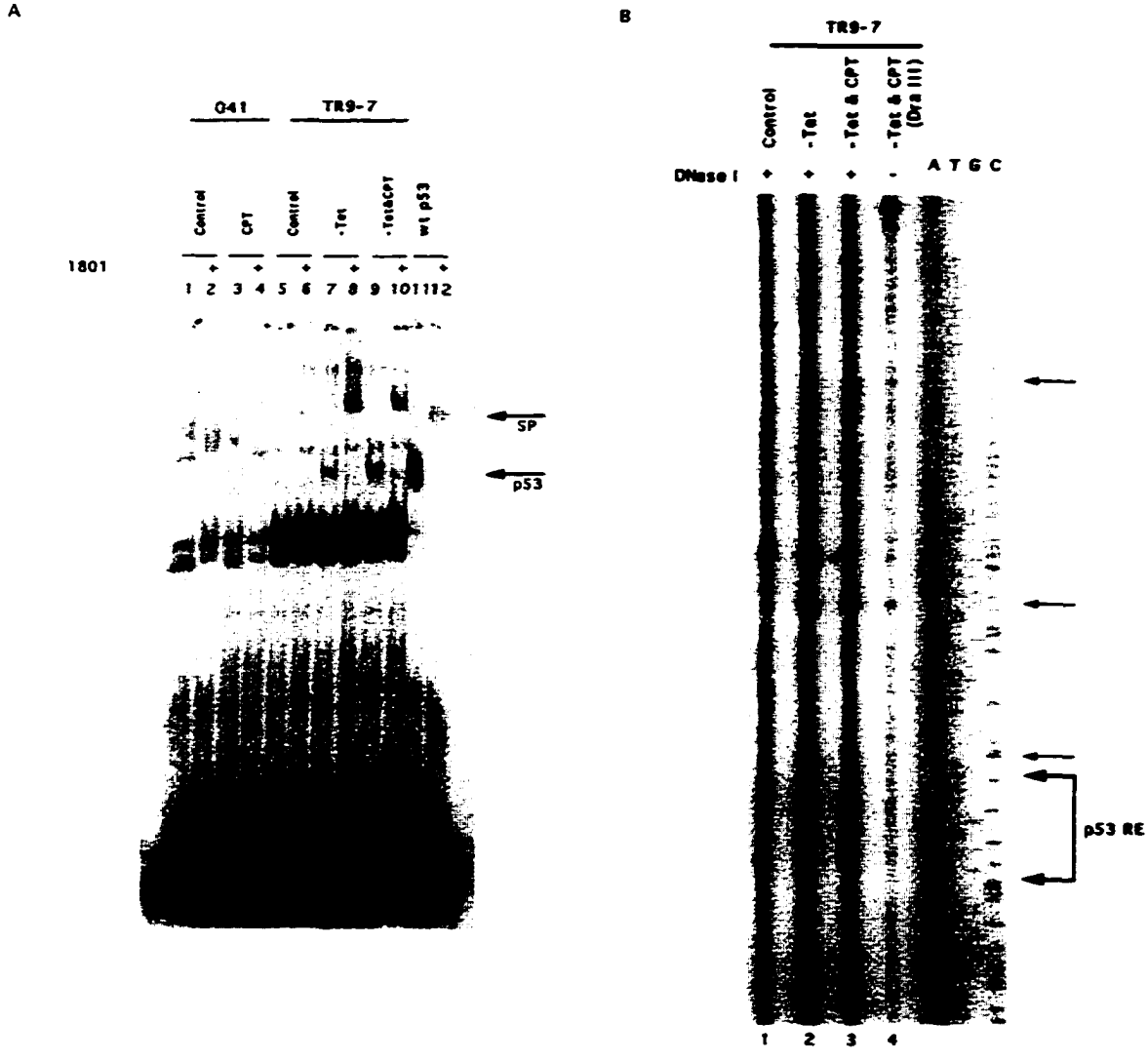


appear to be a PI 3-kinase family member. In fact the addition of wortmannin appeared to facilitate the ability of p53 to activate gadd45 in the absence of DNA damage suggesting that potentially there is a phosphorylation site on p53 that may inhibit the transactivation activity.

4.2.5 DNA binding by p53 was not stimulated by camptothecin treatment

It is believed that p53 activates its target genes in part by the interaction of p53 with its recognition sequences. In fact, *in vitro* studies have demonstrated that the acetylation of p53 at its C-terminus can stimulate the sequence-specific DNA binding activity of p53 (Gu and Roeder, 1997). To see if activation of p53 DNA binding resulted after camptothecin induced DNA damage, electrophoretic mobility shift assays (EMSAs) were carried out with an oligonucleotide containing the p53 super consensus site sequence (SCS) (Halazonetis et al., 1993). The same p53-dependent gel shift species was observed in both the -Tet and -Tet+CPT samples (Fig. 4.6A, indicated by p53 arrow). In addition the p53 specific antibody PAb1801 was able to supershift this protein-DNA complex, further indicating that this complex was p53 dependent (Fig. 4.6A, indicated by SP arrow). The EMSA results showed that p53 could bind to the SCS oligo both in the presence and absence of CPT (Fig. 4.6A, lanes 7-10). Interestingly, the p53 specific antibody PAb421 could not supershift the p53-DNA complex (data not shown) suggesting there was another modification at the C-terminus, such as phosphorylation by protein kinase C (Hupp et al., 1992). We also observed the loss of PAb421 reactivity by Western blot analysis. Since this loss of PAb421 reactivity was detected using denatured and immobilized p53, it can be attributed to a direct steric effect of an added phosphate (Takenaka, et al., 1995). Similarly, we did not observe increased p53 DNA binding ability to the gadd45 oligonucleotide after drug treatment. Although

Figure 4.6: p53 DNA binding activity was not enhanced after DNA damage. (A) The electrophoretic mobility shift assay was carried out at in the absence (lanes; 1, 3, 5,7, 9) or presence of p53 antibodies PAb 1801 (lanes; 2, 4, 6, 8, 10). (B) Genomic DNaseI footprinting was carried out on TR9-7 cells treated with 0.1ug of DNase I. The published putative p53-RE was identified by sequencing genomic gadd45 in a plasmid clone (lanes indicated as ATGC as indicated). Ligation mediated PCR was performed followed by primer extension with [^{32}P] labeled oligonucleotide #3 which hybridized approximately 150 bp downstream from the gadd45 putative p53 binding site. Purified DNA digested with Dra III from DNase I untreated nuclei of TR9-7 (-Tet & CPT) cells was shown in lane 6. Samples were electrophoresed on a 6% urea sequencing gel. This experiment was repeated 3 times.



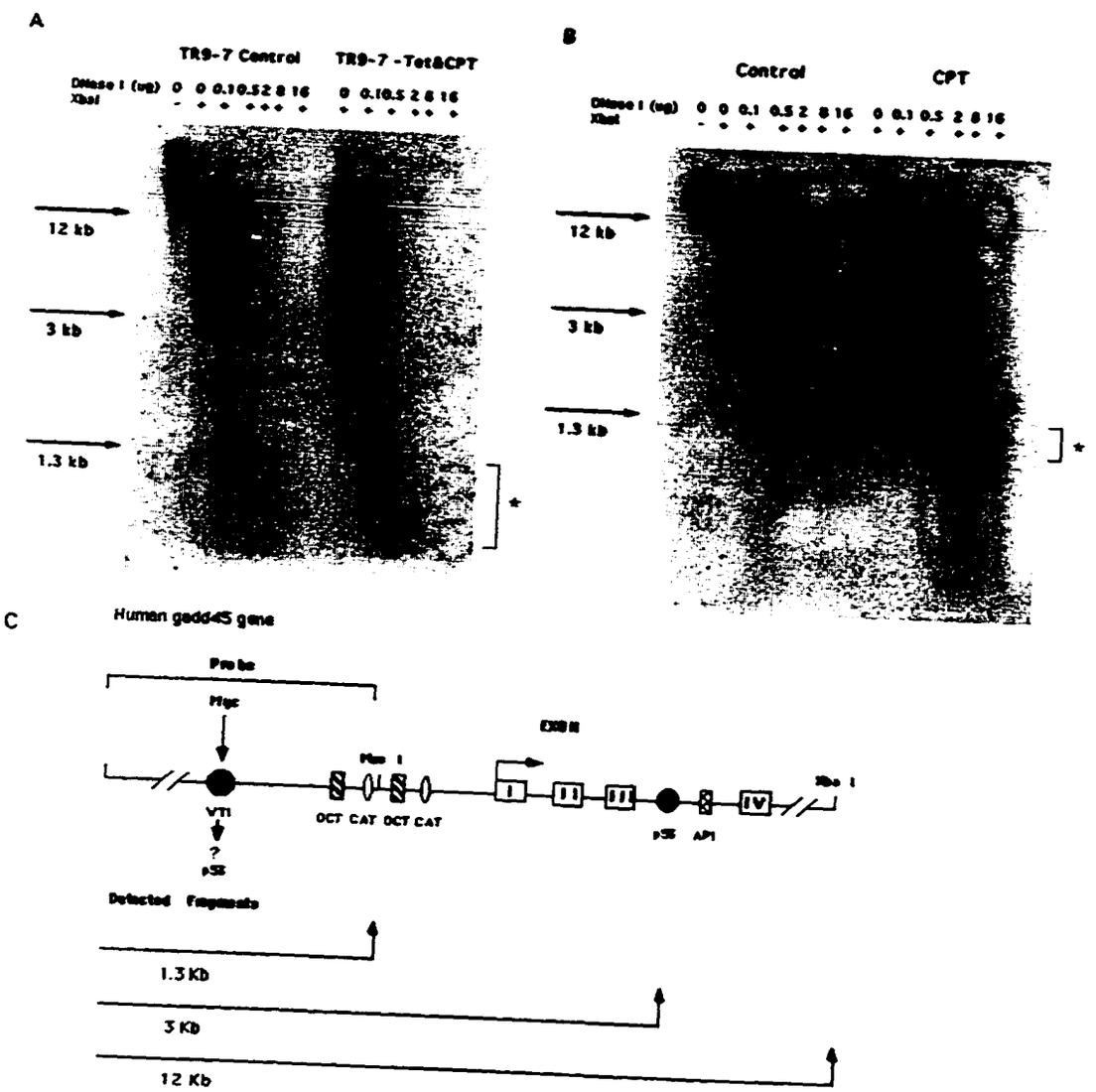
the p53 gel shift results were identical with both the SCS and gadd45 oligonucleotides, the level of background obtained when the gadd45 oligonucleotide was used was high and therefore the SCS results are shown as the representative sample.

Ligation mediated PCR genomic footprinting was performed to determine whether p53 bound to the gadd5 p53-RE *in vivo* and if binding changed upon CPT treatment. We have previously detected protection of the mdm2 p53-RE using this technique (Xiao et al., 1998). The putative p53 RE was identified by sequencing genomic gadd45 (Fig. 4.6B, lanes indicated as ATGC). There was no DNase I protection of the putative p53-RE observed when either the p53 was induced or when it was induced and activated by CPT (Fig. 4.6B, compare lanes 2 &3 to lane 1). Several DNase I hypercuting sites did emerge in a p53-dependent manner. (Fig. 4.6B, lanes 2&3, indicated by arrow) which suggests that p53 might transiently bind to the site and be involved in a modification of the nucleosomal structure in this area. Interestingly, no change in the hypercuting sites was observed when CPT was added. Camptothecin forms a ternary complex with the topoisomerase I on the DNA (Wang 1996a). Gadd45 appears to facilitate topoisomerase activity *in vivo* (Carrier et al., 1999). Therefore, technically we cannot rule out the possibility that there might have been camptothecin mediated DNA cleavage interfering with our DNase I footprints when camptothecin was present. Clearly no increased protection of the putative p53-RE in the gadd45 gene was detected upon activation of gadd45 transcription although protection of the mdm2 p53-REs were detected under these conditions.

4.2.6 Increased DNase I sensitivity was detected at the gadd45 promoter region when camptothecin was present

To determine whether remodeling of the gadd45 gene chromatin was associated with the gene activation Southern blot analysis using a probe to the far end of the expected fragment was carried out under various conditions (Fig. 4.7C). Selective, gene-specific changes in chromatin structure have emerged from DNase I mapping studies focused on the arrangement of nucleosomes around specific genes as a function of their state of transcriptional activity (reviewed in McKnight 1996). Previously we have shown that the p53-RE of the mdm2 gene is constitutively DNase I sensitive (Xiao et al., 1998). We screened for the appearance of DNase I hypersensitive sites at the promoter and the putative p53-RE of the gadd45 gene. The DNase I treated genomes isolated from nuclei of TR9-7 cells grown with or without camptothecin treatment were analyzed (Fig. 4.7A). Two constitutive DNase I hypersensitive sites were observed in the gadd45 gene. One hypersensitive site was observed 3 kb from the Xba I cutting site and corresponded to the putative p53 RE (Fig. 4.7, indicated by the 3 kb arrow). The other hypersensitive site was observed 1.3 kb from the Xba I cutting site and corresponded to the location of the gadd45 promoter region (Fig. 4.7, indicated by the 1.3 kb arrow). The overall DNase I sensitivity at the putative p53 binding sites did not change in the presence of camptothecin. However, the DNase I sensitivity at the gadd45 promoter region was increased when camptothecin was added to the TR9-7 cells (Fig. 4.7A, indicated by *). The 184A1 line is an immortalized human mammary epithelial cell line that contains wild type p53 (Lehman et al., 1993). Using the 184A1 cell line we could rule out the possibility that the increased DNase I sensitivity that resulted in the TR9-7 cells was not due to the artificial nature of the over-expressed p53. In the 184A1 cell line the p53 was only activated by the DNA damage induced by camptothecin, and once again the same

Figure 4.7: p53-mediated activation of the gadd45 gene occurs from a nucleosome free region. DNA in 2 x 10^6 isolated nuclei was digested with increasing amounts of DNase I (0 ug, 0.1 ug, 0.5 ug, 2 ug, 8 ug or 16 ug as indicated above each lane). (A) Nuclei were isolated from TR9-7 cells containing tetracycline (control) or after 24 hrs withdrawal of tetracycline with overlapping 0.1mM camptothecin treatment for the last 4hrs (-Tet&CPT). (B) Nuclei were isolated from 184A1 cells with (CPT) or without (Control) camptothecin for 4 hours. Purified DNA was restricted with XbaI and electrophoresed on 0.8 % agarose gel, probed with a [^{32}P] labeled XbaI-MscI genomic gadd45 probe fragment. (C) Schematic diagram of the genomic structure of the human gadd45 gene, showing known and putative transcription factor binding sites. The corresponding length detected by XbaI-MscI probing to the gadd45 promoter region or putative p53-RE is also shown. This experiment was repeated 2 times.



two constitutive DNase I hypersensitive sites were observed in the gadd45 gene with increased sensitivity at the gadd45 promoter resulting upon camptothecin treatment (Fig. 4.7B). It has been suggested that increased DNase I sensitivity can result as a consequence of the absence of a canonical nucleosome or alternatively it can result from binding of transcription factors which locally distort the DNA within or adjacent to a site (reviewed in Beato and Eisfeld, 1997). Several transcription factors have been found involved in the regulation of gadd45 gene expression through the promoter. Sequence analysis of the gadd45 promoter demonstrates a G-C rich region that contains a consensus sequence for one WT1 and three overlapping Egr-1 sites (Amundson, et al., 1999). It has been reported that this G-C rich region is necessary for p53 dependent activation of the gadd45 promoter through WT1 (Zhan et al., 1998). In addition, myc mediated repression of gadd45 also requires this G-C rich region (Amundson et al., 1998). Our results demonstrate that both the promoter and the putative p53-RE of the gadd45 gene are constitutively hypersensitive to DNase I, indicating that these two regions have accessible chromatin structures. In addition, when gadd45 was turned on, the promoter region appeared to be more dynamic than the putative p53 RE. This suggested that chromatin remodeling and differential association of transcription factors might been involved in gadd45 gene activation from the promoter when the DNA damage signal was present. The mdm2 P2 promoter is constitutively sensitive to DNase I but when p53 activates mdm2 transcription no increase in this sensitivity occurs (Xiao et al., 1998). Therefore, increased DNase I sensitivity at the gadd45 promoter suggests that p53 might function differently at various target genes. Chromatin remodeling may act as modulator to regulate gene expression of a subset of p53 inducible genes.

4.3 Discussion

4.3.1 p53 requires a damage signal to efficiently activate gadd45

DNA damage induces activation of p53 as a transcription factor and also causes the activation of a number of p53-responsive genes. The specific mechanism of how p53 is regulated to activate its many downstream target genes remains unclear. The general paradigm is that upon activation, p53 levels increase concomitant with changes in p53 post-translational modification. DNA damage induces p53 to activate gadd45 transcription but it has been difficult to dissect the type of p53 activation required because DNA damage generally causes both an increase in the level of p53 as well as a number of p53 post-translational modifications (reviewed in Agarwal et al., 1998). Interestingly, we have found that induced p53 in the absence of DNA damage does not significantly activate the gadd45 gene. On the other hand, both the p21/Waf 1 and mdm2 genes were activated. Transcriptional activation of the gadd45 gene required a signal induced by DNA damage and increased p53. Using the p53 inducible cell line TR9-7 we have demonstrated that the addition of the topoisomerase I-targeted DNA damaging agent CPT allowed for the rapid activation of gadd45 without an increase in the intra-cellular p53 level. It has previously been shown that an increase in p53 that occurs without DNA damage is unable to activate gadd45 (Hollander et al., 1993). Therefore, this phenomenon is not restricted to one cell type. We also demonstrated that CPT treatment of TR9-7 cells induced phosphorylation of p53 at serine 15 and acetylation at lysine 382. These changes correlated with the accumulation of gadd45 mRNA. Inhibition of these post-translational modifications by the addition of wortmannin, however, did not inhibit gadd45 activation. This is the first time that efficient gadd45 mRNA induction has been shown to require a

signal initiated by drug induced cellular DNA damage in addition to high levels of wild-type p53.

4.3.2 Are the p53 post-translational modifications induced by DNA damage necessary for gadd45 activation?

ATM kinase activity is increased after DNA damage and this causes phosphorylation of p53 at serine 15 *in vitro* (Banin et al., 1998; Canman et al., 1998). Phosphorylation of serine 15 in AT minus cells is delayed after IR (Siliciano et al., 1997). ATR and DNA-PK can phosphorylate p53 at both serine 15 and serine 37 *in vitro* (Canman et al., 1998; Tibbetts et al., 1999; Lees-Miller et al., 1992). Furthermore, it has been shown that phosphorylation of serine 15 *in vivo* is induced by IR, camptothecin (CPT) and UV irradiation (Shieh et al., 1997; Sciliciano et al., 1997). DNA damage induced phosphorylation at the N-terminus is known to act in two separate pathways. One works at the level of reducing the binding of MDM2 to the p53 protein (Shieh et al., 1997), which inhibits the ability of MDM2 to promote the degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997). The second is involved in increasing the ability of p53 to recruit CBP/p300, followed by increasing the overall level of acetylation of the C-terminus of p53 (Sakaguchi et al., 1998; Lambert et al., 1998). It has been suggested that DNA damage can activate p53 as a transcription factor through signaling for an N-terminal phosphorylation and C-terminal acetylation cascade (Sakaguchi et al., 1998). Gadd45 gene induction by IR is blocked by the protein kinase inhibitor H7, suggesting that gadd45 gene activation is mediated by a kinase (Papathanasiou et al., 1991). In our study inhibition of phosphorylation at serine 15 by wortmannin was observed not to inhibit the DNA damage induced accumulation of gadd45 mRNA. This observation suggests that blocking the phosphorylation of p53 serine 15 by either DNA-PK or ATM

is not sufficient to inhibit p53 mediated gadd45 gene activation. Previous studies have demonstrated that single mutations of individual N-terminal serines do not have significant effects on the p53 transactivation capacity (Fuchs et al., 1995). The possibility cannot be ruled out that wortmannin does not inhibit phosphorylation of the critical amino acids of p53 required for activation of the gadd45 gene. It will be interesting to investigate what effect inhibiting modification at other phosphorylation sites has on the regulation of gadd45 gene activation. The block of acetylation of p53 at lysine 382 also did not have an inhibiting effect on gadd45 gene activation. This suggests that the acetyltransferase activity of p300 associated with p53 may not be necessary to initiate gadd45 induction.

4.3.3 No increase in p53 DNA binding activity occurs co-incident with the activation of gadd45

Post-translational modification of p53 by acetylation is thought to disrupt the interaction between the C- terminal domain and the central domain of p53 (Gu and Roeder, 1997b). This may allow p53 to adopt an active conformation, which enhances the sequence-specific DNA binding activity of the protein. The p53 was acetylated at lysine 382 after camptothecin treatment. Increased DNA binding by p53 after camptothecin treatment was not observed. The increased DNA binding data, documented previously, was obtained by comparing latent bacterially or baculovirus produced p53 to *in vitro* acetylated p53 (Gu and Roeder 1997b; Sakaguchi et al., 1998). The observation that p53 in the TR9-7 cells was not reactive with PAb421 suggests that the protein was phosphorylated at the C-terminus (Takenaka, et al., 1995). *In vitro* phosphorylation of the p53 C-terminal region by protein kinase C can stimulate sequence-specific DNA binding ability while inhibiting PAb 421 reactivity (Hupp et al., 1992). In this way, the p53

protein in TR9-7 cells is conformationally different as compared to latent p53. The C-terminal modification of the p53 in the TR9-7 cell line may be involved in the inability of the p53 to activate gadd45 significantly in the absence of DNA damage. PAb421 reactivity did not emerge after camptothecin treatment, however, and is therefore not required for the activation of the gadd45 gene.

Direct DNA binding of p53 to the gadd45 putative p53-RE was not observed. This suggests that either direct p53 binding to the putative binding element is not necessary for p53 mediated activation or that the interaction of p53 with the gadd45 p53-RE is transient. This was in agreement with (Chin et al., 1997) a previous *in vivo* footprinting study that was unable to detect clear protection at the gadd45 p53-RE. The hyper-cutting seen in the footprinting analysis, that resulted when p53 was induced, strengthens the argument for a transient interaction of p53 with the gadd45 gene. The gadd45 putative p53 binding site was confirmed by comparing homology to a published p53 consensus sequence (Hollander et al., 1993) as well as testing in mobility shift assay, immunoprecipation and transient transfection assays (Kastan et al., 1992). It is possible that p53 may bind to a different region of the gadd45 gene or that p53 binds to the putative binding site prior to the 4 hour drug treatment time point that we examined. Another possibility is that the gadd45 p53-RE is only bound by p53 in a specific cell cycle stage. The TR9-7 cells with induced p53 undergo growth arrest at both G1/S and G2/M and therefore results in a mixed population of cells (Agarwal et al., 1995). If the p53 DNA binding assay were carried out at each cell cycle stage instead of in an exponentially growing population the answer of whether p53 is bound to its putative binding site would be more definitive. p53 can also participate in transcriptional induction of the gadd45 promoter in the absence of direct DNA binding (Zhan et al., 1998). Moreover, while some genotoxic stress does not require p53 to activate gadd45, p53 has

been shown to always have a cooperative activation effect (Zhan et al., 1993). Interestingly, p53 cooperates with WT1 as well as BRCA1 to activate the transcription of the gadd45 gene (Zhan et al., 1998; Harkin et al., 1999). Here barely detectable gadd45 induction by genotoxic stress was observed in the absence of p53, as well as barely detectable induction in the presence of p53 without genotoxic stress. Therefore, p53 must normally be cooperating with some other signal to initiate the rapid and robust activation of gadd45.

4.3.4 A change in DNase I sensitivity at the gadd45 promoter occurs during DNA damage induction

Constitutive DNase I hypersensitivity at the gadd45 promoter and the p53-RE were observed. This suggests that this gene is "pre-primed" for activation and that both these regions require a relaxed chromatin structure in order for the gene to function properly. The observation of increased accessibility for DNase I at the gadd45 promoter region upon DNA damage suggests that chromatin changes occur at the promoter region but the nature of these changes is unclear. It is possible that changes occur because repressing factors are released or because new factors are bound.

Activation of gadd45 expression occurs in a c-myc knockout cell line (Bush et al., 1998). The promoter of the gadd45 gene can be activated by p53 in the absence of the putative p53-RE when WT1 is present (Zhan et al., 1998). The G-C rich region of the promoter required for the WT1/p53 mediated activation is also required for myc mediated gadd45 repression (Amundson et al., 1998; Marhin et al., 1997). The gadd45 promoter lacks a TATA box (Kastan et al., 1992; Marhin et al., 1997). The TATA less promoter organization differs from that of many other p53 target genes, such as waf1,

mdm2 and bax, which may influence how gadd45 is regulated. It is possible that the activation of gadd45 by stress is effected through the release of c-myc repression in addition to p53 recruitment of basal transcription machinery in the absence of a TATA box. This would be reminiscent of the inducible lactose operon system in E. coli that is both positively and negatively regulated.

4.3.5 Differential expression of p53 inducible genes

Mdm2 transcription is reduced by the topoisomerase poison etoposide (Arriola et al., 1999). We have demonstrated that reduction of mdm2 expression occurs after camptothecin treatment as well. Arriola et al. (1999) proposed that mdm2 expression may be inhibited by bulky adduct damage to the template since both the P1 and P2 promoters of mdm2 are DNase I hypersensitive. However, this suggestion is not consistent with our observation that the gadd45 promoter (which is also constitutively DNase I hypersensitive) is turned on in the presence of camptothecin. The model proposed by Wu and Levine (1997) is prefered where DNA damage induces a repressor specific for mdm2, which will inhibit mdm2 transcription. It should be noted that to fully analyze p53 transcriptional activity, the analysis of one target gene (or one means of p53 activation) is not enough. Co-ordinate repression and activation may work to differentially regulate the complex pattern of expression of p53 target genes. In an inducible BRCA1 system, gadd45 induction is coincident with BRCA1 expression (Harkin et al., 1999). This suggests that BRCA1 may be a more powerful activator of gadd45 than p53. Additionally, DNA damage may activate endogenous BRCA1 so that it readily cooperates with p53 to activate gadd45 transcription. Many hypothesis for the mechanisms of p53 mediated gadd45 gene activation remain to be tested. The phosphorylation-acetylation cascade involving p53 post-translational modifications at

serine 15 and lysine 382 does not appear to be critical for the regulation of p53-mediated activation of gadd45 although it may be critical for other functions of p53. Gadd45 has recently been reported to bind and activate an upstream regulator of JNK/SAPK thus triggering JNK/SAPK-dependent apoptosis (Takekawa and Saito, 1998; Harkin et al., 1999). When p53 is induced by the withdrawal of tetracycline in TR9-7 cells it directs the cells to undergo G1/S and G2/M arrest (Agarwal et al., 1995). Therefore it is not surprising that no detectable activation of gadd45 was observed in cells with induced p53. It is possible that in the presence of CPT a program directing the cells towards apoptosis is turned on. This might necessitate the activation of gadd45.

Chapter V

Summary and Further Directions

5.1 Summary

The work that is reported in this thesis has provided strong evidence leading to the following statements. p53 binds specifically to the murine mdm2 P2 promoter in nuclear chromatin and this binding correlates with mdm2 endogenous mRNA induction. The mdm2 P2 promoter is maintained in a nucleosome free state and activation of the mdm2 gene does not require chromatin remodeling. Additionally, the p53 target genes p21, gadd45, and mdm2 are differentially activated in the presence and absence of DNA damage. Both induced p53 and DNA damage signaling are necessary to activate the gadd45 gene. While the mdm2 gene is activated more efficiently in the absence of DNA damage the p53-mediated gadd45 induction by camptothecin did not require a further increase in nuclear p53 levels, or DNA binding activity, or phosphorylation of p53 at serine 15 and acetylation of p53 at lysine 382. Finally, both the gadd45 promoter and its putative p53-RE are constitutively DNase I hypersensitive detected by indirect Southern blot analysis, but after camptothecin treatment increased DNase I sensitivity was only detected at the gadd45 promoter region.

5.1.1 p53 binds specifically to the mdm2 P2 promoter in nuclear chromatin.

To investigate the *in vivo* DNA binding activity of p53, we carried out ligation-mediated *in vivo* footprinting in two cell lines, a p53-null cell line (10-1) and a temperature-sensitive p53-Val135 overexpressing line (3-4). At 32°C, p53-Val135 assumes a wild-type conformation while at 37°C, this p53 is conformationally mutant. p53-dependent DNase I protection at the mdm2 P2 promoter region was compared at 32°C and 37°C in 10-1 and 3-4 cells. We observed p53-dependent protection of the 2 putative p53-REs as well as the adjacent TATA box when 3-4 cells were shifted to 32°C

for 4 hours. No changes were observed between the protection patterns at p53 RE-1 or p53 RE-2 in 3-4 cells maintained at 37°C. Northern blot analysis indicated that a simultaneous increase of mdm2 mRNA occurred with binding of p53 to its RE in nuclear chromatin. In addition, protein analysis and gel-shift experiments demonstrated that the p53-dependent DNase I protection detected by genomic footprinting of the p53-REs correlated with an increase in the nuclear p53 level and the p53 DNA binding activity. Interestingly the protection pattern with purified p53 on naked DNA was not as large or clear as that detected by genomic footprinting. Therefore, these results suggested that the chromatin structure of the mdm2 gene organizes the P2 promoter region into a sequence, which acts as an optimal p53 DNA binding site.

5.1.2 The mdm2 P2 promoter is maintained in a nucleosome free state and activation of the mdm2 gene does not require chromatin remodeling.

Indirect Southern blot analysis revealed constitutive nuclease-hypersensitivity at both the P1 and P2 promoters of the mdm2 gene, indicating that both promoters are located in altered chromatin conformations that are most likely nucleosome free. Using a micrococcal nuclease sensitivity assay further proved the constitutive nuclease-hypersensitivity at the mdm2 P2 promoter. Although p53 binding to the P2 promoter region in chromatin was demonstrated, no overall nuclease sensitivity change was observed at the corresponding time point. These data indicate that an altered chromatin structure exists at the mdm2 P2 promoter and that the binding of p53 does not significantly influence the overall chromatin structure of this region.

5.1.3 p53 target genes are differentially activated in the presence and absence of DNA damage, and both induced p53 and DNA damage signaling are necessary to activate gadd45.

We have utilized the tetracycline-regulated wild-type p53 expression cell line TR9-7 to investigate the p53-mediated transcription profile of gadd45, p21 and mdm2 in the presence and absence of DNA damage. When TR9-7 cells are grown in the presence of tetracycline the p53 protein is expressed at a very low level. Increased p53 protein can be detected by Western blot analysis after the removal of tetracycline. Northern blot analysis was used to demonstrate that when p53 was induced in the absence of DNA damage, p21 and mdm2 mRNA levels were greatly increased while a change in the gadd45 mRNA level was barely detectable. However, an increased gadd45 mRNA level was observed after adding the topoisomerase I-targeted DNA damage agent camptothecin (CPT). However, in the absence of p53 camptothecin did not significantly activate the gadd45 gene. In addition, mdm2 mRNA levels decreased upon DNA damage. We also analyzed the camptothecin specific activation of gadd45 using real-time PCR with a molecular beacon for gadd45. We observed a 7 fold activation of gadd45 in the presence of camptothecin.

5.1.4 p53-mediated gadd45 induction by camptothecin does not require a further increase in nuclear p53, DNA binding activity, or phosphorylation of p53 at serine 15 and acetylation of p53 at lysine 382.

To investigate the mechanism of activation of the gadd45 gene mediated by p53 and camptothecin, we have analyzed the possibilities of increasing p53 stability, specific p53 post-translational modifications and p53 DNA binding activity. Western blot

analysis indicated that the addition of camptothecin did not cause a further increase in the p53 nuclear protein level. After CPT treatment, phosphorylation of p53 at serine 15 and acetylation at lysine 382 were detected, however inhibition of the modifications of serine 15 and lysine 382 by wortmannin *in vivo* was not sufficient to block the transactivation of gadd45 gene. Additionally, no overall increase in p53 DNA binding activity resulted after camptothecin treatment.

5.1.5 Chromatin remodeling was observed at the gadd45 promoter region when the gadd45 gene was turned on.

Using ligation mediated PCR genomic footprinting we were unable to detect direct DNA binding at the putative p53 binding site. Interestingly, constitutive DNase I hypersensitivity was detected by indirect Southern blot analysis at both the gadd45 promoter and the putative p53 RE, but after camptothecin treatment increased sensitivity was only detected at the gadd45 promoter region. These data suggest that p53 requires a damage signal to induce changes at the gadd45 promoter which enable p53 to activate transcription. In addition, the observed chromatin remodeling at the gadd45 promoter region may be essential for p53 to activate gadd45 transcription.

5.2 Further Directions

The work described in this thesis has laid a solid foundation for continuous work in the following two areas: (1) to characterize the protein complexes that associate at the mdm2 P2 promoter region *in vivo*, (2) to analyze the molecular basis of p53-mediated gadd45 activation at the gadd45 promoter.

5.2.1 To characterize the protein complexes that associate at the mdm2 P2 promoter region *in vivo*

We have demonstrated that p53 is able to bind to its intronic binding sites of mdm2 *in vivo* and induce mdm2 gene expression from the P2 promoter. In addition, we have also shown that the mdm2 P2 promoter is constitutively nucleosome free. However, the specific mechanism of how p53 functions to activate the mdm2 gene when it binds to its REs remains unclear. Binding of the basal transcriptional machinery complex to the promoter is required for many eukaryotic cells gene expression. Therefore, regulation of this binding is an important determinant of promoter activity. Whether p53 is able to recruit the basal transcriptional machinery complex to the mdm2 P2 promoter and enhance transcription will be investigated. We have observed that nuclear extracts from 3-4 cells maintained at 32°C for 4 hours migrated slower than the purified p53-DNA complex in the EMSA assays with the mdm2 oligonucleotide. This observation suggests that additional proteins might associate with p53 in 3-4 cells when it binds to DNA. The putative components of this p53 complex may be either the transcriptional machinery recruited by p53 to the mdm2 P2 promoter or factors that are involved in modifying the p53 DNA binding activity. In addition, nuclear proteins that associate at this region may lead to the formation and maintenance of the mdm2 P2 promoter as a nucleosome free region. Hence, a logical step would be to characterize the protein complex that associates with the mdm2 P2 promoter region in 10-1 and 3-4 cells maintained at 32°C for 4 hours.

a. To prove the recruitment of basal transcriptional machinery by p53 to the mdm2 promoter region *in vivo*

We have observed that a change in protection at the TATA box region in 3-4 cells shifted to 32°C for 4 hours in the *in vivo* footprinting experiment. But whether this TATA-box footprint is actually due to TBP binding is still not definitive. Recently, using the chromatin immunoprecipitation (CHIP) within formaldehyde in situ cross-linked cells, specific proteins that bind to interested region of chromatin have been detected (Cosma et al., 1999). The chromatin is sonicated to an average DNA size of 0.5-1 kb in cross-linked cells. Specific DNA fragments immunoprecipitated by specific antibody from whole cells are quantified by PCR. Using either anti-p53 or anti-TBP antibody we will be able to determine the order of p53 and TBP binding to the promoter region of the mdm2 gene (Kuras and Struhl 1999).

Another method to test the increased recruitment of TBP by transcription activators to a TATA box *in vivo* was reported by Klein and Struhl in 1994. A TBP derivative with altered TATA-element binding ability was used to detect the rate of initiation of transcription from a chromatin template (Klein and Struhl 1994). The activity of TBP derivative (TBP^{m3}) is distinguished from the endogenous wild-type TBP by its ability to permit transcription from a promoter containing a mutated TATA element (T<u>G</u>TAAA). The expression of TBP^{m3} is regulated by an inducible promoter such as a copper inducible promoter. Therefore, the construct containing the T<u>G</u>TAAA element and either lacking or possessing the p53 REs of the mdm2 gene along with the luciferase reporter gene will be introduced to 3-4 cells along with the inducible TBP^{m3} construct. The co-transfected cells will be grown at 37°C for 2 days and shifted to 32°C for 4 hours along with inducing TBP^{m3} by adding copper. The luciferase activity will be measured

from cells either lacking or possessing the p53-REs of mdm2 gene. The luciferase activity after induction of TBP^{m3} is thought to reflect the rate at which TBP productively associated with the chromatin template *in vivo*.

b. To identify a factor that is able to assemble mdm2 P2 promoter into a positioned nucleosome

In tumors with overexpression of mdm2 along with wild-type p53, reduction of the mdm2 gene expression could be an important goal for cancer therapy. If the nucleosome disrupting factor can be regulated by either a dominant-negative mutant or injection of a specific antibody to form a positioned nucleosome at the mdm2 promoter, the transcription of mdm2 might be repressed accordingly. Another approach will be to find and overexpress a chromatin assembly factor that can help in forming a positioned nucleosome at the mdm2 promoter. It has been shown that the ATP-utilizing chromatin assembly and remodeling factor (ACF) was purified from Drosophila embryo extracts (Ito et al., 1997). The *in vitro* chromatin assembly reaction was carried out with relaxed plasmid DNA, purified core histones, ATP, purified core histone chaperone and chromatin assembly factor. The extent and quality of chromatin assembly were determined by using micrococcal nuclease digestion and DNA supercoiling assays. The method that was performed for ACF purification can be utilized in 10-1 and 3-4 cell lines to find a factor that is able to assemble the mdm2 P2 promoter without a positioned nucleosome. Nuclear proteins will be separated by SP Sepharose, Q Sepharose, P11 Phosphocellulose columns and tested for nucleosome assembly activity. The pattern of micrococcal nuclease digestion on protein-nucleosome complex will be used to demonstrate the activity of assembly of nucleosome. The fractions containing nucleosome-assembly activity will be loaded onto a hydroxyapatite column. The peak

fractions from hydroxyapatite column are subjected to glycerol gradient sedimentation followed source 15Q chromatography and POROS heparin chromatography. Each fraction will be subjected to micrococcal nuclease digestion assay.

5.2.2 To analyze the molecular basis of p53-mediated gadd45 activation at the gadd45 promoter

We have shown that a DNA damage signal was required for p53-dependent gadd45 gene activation. According to our DNase I sensitivity assay, the promoter region of the gadd45 gene appears to be the one of the controlling elements of p53-mediated gadd45 activation because an increased DNase I sensitivity was detected at the gadd45 promoter region upon CPT treatment. It should be noted that the activity of the p53 transcription factor is profoundly influenced by co-activator and co-repressor proteins that associate with p53 (reviewed in Prives and Hall, 1999). A gadd45 promoter deletion assay has demonstrated that 253 bp upstream to the transcriptional start site was required for p53-dependent gadd45 gene expression in response to DNA damage (Zhan et al., 1998). Interestingly, the gadd45 promoter lacks both a TATA box and a pyrimidine-rich initiator element (Inr^-) (Marhin et al., 1997). It has been suggested that, for $TATA^-Inr^-$ promoters, activators bound to either promoter proximal or distal enhancer elements could recruit TFIID to the promoter, and support transcription initiation from a multitude of start sites (reviewed in Burley and Roeder 1996). Sequence analysis indicated that several putative transcription factor binding sites were located in this region, including a G-C rich region which contains a novel 20 bp symmetrical motif with two perfect overlapping WT1-EGR1 consensus sequences (Zhan et al., 1998). Two OCT sites and two CAT sites are located within this upstream region (Hollander et al., 1993). Synergistic activation by transcriptional co-activators has been found in many cases. In

addition, it has been shown that myc can repress endogenous gadd45 gene activation (Marhin et. al., 1997; Bush et al., 1998), therefore we would like to investigate the hypothesis that the activation of gadd45 by DNA damage is effected through the release of myc repression.

In this study, we have examined the relationship between camptothecin mediated gadd45 gene expression and the phosphorylation status of p53. Another aspect of these interrelated pathways that has not been addressed is whether different type of DNA damage cause differential adaptive responses of p53. DNA damage agents cause DNA damage by a variety of mechanisms. IR causes DNA double-strand breaks via free radical formation or direct damage to the DNA while UV induces bulky lesions in DNA including pyrimidine dimers (reviewed in Agarwal et al., 1998). Actinomycin D and Adriamycin are DNA intercalators which inhibit topoisomerases. Etoposide is an inhibitor of topoisomerase II. 5-Fluorouracil can incorporate into DNA and inhibit DNA synthesis. Alkylating agents mitomycin C (MC) and cisplatin act as DNA cross-linking agents (Tishler, et al., 1993). It has been reported that both DNA interstrand and intrastrand cross-linked adducts are formed readily upon exposure of DNA to MC (Tomasz and Palom 1997). Interestingly, one analogue of MC, decarbamoyl mitomycin C (DMC) only produces DNA monoadducts and is unable to form cross-links (Kim and Rockwell, 1995). By using these two drugs we will be able to determine whether DNA cross-links are necessary for the induction of the gadd45 gene or if the bulky chemical-DNA adduct which results in both cases are sufficient. In the absence of DNA crosslinkimg it is assumed that double strand DNA breaks do not occur and DMC is less toxic than MC (Kim and Rockwell, 1995). It is known that cells from AT patients are defective in their response to IR, but show a normal response to UV radiation (Wang et al., 1999). In addition, it has been shown that although phosphorylation of p53 at serine

20 is rapidly induced by IR, its induction by UV is significantly delayed (Shieh, et al., 1999). Phosphorylation of p53 at serine 392 is induced by UV and not IR (reviewed in Giaccia and Kastan, 1998). Detection of bulky lesions including pyrimidine dimers is independent of ATM (Dumaz et al., 1997). All these observations suggest different DNA damage lead to activation of different signal transduction pathways that activate of p53. It will be essential to understand how p53 integrates upstream signaling pathways emanating from diverse genotoxic stresses. Do these different pathways culminate in activation of the same set of p53 target genes? We will test to see what drugs in addition to camptothecin allow for the activation of gadd45.

a. To determine a transcriptional co-activator of p53 for up-regulation of gadd45 gene

We will perform *in vitro* DNase I footprinting assays with nuclear extracts from the 041 and TR9-7 cells treated with different drugs eliciting different downstream gadd45 activator as an initial effort to distinguish possible proteins binding to this region when the gadd45 gene is induced in a p53-dependent manner upon DNA damage. Another attempt to understand the activator of gadd45 will utilize reporter constructs. Reporter constructs which contain site-specific mutations at the different putative binding sites will be made and transiently transfected into TR9-7 cells both in the presence and absence of DNA damage. The relative activity of each reporter will be able to help elucidate the control factor that regulates the p53 mediated gadd45 gene induction.

b. To test the possibility that sequestration of the negative regulatory factor c-myc, by p53 upon DNA damage, will lower the inhibitory threshold of gadd45

p53 is frequently mutated, deleted, or inactivated in a variety of tumors and tumor cell lines that exhibit myc deregulation or amplification. Several examples are the myeloid leukemia cell line HL60, which has amplification of the c-myc gene and deletion of p53; small cell lung carcinomas, which have amplification of L- or c-myc and p53 mutations; and Burkitt lymphomas and pre-B and T cell acute lymphoblastic leukemias, which have deregulation of c-myc and p53 mutations (Wagner, et al., 1994).

The molecular mechanism of myc mediated repression of the gadd45 promoter is still unknown. Some repressors inhibit activated transcription by competing with activators for overlapping DNA-binding sites. Other repressors interact with activators to mask activation domains or compete with basal transcription factors for interaction. Sequence analysis of the gadd45 promoter revealed that its regulatory region lacks canonical myc binding sites (Marhin et al., 1997). However, myc may interact with the promoter region indirectly through protein-protein interactions since it has been reported that WT1 and p53 can form a complex and as such bind to WT1-binding sites (Zhan et al., 1998). The association of protein complexes at the gadd45 promoter region *in vivo* can be used for analyzing the regulation of the gadd45 gene by p53. In addition, the Myc protein may compete with p53 for binding with WT1 at the gadd45 promoter region. It is not clear whether WT1 can form a complex with the Myc protein. Therefore, a co-immunoprecipitation assay with anti-WT1 antibody will be performed with nuclear extracts from TR9-7 cells to estimate the ability of the complex formation. In order to examine the extent of Myc protein binding to the gadd45 promoter region in the presence and absence of DNA damage, an EMSA assay using a WT1 consensus sequence will be

carried out with nuclear extracts from TR9-7 cells in the presence and absence of DNA damage. It has been shown that this G-C rich WT1 consensus sequence of the gadd45 promoter required for the WT1/p53 mediated activation is also required for myc mediated gadd45 repression (Amundson et al., 1998; Marhin et al., 1997). The anti-myc antibody-mediated supershift will be used as an indicator of the myc-DNA complex. The anticipated results will be a reduced association of Myc protein to this G-C rich region when gadd45 mRNA accumulates in a p53-dependent manner upon DNA damage. This competitive DNA binding may work in the same way as p53 blocking the SV40 large T antigen replication function by preventing T antigen from binding to the viral replication origin (Gannon and Lane 1987; Wang et al., 1989; Friedman et al., 1990; Bargonetti et al. 1991).

These are just a few ideas for project that extend the findings of this work. Many questions will continue arise as answers emerge. The road to understanding the complex regulation of p53 in the all is long, and continuing to travel it should lead to many new discovers.

Chapter VI

Appendix

6.1 Statistics Analysis

6.1.1 p53 nuclear protein levels in 3-4 cells

39C	37C	32C 2hr	32C 4hr	32C 24hr
1	1.47	0.96	1.83	0.48
1	1.07	1.5	2.01	1.28
1	0.99	0.67	1.52	0.39

Descriptive statistics

Column1		*Column2*		*Column3*		*Column4*	
Mean	1	Mean	1.176667	Mean	1.043333	Mean	1.786667
Standard E	0	Standard E	0.148474	Standard E	0.243196	Standard E	0.143101
Median	1	Median	1.07	Median	0.96	Median	1.83
Mode	1	Mode	#N/A	Mode	#N/A	Mode	#N/A
Standard D	0	Standard D	0.257164	Standard D	0.421228	Standard D	0.247857
Sample Va	0	Sample Va	0.066133	Sample Va	0.177433	Sample Va	0.061433
Kurtosis	#DIV/0!	Kurtosis	#DIV/0!	Kurtosis	#DIV/0!	Kurtosis	#DIV/0!
Skewness	#DIV/0!	Skewness	1.545393	Skewness	0.85541	Skewness	-0.762695
Range	0	Range	0.48	Range	0.83	Range	0.49
Minimum	1	Minimum	0.99	Minimum	0.67	Minimum	1.52
Maximum	1	Maximum	1.47	Maximum	1.5	Maximum	2.01
Sum	3	Sum	3.53	Sum	3.13	Sum	5.36
Count	3	Count	3	Count	3	Count	3

39C vs. 37C
t-Test: Paired Two Sample for Means

	Variable 1	*Variable 2*
Mean	1	1.176667
Variance	0	0.066133
Observatio	3	3
Pearson Co	#DIV/0!	
Hypothesiz	0	
df	2	
t Stat	-1.189885	
P(T<=t) one	0.178095	
t Critical on	2.919987	
P(T<=t) two	0.356191	
t Critical tw	4.302656	

39C vs. 32C 2hr
t-Test: Paired Two Sample for Means

	Variable 1	*Variable 2*
Mean	1.043333	1.176667
Variance	0.177433	0.066133
Observatio	3	3
Pearson Co	-0.016001	
Hypothesiz	0	
df	2	
t Stat	-0.464645	
P(T<=t) one	0.343931	
t Critical on	2.919987	
P(T<=t) two	0.687862	
t Critical tw	4.302656	

39C vs. 32C 4hr
t-Test: Paired Two Sample for Means

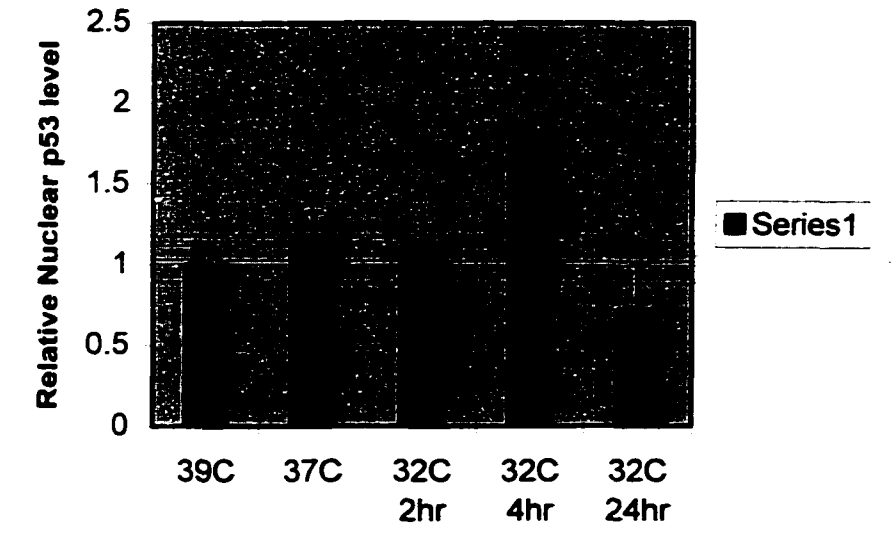
	Variable 1	*Variable 2*
Mean	1	1.786667
Variance	0	0.061433
Observatio	3	3
Pearson Co	#DIV/0!	
Hypothesiz	0	
df	2	
t Stat	-5.497299	
P(T<=t) one	0.015767	
t Critical on	2.919987	
P(T<=t) two	0.031534	
t Critical tw	4.302656	

39C vs. 32C 24hr
t-Test: Paired Two Sample for Means

	Variable 1	*Variable 2*
Mean	1	0.716667
Variance	0	0.240033
Observatio	3	3
Pearson Co	#DIV/0!	
Hypothesiz	0	
df	2	
t Stat	1.001665	
P(T<=t) one	0.211005	
t Critical on	2.919987	
P(T<=t) two	0.422009	
t Critical tw	4.302656	

Column5	
Mean	0.716667
Standard E	0.282862
Median	0.48
Mode	#N/A
Standard D	0.489932
Sample Va	0.240033
Kurtosis	#DIV/0!
Skewness	1.666528
Range	0.89
Minimum	0.39
Maximum	1.28
Sum	2.15
Count	3

39C	37C	32C 2hr	32C 4hr	32C 24hr
1	1.176667	1.043333	1.786667	0.716667



6.1.2 mdm2 mRNA induction upon temperature shift in 3-4 cells

37C	32C 4hr
1	16
1	14
1	16

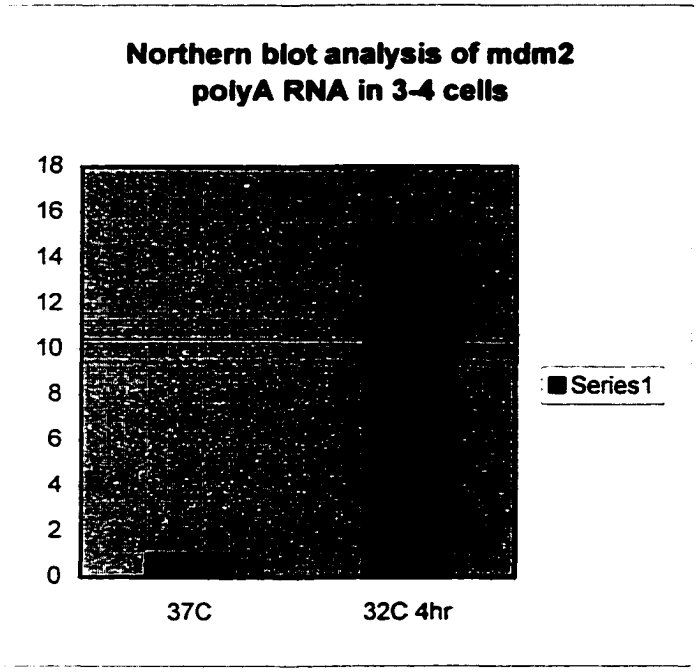
Descriptive statistics

Column1		*Column2*	
Mean	1	Mean	15.33333
Standard Error	0	Standard Error	0.666667
Median	1	Median	16
Mode	1	Mode	16
Standard Deviation	0	Standard Deviation	1.154701
Sample Variance	0	Sample Variance	1.333333
Kurtosis	#DIV/0!	Kurtosis	#DIV/0!
Skewness	#DIV/0!	Skewness	-1.732051
Range	0	Range	2
Minimum	1	Minimum	14
Maximum	1	Maximum	16
Sum	3	Sum	46
Count	3	Count	3

t-Test: Paired Two Sample for Means

	Variable 1	*Variable 2*
Mean	1	15.33333333
Variance	0	1.333333333
Observations	3	3
Pearson Correlation	#DIV/0!	
Hypothesized Mean	0	
df	2	
t Stat	-21.5	
P(T<=t) one-tail	0.001078	
t Critical one-tail	2.919987	
P(T<=t) two-tail	0.002156	
t Critical two-tail	4.302656	

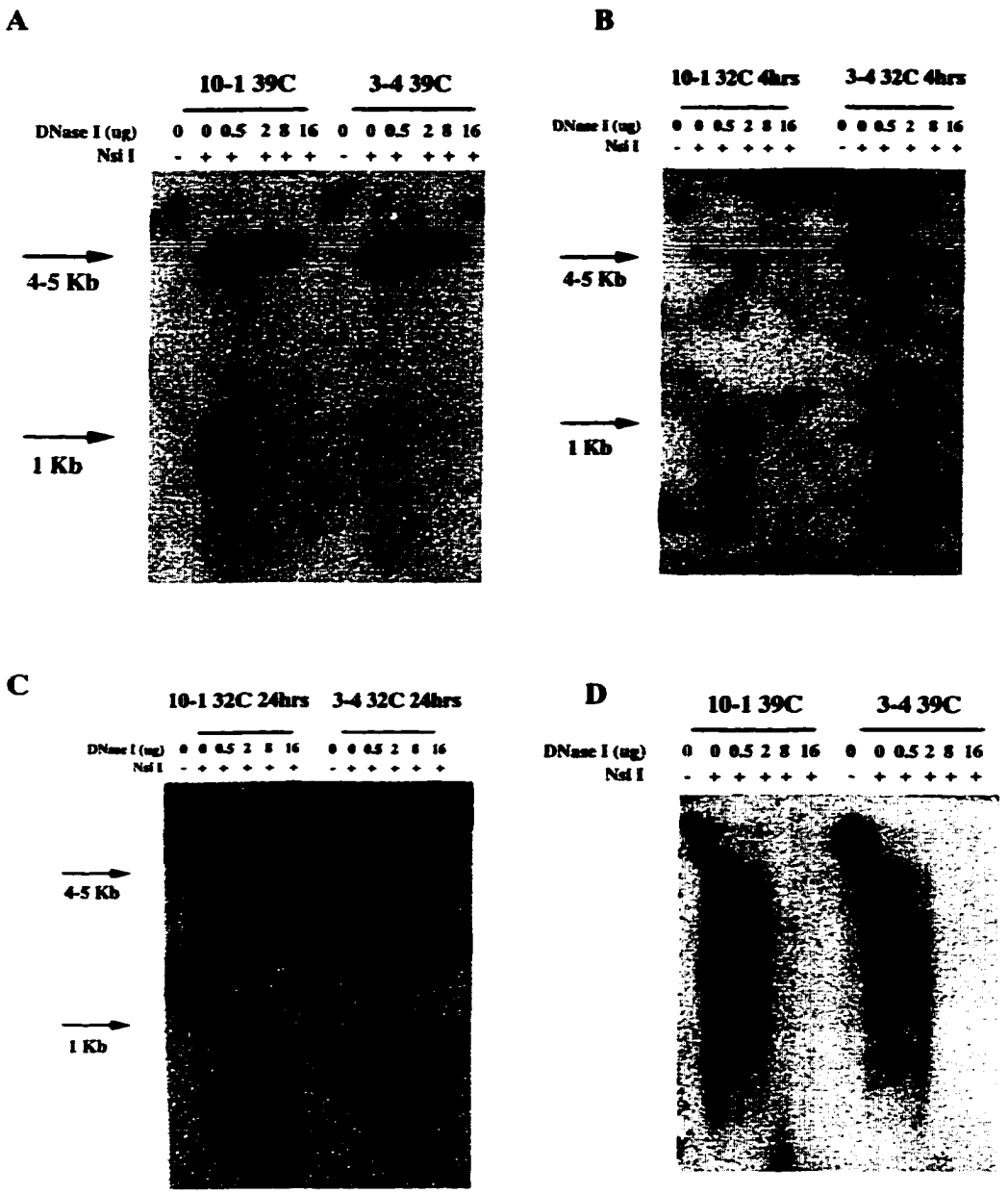
37C	32C 4hr
1	15.33333



6.2 Supplement Figures

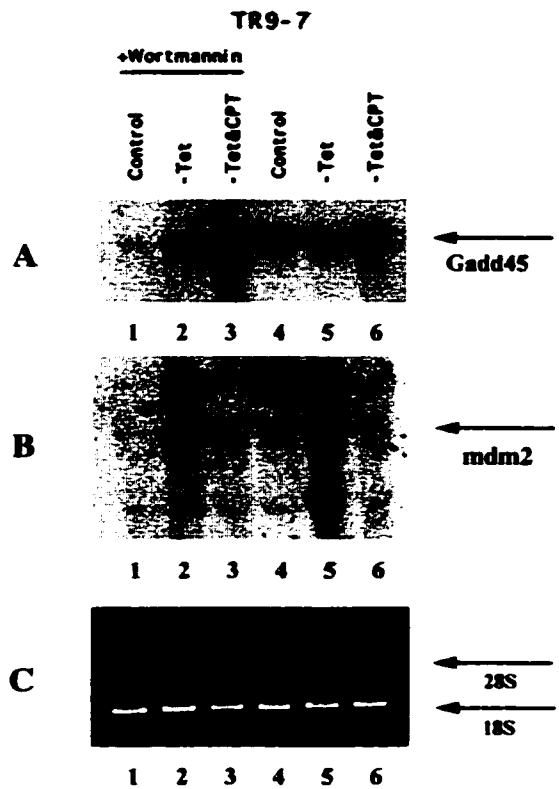
6.2.1 Murine mdm2 P2 promoter region is resistance to DNase I digestion at 39°C

DNA in 2 x 10^6 isolated nuclei was digested with increasing amounts of DNase I (0 ug, 0.5 ug, 2 ug, 8 ug or 16 ug as indicated above each lane). Nuclei were isolated from 10-1 and 3-4 cells maintained at 39°C (A), 32°C for 4 hours (B) or 32°C for 24 hours (C). Purified DNA was restricted with Nsi I and electrophoresed on 0.8 % agarose gel, probed with a [^{32}P] labeled Apa I-Nsi I genomic mdm2 probe fragment (A, B & C). The arrows indicate the hypersensitive sites in the mdm2 gene. (D) The blot of 39°C was reprobed with GAPDH as a control of loading.



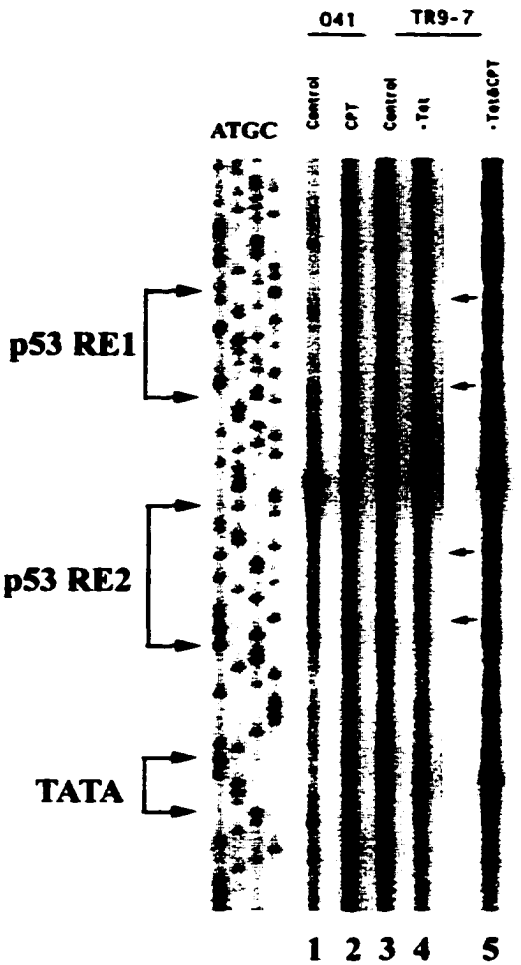
6.2.2 Northern blot analysis shows wortmannin does not inhibit the DNA damage induced accumulation of gadd45 mRNA

The RNA extraction was made from cells grown in media with or without wortmannin. Northern blot analysis of the gadd45 mRNA was carried out by separating 25 ug of cytoplasmic RNA in 1% formaldehyde-agarose gel and transferring to a nylon membrane. The blot was hybridized with full-length cDNA probes for gadd45 (A) and mdm2 (B). (C) The total RNA of each sample was shown by the ethidium stained agarose gel.



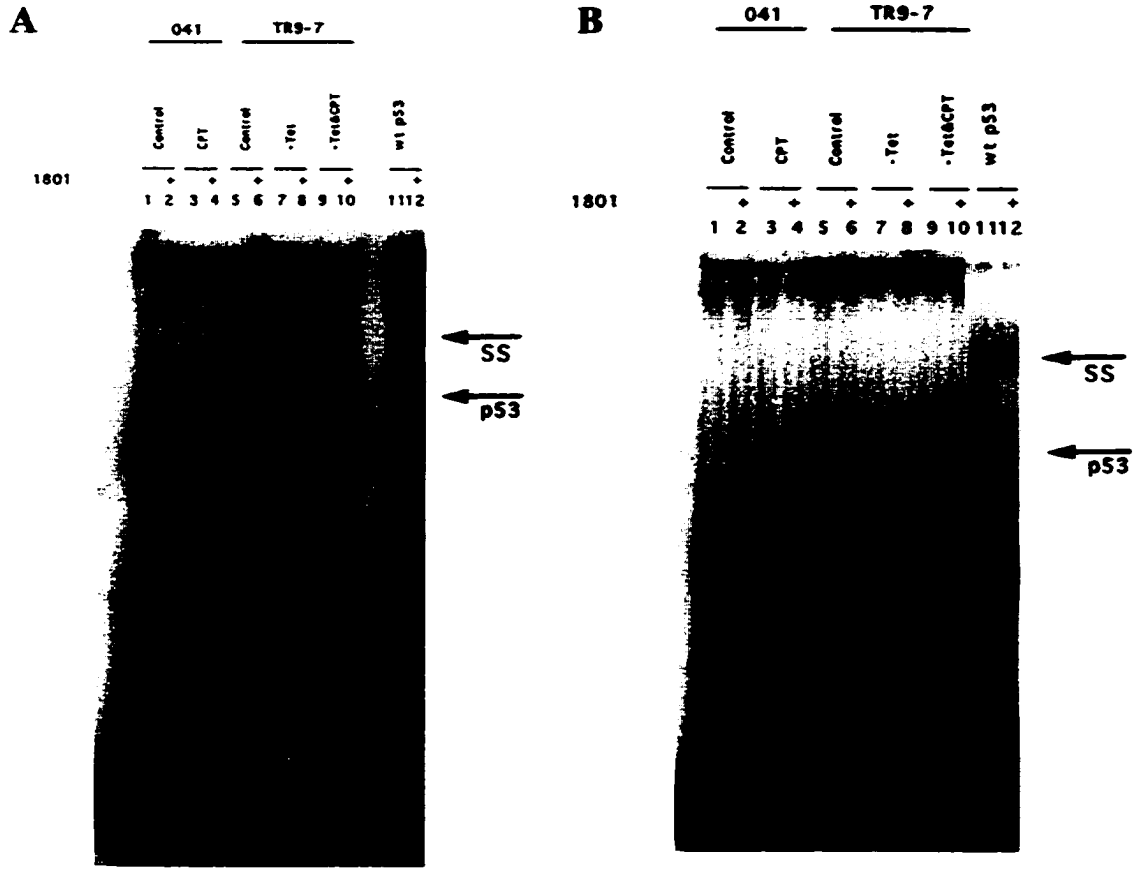
6.2.3 LM-PCR footprints at mdm2 P2 promoter region in 041 and TR9-7 cells

The published p53-REs and adjacent TATA box were identified by sequencing genomic mdm-2 in a plasmid clone (lanes indicated as ATGC) and are indicated on the left. DNA in 2 $x10^6$ isolated nuclei from 041 and TR9-7 cells was digested with 0.1ug of DNase I and purified.



6.2.4 EMSA of gadd45 and mdm2 oligonucleotide with 041 and TR9-7 nuclear extracts in the presence of PAb 1801

The electrophoretic mobility shift assay was carried out at in the absence (lanes; 1, 3, 5,7, 9) or presence of p53 antibodies PAb 1801 (lanes; 2, 4, 6, 8, 10) on either gadd45 oligo (A) or mdm2 oligo (B).



Chapter VII

References

Agarwal, M. L., Agarwal, A., Taylor, W. R., and Stark, G. R. (1995). p53 controls both G2/M and G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proc. Natl. Acad. Sci. USA **92**, 8493-8497.

Agarwal, M. L., Taylor, W. R., Chernov, M. V., Chernova, O. B., and Stark, G. R. (1998). The p53 network. J. Biol. Chem. **273**, 1-4.

Amundson, S. A., Zhan, Q., Penn, L. Z., and Fornace Jr., A. J. (1998). Myc suppresses induction of the growth arrest genes gadd34, gadd45, and gadd153 by DNA-damaging agents. Oncogene **17**, 2149-2154.

Arriola, E., Lopez, A. R., and Chresta, C. M. (1999). Differential regulation of $p21^{waf-1/cip-1}$ and Mdm2 by etoposide: etoposide inhibits the p53-mdm2 autoregulatory feedback loop. Oncogene **18**, 1081-1091.

Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., vanTuinen, P., Ledbetter, D. H., Barker, D. F., Nakamura, Y., White, R., and Vogelstein, B. (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science **244**, 217-221.

Banin. S., Moyal, L., Shieh, S. Y., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science **281**, 1674-1677.

Barak, Y., Juven, T., Haffner, R., and Oren, M. (1993). mdm2 expression is induced by wild type p53 activity. EMBO J. **12**, 461-468.

Barak, Y., Gottlieb, E., Juven-Gershon, T., and Oren, M. (1994). Regulation of mdm2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. Genes & Dev. **8**, 1739-1749.

Bargonetti, J., Friedman, P. N., Kern, S. E., Vogelstein, B., and Prives, C. (1991). Wild-type but not mutant p53 immunopurified protein binds to sequences adjacent to the SV40 origin of replication. Cell **65**, 1083-1091.

Bargonetti, J., Manfredi, J. J., Chen, X., Marshak, D. R., and Prives, C. (1993). A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein. Gene & Dev. **7**, 2565-2574.

Bates, S., and Vousden, K. H. (1999). Mechanisms of p53-mediated apoptosis. Cell. Mol. Life Sci. **55**, 28-37.

Beato, M. and Eisfeld, K. (1997). Transcription factor access to chromatin. Nucleic Acids Res. **25**, 3559-3563.

Bischoff, J. R., Friedman, P. N., Marshak, D. R., Prives, C., and Beach, D. (1990). Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. Proc. Natl. Acad. Sci. U. S. A **87**, 4766-4770.

Boyes, J. and Felsenfeld, G. (1996). Tissue-specific factors additively increase the probability of the all-or-none formation of a hypersensitive site. EMBO J. **15**, 2496-2507.

Brain, R., and Jenkins, J. R. (1994). Human p53 directs DNA strand reassociation and photolabelled by 8-azido ATP. Oncogene **9**, 1775-1780.

Buckbinder, L., Talbott, R., Valesco-Miguel, S., Takenaka, I., Faha, B., Seizinger, B. R., and Kley, N. (1995). Induction of the growth inhibitor IGF-binding protein 3 by p53. Nature **377**, 646-649.

Burley, S. K., and Roeder, R. G. (1996). Biochemistry and structural biology of transcription factor IID (TFIID). Annu. Rev. Biochem. **65**, 769-799.

Bush, A., Mateyak, M., Dugan, K., Obaya, A., Adachi, S., Sedivy, J., and Cole, M. (1998). c-myc null cells misregulate cad and gadd45 but not other proposed c-myc targets. Genes & Dev. **12**, 3797-3802.

Caelles, C., Heimberg, A., and Karin, M. (1994). p53 dependent apoptosis in the absence of p53-target genes. Nature **370**, 220-223.

Cahilly-Snyder, L., Yang-Feng T., Francke U., and George, D. L. (1987). Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. Somat. Cell Mol. Genet. **13**, 235-244.

Canman, C. E., Lim, D, Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M., and Siliciano, J. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science **281**, 1677-1679.

Carrier, F., Smith, M. L., Bae, I., Kilpatrick, K. E., Lansing, T. J., Chen, C-Y., Engelstein, M., Friend, S. H., Henner, H. D., Gilmer, T. M., Kastan, M. B., and Fornace Jr., A. J. (1994). Characterization of human Gadd45, a p53-regulated protein. J. Biol. Chem. **269**, 32672-32677.

Carrier, F., Georgel, P. T., Pourquier, P., Blake, M., Kontny, H. U., Antinore, M. J., Gariboldi, M., Myers, T. G., Weinstein, J. N., Pommier, Y., and Fornace Jr., A. J. (1999). Gadd45, a p53-reponsive stress protein, modifies DNA accessibility on damaged chromatin. Mol. Cell. Biol. **19**, 1673-1685.

Chen, C., Oliner, J. D., Zhan, Q., Fornace, A. J. Jr., Vogelstein, B., and Kastan, M. B. (1994). Interactions between p53 and MDM2 in a mammalian cell cycle checkpoint pathway. Proc. Natl. Acad. Sci. USA **91**, 2684-2688.

Chen, J., Wu, X., Lin, J., and Levine, A. J. (1996). mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. Mol. Cell. Biol. **16**, 2445-2452.

Chen, P. L., Chen, Y., Bookstein, R., and Lee, W. H. (1990). Genetic mechanisms of tumor suppression by the human p53 gene. Science **250**, 1576-1579.

Chen, S., Marechal, V., and Levine, A. J. (1993). Mapping of the p53 and mdm-2 interaction domains. Mol. Cell. Biol. **13**, 4107-4114.

Chen, X., Farmer, G., Zhu, H., Prywes, R., and Prives, C. (1993). Cooperative DNA binding of p53 with TFIID (TBP): a possible mechanism for transcriptional activation. Genes & Dev. **7**, 1837-1849.

Chen, X., Bargonetti, J., and Prives, C. (1995) p53, though p21(WAF1/CIP1), induce cyclin D1 synthesis. Cancer Research **55**, 4257-4263.

Chen, X., Ko, L. J., Jayaraman, L., and Prives, C. (1996). p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. (1996). Genes & Dev. **10**, 2438-2451.

Chin, P.L., Momand, J. and Pfeifer, G. P. (1997). *In vivo* evidence for binding of p53 to consensus binding sites in the p21 and Gadd45 genes in response to ionizing radiation. Oncogene **15**, 87-99.

Cho, Y., Gorina, S., Jeffery, P. D., and Pavletich, N. P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science **265**, 346-355.

Clore, G. M., Omichinski, J. G., Sakaguchi, K., Zambrano, N., Sakamoto, H., Appella, E., and Gronenborn, A. M. (1994). High-resolution structure of the oligomerization domain of p53 by multimensional NMR. Science **265**, 386-391.

Cosma, M. P., Tanaka, T., and Nasmyth, K. (1999). Ordered recruitment of transcription and chromatin remodeling factors to a cell-cycle and developmentally regulated promoter. Cell **97**, 299-311.

Cox, L. S., Hupp, T., Midgley, C. A., and Lane, D. P. (1995a) A direct effect of activated human p53 on nuclear DNA replication. EMBO **14**, 2099-2105.

Cox, L. S. and Lane D. P. (1995b). Tumor suppressors, kinases and clamps: how p53 regulates the cell cycle in response to DNA damage. BioEssays **17**, 501-508.

Cross, S. M., Sanchez, C. A., Morgan, C. A., Schimke, M. K., Ramel, S., Idzerda, R. L., Raskind, W. H., and Reid, B. (1995). A p53-dependent mouse spindle checkpoint. Science 267, 1353-1356.

de Laat, W. L., Jaspers, N. G. J., and Hoeijmakers, J. H. J. (1999). Molecular mechanism of nucleotide excision repair. Genes & Dev. **13**, 768-785.

de Stanchina, E., McCurrach, M. E., Zindy, F., Sjhieh, S Y., Ferbryre, G., Samuelson, A. V., and Prives, C., Roussel, M. F., Sherr, C. J., and Lowe, S. (1998). E1A signaling to p53 involves the p19(ARF) tumor suppressor. Genes & Dev. **12**, 2434-2443.

Dang, C. V., and Lee, W. M. F. (1989). Nuclear and nucleolar targeting sequences of c-erb-A, c-myb, N-myc, p53, HSP70, and HIV tat proteins. J. Biol. Chem., **264**, 18019-18023.

Deng, C., Zhang, P., Harper, J W., Elledge, S J., and Leder, P. (1995). Mice lacking $p21^{CIP/WAF1}$ undergo normal development, but are defective in G1 checkpoint control. Cell **82**, 675-684.

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A. J., Butel, J. S., and Bradely, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. Nature **356**, 215-221.

Dumaz, N., Duthu, A., Ehrhart, J. C., Drougard, C., Appella, E., Anderson, C. W., May, P., Sarasin, A., and Daya-Grosjean, L. (1997). Prolonged p53 protein accumulation in trichothiodystrophy fibroblasts dependent on unpaired pyrimidine dimers on the transcribed strands of cellular genes. Mol. Carcinog. **20**, 340-347.

Dutta, A., Ruppert, J. M., Aster, J. C., and Winchester, E. (1993). Inhibition of DNA replication factor RPA by p53. Nature **365**, 79-82.

El-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992). Definition of a consensus binding site for p53. Nature Genetics **1**, 45-49.

El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. E., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993). WAF-1, a potential mediator of p53 tumor suppression. Cell **75**, 817-825.

El-Deiry, W. S., Tokino, T., Waldman, T., Oliner, J. D., Velculescu, V. E., Burrell, M., Hill, D. E., Healy, E., Rees, J. L., Hamiliton S. R., Kinzler, K. W., and

Vogelstein, B. (1995). Topological control of p21 WAF1/CIP1 expression in normal and neoplastic tissues. Cancer Res. 55, 2910-2919.

Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O., and Oren, M. (1989). Wild-type p53 can inhibit oncogene-mediated focus formation. Proc. Nati. Acad. Sci. **86**, 8763-8767.

Elledge, R. M., and Lee, W. H. (1995). Life and death by p53. BioEssays **17**, 923-930.

Fakharzadeh, S. S., Trusko, S., and George, D. L. (1991). Tumorigenic potential associated with enhanced expression of gene that is amplified in a mouse tumor cell line. EMBO J. **10**, 1565-1569.

Fields, S. and Jang, K. (1990). Presence of a potent transcription activating sequence in the p53 protein. Science **249**, 1046-1048.

Finaly, C. A., Hinds, P. W., and Levine, A. J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. Cell **57**, 1083-1093.

Finlay, C. A. (1993). The mdm-2 oncogene can overcome wild-type p53 suppression of transformed cell growth. Mol. Cell Biol. **13**, 301-306.

Fornace, A. J. Jr., Alamo, I. J., and Hollander, M. C. (1988). DNA damage-inducible transcripts in mammalian cells. Proc. Natl. Acad. Sci. U. S. A. **85**, 8800-8804.

Fornace, A. J. Jr., Nebert, D. W., Hollander, M. C., Luethy, J. D., Papathanasiou, M., Fargnoli, J., and Holbrook, N. J. (1989). Mammalian genes coordinately regulated by growth arrest signals and DNA damaging agents. Mol. Cell. Biol. **9**, 4196-4203.

Fornace, A. J. Jr. (1992). Mammalian genes induced by radiation: activation of genes associated with growth control. Annu. Rev. Genet. **26**, 507-526.

Forus, A., Florenes, V. A., Maelandsmo, G. M., Fodstad, O., and Myklebost, O. (1994). The protooncogene CHOP/GADD153, involved in growth arrest and DNA damage response , is amplified in a subset of human sarcomas. Cancer Genet. Cytogenet. **78**, 165-171.

Fort, P., Marty, L., Piechaczyk, M., Sabrouty, S. L., Dani, C., Jeanteur, P., and Blanchard, J. M. (1985). Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigentic family. Nucleic Acids Res. **13**, 1431-1442.

Freedman, D. A., and Levine, A. J. (1998). Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6. Mol. Cell. Biol. **18**, 7288-7293.

Freedman, D. A., and Levine A. J. (1999). Regulation of the p53 protein by the MDM2 oncoprotein-Thirty-eight G. H. A. clowes memorial award lecture. Cancer Res. **59**, 1-7.

Friedman, P. N., Kern, S. E., Vogelstein, B., and Prives, C. (1990). Wild-type, but not mutant, human p53 proteins inhibit the replication activities of simian virus 40 large tumor antigen. Proc. Natl. Acad. Sci. USA **87**, 9275-9279.

Friedman, P. N., Chen, X., Bargonetti, J., and Prives C. (1993). The p53 protein is an unusually shaped tetramer that binds directly to DNA. Proc. Natl. Acad. Sci. USA **90**, 3319-3323.

Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E., Shay, J. W. (1992). A transcriptionally active DNA-binding site for human p53 protein complexes. Mol. Cell Biol. **12**, 2866-2871.

Gallagher, W. M., and Brown, R. (1999). p53-oriented cancer therapies: current progress. Annals of Oncology **10**, 139-150.

Gannon, J. V., and Lane, D. P. (1987). p53 and DNA polymerase a compete for binding to SV40 T antigen. Nature **329**, 456-458.

Gannon, J. V., and Lane, D. P. (1991). Protein synthesis required to anchor a mutant p53 protein which is temperature-sensitive for nuclear transport. Nature **349**, 802-806.

Giaccia, A. J., and Kastan, M. B. (1998). The complexity of p53 modulation: emerging patterns from divergent signals. Genes & Dev. **12**, 2973-2983.

Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999). Bcl-2 family members and the mitochondria in apoptosis. Genes & Dev. **13**, 1899-1911.

Gobert, C., Bracco, L., Rossi, F., Olivier, M., Tazi, J., Lavelle, F., Larsen, F., Larsen, A. K., and Riou, J. F. (1996). Modulation of DNA topoisomerase I activity by p53. Biochemistry **35**, 5778-5786.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. **89**, 5547-5551.

Gross, D. S. and Garrard, W. T. (1988). Nuclease hypersensitive sites in chromatin. Ann. Rev. Biochem. **57**, 159-197.

Gross, D. S., Adams, C. C., Lee, S., and Stentz, B. (1993). A critical role for heat shock transcription factor in establishing a nucleosome-free region over the TATA-initiation site of the yeast HSP82 heat shock gene. EMBO J. **12**, 3931-3945.

Grunstein, M. (1990). Histone function in transcription. Annu. Rev. Cell. Biol. **6**, 643-678.

Gu, W., Shi, X., and Roeder, R. G. (1997a). Synergistic activation of transcription by CBP and p53. Nature, **387**, 819-822.

Gu, W., and Roeder, R. G. (1997b). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell **90**, 595-606.

Gujuluva, C. N., Baek, J. H., Shin, K. H., Cherrick, H. M., and Park, N. H. (1994). Effect of UV-irradiation on cell cycle, viability and the expression of p53,

gadd153 and gadd45 genes in normal and HPV-immortalized human oral keratinocytes. Oncogene **9**, 1819-1827.

Halazonetis, T. D., Davis, L. J., and Kandil, A. N. (1993). Wild-type p53 adopts a 'mutant'-like conformation when bound to DNA. EMBO J. **12**, 1021-1028.

Hall, P. A., Kearsey, J. M., Coates, P. J., Norman, D. G., Warbrick, E., and Cox, L. S. (1995). Characterization of the interaction between PCNA and Gadd45. Oncogene **10**, 2427-2433.

Han, M., and Grunstein, M. (1988). Nucleosome loss activities yeast downstream promoters in vivo. Cell **55**, 1137-1145.

Harkin, D. P., Bean, J. M., Miklos, D., Song, Y-H., Truong, V. B., Englert, C., Christians, F. C., Ellisen, L. W., Maheswaran, S., Oliner, J. D., and Haber, D. A. (1999). Induction of gadd45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1. Cell **97**, 575-586.

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, H., and Elledge, S. J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75**, 805-816.

Harvey, D., and Levine, A. J. (1991). p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. Genes & Dev. **5**, 2375-2385.

Harvey, M., Sands, A. T., Weiss, R. S., Hegi, M. E., Wiseman, R. W., Pantazis, P., Giovanella, B. C., Tainsky, M. A., Bradley, A., and Donehower, L. A. (1993). *In vitro* growth characteristics of embryo fibroblasts isolated from p53 deficient mice. Oncogene **8**, 2457-2467.

Harvey, M., Vogel, H., Morris, D., Bradley, A., Bernstein, A., and Donehower, L. A. (1995). A mutant p53 transgene accelerates tumor development in heterozygous but not nullizygous p53-deficient mice. Nature Genet. **9**, 305-311.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature **387**, 296-299.

Hecker, D., Page, G., Lohrum, M., Weiland, S., and Scheidtmann, K. H. (1996). Complex regulation of the DNA-binding activity of p53 by phosphorylation : differential effects of individual phosphorylation sites on the interaction with different binding motifs. Oncogene **12**, 953-961.

Hermeking, H., Lengauer, C., Polyak, K., He, T., Zhang, L., Thiagalingam, A., Kinzler, K., and Vogelstein, B. (1997). 14-3-3σ is a p53-regulated inhibitor of G2/M progression. Mol. Cell **1**, 3-11.

Hinds, P., Finlay, C., and Levine, A. J. (1989). Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. J. Virol. **63**, 739-746.

Hollander, M., Alamo, I., Jackman, J., Wang, M. G., Mcbride, O. W., and Fornace, A. J. Jr. (1993). Analysis of the mammalian gadd45 gene and its response to DNA damage. J. Biol. Chem. **268**, 24385-24393.

Hollstein, M., Rice, K., Greenblatt, M. S., Soussi, T., Fuchs, R., Sorlie, T., Hovig, E., Smith-Sorensen, B., Montesano, R., and Harris, C. C. (1994). Database of p53 gene somatic mutations in human tumors and cell lines. Nucleic Acids Res. **22**, 3551-3555.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett. **420**, 25-27.

Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1992). Regulation of the specific DNA binding function of p53. Cell **71**, 875-886.

Hupp, T. R., Sparks, A., and Lane, D. P. (1995). Small peptides activate the latent sequence-specific DNA binding function of p53. Cell **83**, 237-245.

Hupp, T. R. (1999). Regulation of p53 protein function through alterations in protein-folding pathways. Cell. Mol. Life Sci. **55**, 88-95.

Ito, T., Bulger, M., Pazzin, M. J., Kobayasshi, R., and Kadonaga, J. T. (1997). ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. Cell **90**, 145-155.

Imbalzano, A. N., Kwon, H., Green, M. R., Kingston, R. E. (1994). Facilitated binding of TATA-binding protein to nucleosomal DNA. Nature **370**, 481-485.

Janknecht, R., and Hunter, T. (1996). A growing coactivator network. Nature **383**, 22-23.

Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995). Rescue of embryonic lethality in mdm2-deficient mice by absence of p53. Nature **378**, 206-208.

Juven, T., Barak, Y., Zauberman, A., Deorge, D. L., and Oren, M.

Jayaraman, L., Moorthy, N. C., Murthy, K. G. K., Manley, J. L., Bustin, M., and Prives, C. (1998). High mobility group protein-1(HMG-1) is a unique activator of p53. Genes & Dev. **12**, 462-472.

Jeong, S. W., Lauderdale, J. D., and Stein, A. (1991). Chromatin assembly on plasmid DNA *in vitro*. Apparent spreading of nucleosome alignment from one region of pBR327 by histone H5. J. Mol. Biol., **222**, 1131-1147.

Juven, T., Barak, Y., Zauberman, A., Deorge, D. L., and Oren, M. (1993). Wild type p53 can mediate sequence-specific transactivation of an internal promoter within the mdm2 gene. Oncogene **8**, 3411-3416.

Juven-Gershon, T., and Oren, M. (1999). Mdm2: The ups and Downs. Mol. Med., **5**, 71-83.

Kadonaga, J. T. (1998). Eucaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. Cell **92**, 307-313.

Kao, C. C., Yew, P. R., and Berk, A. J. (1990). Domains required for *in vitro* association between the cellular and p53 and the adenovirus 2 E1B 55kD proteins. Virology **179**, 806-814.

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. (1991). Participation of p53 protein in the cellular response to DNA damage. Cancer Res. **51**, 6304-6311.

Kastan, M. B., Zhan, Q., El-Deiry, Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J. Jr. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell **71**, 587-597.

Kern, S. E, Kinzler, K. W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C., and Vogelistein, B. (1991). Identification of p53 as a sequence-specific DNA-binding protein. Science **252**, 1708-1711.

Kim, S. Y. and Rockwell, S. (1995). Cytotoxic potential of monoalkylation products between mitomycins and DNA: studies of decarbamoyl mitomycin C in wild-type and repair-deficient cell lines. Oncology Res. **7**, 39-47.

Klein, C., and Struhl, K. (1994). Increased recruitment of TATA-binding protein to the promoter by transcriptional activation domains *in vivo*. Science **266**, 280-282.

Knippschild, U,. Milne, D. W., Campbell, L. E., DeMaggio, A. J., Christenson, E., Hoekstra, M. F., and Meek, D. W. (1997). p53 is phosphorylated *in vitro* and *in vivo* by the delta and epsilon isoforms of casein kinase 1 and enhances the level of casein kinase 1 delta in response to topoisomerase-directed drugs. Oncogene **15**, 1727-1736.

Ko, L. J., and Prives, C. (1996) p53: puzzle and paradigm. Genes & Dev. **10**, 1054-1072.

Kondo, S., Barnett G. H., Hara, H., Morimura, T., and Takeuchi, J. (1995). MDM2 protein confers the resistance of a human glioblastoma cell line to cisplatin-induced apoptosis. Oncogene **8**, 2001-2006.

Kubbutat, M. H. G., Jones, S. N., and Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. Nature **387**, 299-302.

Kunkel, G. R., and Martinson, H. G. (1987). Nucleosome will not form on double-stranded RNA or over poly (dA). poly (dT) tracts in recombinant DNA. Nucleic Acids Res. 9, 6869-6888.

Kuras, L. and Struhl, K. (1999). Binding of TBP to promoters in vivo is stimulated by activators and requires Pol II holoenzymes. Nature **399**, 609-613.

Kussie, P. H., Gorina, S., Marechal V., Elenbass B., Moreau, J., Levine, A J., and Pavletich, N. P. (1996). Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science **274**, 948-953.

Lambert, P. F., Kashanchi, F., Radonovich, M. F., Shiekhattar, R. and Brady, J. N. (1998) Phosphorylation of p53 serine 15 increases interaction with CBP. J. Biol. Chem. **273**, 33048-33053.

Landers, J. E., Haines, D. S., Strauss, J. F., and George, D. L. (1994). Enhanced translation: a novel mechanism of mdm2 oncogene overexpression identified in human tumor cells. Oncogene **9**, 2745-2750.

Lee, S., Elenbaas, B., Levine, A. J., and Griffith, J. (1995). p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. Cell **81**, 1013-1020.

Lees-Miller, S. P. Sakaguchi, K., Ullrich, S. J., Appella, E., and Anderson, C. W. (1992). Human DNA-activated protein kinase phosphorylates serine 15 and 37 in the amino-terminal transactivation domain of human p53. Mol. Cell. Biol. **12**, 5041-5049.

Lehman, T. A., Modali, R., Boukamp, P., Stanek, J., Bennett, W. P., Welsh, J. A., Metcalf, R. A., Stampfer, M. R., Fusenig., N., Rogan, E. M., and Harris, C. C. (1993). p53 mutations in human immortalized epithelial cell lines. Carcinogenesis **14**, 833-839.

Leveillard, T., Andera, L., Bissonnette, N., Schaeffer, L., Bracco, L., Egly, J-M., and Waslyk, B. (1996). Functional interactions between p53 and the TFIIH complex are affected by tumour-associated mutations. EMBO J. **15**, 1615-1624.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. Cell **88**, 323-331.

Lill, N. L., Grossman, S. R., Ginsberg, D., DeCaprio, J., and Livingston, D. M. (1997). Binding and modulation of p53 by p300/CBP coactivators. Nature **387**, 823-826.

Lin, J., Chen, J., Elenbaas, B., and Levine, A. J. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55kD protein. Genes & Dev. **8**, 1235-1246.

Linzer, D., and Levine, A. J. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell **17**, 43-52.

Liu, X., Miller, C. W., Koeffler, P.H., and Berk, A. J. (1993). The p53 activation domain binds the TATA box -binding polypeptide in holo-TFIID, and a neighboring p53 domain inhibits transcription. Mol. Cell. Biol. **13**, 3291-3300.

Liu, L., Scolnick, M., Trievel, R. C., Zhang, H. B., Marmorstiein, R., Halazonetis, T. D., and Berger, S. L. (1999). p53 sites acetylated *in vitro* by PCAF and p300 are acetylated *in vivo* in response to DNA damage. Mol. Cell. Biol. **19**, 1202-1209.

Livingstone, L. R., White, A., Sprouse, J., Livanos, E., Jacks, T., and Tlsty, T. D. (1992). Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell **70**, 923-935.

Lu, H., Fisher, R. P., Bailey, P., and Levine, A. J. (1997). The CDK-cycH-p36 complex of transcription factor IIH phosphorylates p53, enhancing its sequence-specific DNA binding activity *in vitro*. Mol. Cell. Biol. **17**, 5923-5934.

Lu, H. and Levine, A. J. (1995). Human TAFII31 protein is a transcriptional coactivator of the p53 protein. Proc. Natl. Acad. Sci. USA **92**, 5154-5158.

Lundgren, K., Montes de Oca Luna, R., Mcneill, Y B., Emerick, E. P., Spencer, B., Barfield, C R., Lozano, G., Rosenberg, M. P., and Finaly, C. A. (1997). Targeted expression of MDM2 uncouples S phase from mitosis and inhibits mammary gland development independent of p53. Genes & Dev. **11**, 714-725.

Manganelli, R., Dubnau, E., Tyagi, S., Kraner, F. R., and Smith, I. (1999). Differential expression of 10 sigma factor genes in Mycobaterium tuberculosis. Mol. Microbiology **31**, 715-724.

Marhin, W. W., Chen, S., Facchini, L. M., Fornace Jr, A. J., and Penn, L. Z. (1997). Myc represses the growth arrest gene gadd45. Oncogene **14**, 2825-2834.

Martinez, J., Georgoff, I., Martinez, J., and Levine, A. J. (1991). Cellular localization and cell cycle regulation by temperature-sensitive p53 protein. Genes & Dev. **5**, 151-159.

Mayr, G. A., Reed, M., Wang, P., Wang, Y., Schwedes, J. F., and Tegtmeyer. (1995). Serine phosphorylation in the NH2 terminus of p53 facilitates transactivation. Cancer Res. **55**, 2410-2417.

Martin, K., Trouche, D., Hagemeler, C., Sorensen, T. S., La Thangue, N. B., and Kouzarides, T. (1995). Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. Nature **375**, 691-694.

McKnight, S. (1996). Transcription revisited: a commentary on the 1995 Cold Spring Harbor laboratory meeting, "mechanisms of eukaryotic transcription". Genes & Dev. **10**, 367-381.

McPherson, C. E., Shim, E., Friedman, D. S., and Zaret, K. S. (1993). An active tissue-specific enhancer and bound transcription factors existing in a precisely positioned nucleosomal array. Cell **75**, 387-398.

McVey, D., Brizuela, L., Mohr, I., Marshark, D. R., Gluzman, Y., and Beach, D. (1989). Phosphorylation of large tumor antigen by cdc2 stimulates SV40 DNA replication. Nature **341**, 503-507.

Meek, D. W., Simon, S., Kikkawa, U., and Eckhart, W. (1990). The p53 tumour suppressor protein is phosphorylated at serine 389 by casein kinase II. EMBO J. **9**, 3253-3260.

Meek, D. W. (1998). Multisite phosphorylation and the integration of stress signals at p53. Cell Signal **10**, 159-166.

Michalovitz, D., Halevy, O., and Oren, M. (1990). Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell **62**, 671-680.

Milczarek, G. J., Martinez, J., and Broden, G. T. (1997). p53 phosphorylation : biochemical and functional consequences. Life Sci. **60**, 1-11.

Miyashita, T., and Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell **80**, 293-299.

Moll, U., LaQuaglia, M., Benard, J., and Riou, G. (1995). Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. Proc. Natl. Acad. Sci. USA **92**, 4407-4411.

Momand, J., Zambetti, G., Olson, D.C., George, D., and Levine, A. J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell **69**, 1237-1245.

Momand, J. and Zambetti, G. P. (1997). Mdm2: "big brother" of p53. J. Cell. Bioch. **64**, 343-352.

Montes de Oca Luna, R., Wagner, D. S., and Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 378, 203-206.

Montes de Oca Luna, R., Tabor A. D., Eberspaecher, H., Hulboy, D. (1996). The organization and expression of the mdm2 gene. Genomics **33**, 352-357.

Mueller, P. R., and Wold, B. (1989). *In vivo* footprinting of a muscle specific enhancer by ligation mediated PCR. Science **246**, 780-786.

Mummenbrauuer, T., Jannus, F., Muller, B., Wiesmuller, L., Deppert, W., and Grosse, F. (1996). p53 protein exhibits 3'-to-5' exonuclease activity. Cell **85**, 1089-1099.

Nelson, W. G., and Kastan, M. B. (1994). DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol. Cell. Biol. **14**, 1815-1823.

Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R.. Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C., and Vogelstein, B. (1989). Mutations in the p53 gene occur in diverse human tumor types. Nature **342**, 705-708.

Oberosler, P., Hloch, P., Ramsperger, U., and Stahl, H. (1993). p53-catalyzed annealing of complementary single-stranded nucleic acids. EMBO J. **12**, 2389-2396.

Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., and Vogelstein, B. (1992). Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature **358**, 80-83.

Oliner, J. D., Pietenpol, J. A., Thiagalingam,S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumor suppressor p53. Nature **362**, 857-860.

Olson, D. C., Marechal, V., Momand, J., Chen, J., Rimocki, C., and Levine, A. J. (1993). Identification and characterization of multiple mdm2 protein and mdm2-p53 protein complexes. Oncogene **8**, 2353-2360.

Otto, A., and Deppert, W. (1993). Upregulation of mdm2 expression in METH A tumor cells tolerating wild-type p53. Oncogene **8**, 2591-2603.

O'Reilly, D. R., and Miller, L. K. (1988). Expression and complex formation of simian virus 40 large T antigen and mouse p53 in inset cells J. Virol., 63, 3109-3119.

Papathanasiou, M. A., Kerr, N. C., Robbins, J. H., Mcbride, O. W., Alamo, I. J., Barrett, S. F., Hickson, I. D., and Fornace, A. J. Jr. (1991). Induction by ionizing radiation of the gadd45 gene in cultured human cells: lack of mediation by protein kinase C. Mol. Cell. Biol. **11**, 1009-1016.

Pavletich, N. P., Chambers, K.A., and Pabo, C. O. (1993). The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. Genes & Dev. **7**, 2556-2564.

Perry, M. E., Commane, M., and Stark, G. R. (1992). Simian virus 40 large tumor antigen alone or two cooperating oncogenes convert REF52 cells to a state permissive for gene amplification. Proc. Natl. Acad. Sci. U. S. A. **89**, 8112-8116.

Picksley, S. M., Vojtesek, B., Sparks, A., and Lane, P. (1994). Immunochemical analysis of the interaction of p53 with MDM2; fine mapping of the MDM2 binding site on p53 using synthetic peptides. Oncogene **9**, 2523-2529.

Piette, J., Neel, H., and Marechal, V. (1997). Mdm2: keeping p53 under control. Oncogene **15**, 1001-1010.

Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H., Cordon-Cardo, C., and DePinho, R. A. (1998). The Ink4a tumor suppressor gene product, $p19^{Arf}$, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell **92**, 713-723.

Powis, G., Bonjouklian, R., Berggren, M. M., Gallegos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W. F., Dodge, J., Gindley, G., and Vlahos, C. J. (1994). Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. Cancer Res. **54**, 2419-2423.

Price, B., and Youmell, M. B. (1996). The phosphatidylinositol 3-kinase inhibitor wortmannin sensitizes murine fibroblasts and human tumor cells to radiation and block induction of p53 following DNA damage. Cancer Res. **56**, 246-250.

Prives, C. (1994). How loop, β sheets, and α helices help us to understand p53. Cell **78**, 543-546.

Prives, C. (1998). Signaling to p53: Breaking the MDM2-p53 circuit. Cell **95**, 5-8.

Prives, C. and Hall, A. (1999). The p53 pathway. J. Pathol. **187**, 112-126.

Raff, M. C. (1992). Social controls on cell survival and cell death. Nature 356, 397-400.

Reed, J. C. (1994). Bcl-2 and the regulation of programmed cell death. J. Cell Biol. **124**, 1-6.

Reed, M., Woelker, B., Wang, P., Wang, Y., Anderson, M. E., and Tegtmeyer, P. (1995). The C-terminal domain of p53 recognizes DNA damaged by ionizing radiation. Proc. Natl. Acad. Sci. **92**, 9455-9459.

Renzing, J., and Lane, D. P. (1995). p53-dependent growth arrest following calcium phosphate -mediated transfection of murine fibroblasts. Oncogene **10**, 1865-1868.

Roth, J., Dobbelstein, M., Freedman, D. A., Shenk,T., and Levine, A. J. (1998). Nucleo-cytoplasmic shuttling of the hdn2 oncoprotein regulates the levels of p53 protein via a patheay used by the human immunodeficiency virus rev protein. EMBO J. **17**, 554-564.

Ruppert, M., and Stillman, B. (1993). Analysis of a protein-binding domain of p53. Mol. Cell. Biol. **13**, 3811-3820.

Sakaguchi, K., Herrera, J. E., Satio, S. S., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W. and Appella, E. (1998). DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes & Dev. **12**, 2831-2841.

Saluz, H. P., and Jost, J-P. (1993). Approaches to characterize protein-DNA interactions *in vivo*. Critical Reviews in Eukaryotic Gene Expression **3**, 1-29.

Seto, E., Usheva, A., Zambetti, G. P., Horikoshi, N., Weinmann, R., Levine, A. J., and Shenk, T. (1992). Wild-type p53 binds to the TATA-binding protein and repress transcription. Proc. Natl. Acad. Sci. **89**, 12028-12032.

Sherr, C. J., and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes & Dev. **13**, 1501-1512.

Shieh, S., Ikeda, M., Taya, Y. and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell **91**, 325-334.

Shieh, S., Taya, Y., and Prives, C. (1999). DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, ser20, requires tetramerization. EMBO J. **18**, 1815-1823.

Siliciano, J. D., Canman, C. E., Taya, Y., Sakaguchi, K., Appella, E. and Kastan. M. B. (1997). DNA damage induces phosphorylation of the amino terminus of p53. Genes & Dev. **11**, 3471-3481.

Sigalas, I., Calvert, A. H., Anderson, J. J., Neal, D. E., and Lunec, J. (1996). Alternatively spliced mdm2 transcripts with loss of p53 binding domain sequences: transforming ability and frequent detection in human cancer. Nature Med. **2**, 912-917.

Smith, M. L., Chen, I. T., Zhan, Q., Bae, I., Chen, C-Y., Gilmer, T. M., Kastan, M. B., O'Connor, P. M., and Fornace Jr, A. J. (1994). Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science **226**, 1376-1379.

Stenger, J. E., Tegtmeyer, P., Mayr, G. A., Reed, M. W., Wang, Y. P., Hough, P. V. C., and Mastrangelo, I. A. (1994). p53 oligomerization and DNA looping are linked with transcriptional activation. EMBO J. **13**, 6011-6020.

Stott, F. J., Bates S., James, M. C., McConnel, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H., and Peters, G. (1998). The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. EMBO J. **17**, 5001-5014.

Stryer, L. 1988. Biochemistry, 3rd edition.

Struhl, K. (1996). Chromatin structure and RNA polymerase II connection: implications for transcription. Cell **84**, 179-182.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. Genes & Dev. **12**, 599-606.

Studitsky, V. M., Clark, D. J. and Felsenfeld. (1995). Overcoming a nucleosomal barrier to transcription. Cell **83**, 19-27.

Sturzbecher, H. W., Brain, R., Addision, C., Rudge, K., Remm, M., Grimaldi, M., Keenan, E., and Jenkins. (1992). A C-terminal α-helix plus basic region motif is the major structural determinant of p53 tetramerization. Oncogene **7**, 1513-1523.

Takekawa, M., and Saito, H. (1998). A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell **95**, 521-530.

Takenaka, I., Morin, F., Seizinger, B. P., and Kley, N. (1995). Regulation of the sequence-specific DNA binding function of p53 by protein kinase C and protein phosphatase. J. Biol. Chem. **270**, 5405-5411.

Taunton, J., Hassig, C. A., and Schreiber, S. L. (1996). A mammalian histone deaceylase related to yeast transcriptional regulator Rpd3p. Science **272**, 408-411.

Thut, C. J., Chen, J. L., Klemm, R., and Tjian, R. (1995). p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. Science **267**, 100-104.

Thut, C. J., Goodrich, J. A., and Tjian, R. (1997). Repression of p53-mediated transcription by MDM2: a dual mechanism. Genes. Dev., **11**, 1974-1986.

Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S., Taya, Y., Prives, C., and Abraham, R. T. (1999). A role for ATR in the DNA damage-induced phosphorylation of p53. Genes & Dev. **13**, 152-157.

Tishler, R. B., Calderwood, S. K., Coleman, C. N., and Price, B. D. (1993). Increases in sequence specific DNA binding by p53 following treatment with chemotherapeutic and DNA damaging agents. Cancer Res., **53**, 2212-2216.

Tokino, T., Thiagalingam, S., el-Deiry, W. S., Waldman, T., Kinzlor, K. W., and Vogeistein, B. (1994). p53 tagged sites from human genomic DNA. Hum. Mol. Genet. **3**, 1537-1542.

Tomasz, M. and Palom, Y. (1997). The mitomycin bioreductive antitumor agents: cross-linking and alkylation of DNA as the molecular basis of their activity. (1997). Pharmacol. Ther. **76**, 73-87.

Truant, R., Xiao, H., Ingles, C. J, and Greenblatt, J. (1993) Direct interaction between the transcriptional activation domain of human p53 and the TATA box-binding protein. J. Biol. Chem. **268**, 2284-2287.

Truss, M., Bartsch, J., Schelbert, A., Hache, R. J. G., and Beato, M. (1995). Hormone induces binding of receptors and transcription factors to a rearranged nucleosome on the MMTV promoter *in vivo*. EMBO J. **14**, 1737-1751.

Tsukiyama, T., Becker, P. B., and Wu, C. (1994). ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor. Nature **367**, 525-532.

Tsukiyama, T., and Wu, C. (1995). Purification and properties of an ATP-dependent nucleosome remodeling factor. Cell **83**, 1011-1020.

Tyagi, S., and Kramer, F. R. (1996). Molecular beacons: probes that fluoresce upon hybridization. Nature Biotechnol. **14**, 303-308.

Ui, Michio, Okada, T., Hazeki, K., and Hazeki, O. (1995). Wortmannin as a unique probe for an intracellular signaling protein, phosphoinositide 3-kinase. TIBS. **20**, 303-307.

van Holde, K., Zlatanova, J., Arents, G., and Moudrianakis, E. (1995). Chromatin Structure and Gene Expression (Elgin, S. C. R., ed) pp. 1-26. Oxford University Press, Oxford, UK

van Holde, K. E. (1997). Chromatin structure and regulation of gene expression minireview series. J. Bio. Chem. **272**, 26073.

Wade, P. A., Pruss, D., and Wolffe, A. P. (1997). Histone acetylation: chromatin in action. TIBS **22**, 128-132.

Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature **369**, 574-578.

Wagner, A. J., kokontis, J. M., and Hay, N. (1994). Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce $p21^{waf1/cip1}$. Genes & Dev. **8**, 2817-2830.

Wallrath, L. L., Lu, Q., Granok, H., and Elgin, S. C. R. (1994). Architectural variations of inducible eukaryotic promoters: preset and remodeling chromatin structures. BioEssays **16**, 165-170.

Wang, E. H., Fridman, P. N., and Prives, C. (1989). The murine p53 protein blocks replication of SV40 DNA replication *in vitro* by inhibiting the initiation functions of SV40 large T antigen. Cell, **57**, 379-392.

Wang, J. C. (1991). DNA topoisomerase: Why so many? J. Biol. Chem. **266**, 6659-6662.

Wang, J. C. (1996a). DNA topoisomerases. Annu. Rev. Biochem. **65**, 635-692.

Wang, Y., and Prives, C. (1995). Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin- dependent kinase. Nature **376**, 88-91.

Wang, Y., Reed, M., Stenger, J. E., Mayr, G., Anderson, M.E., Schwedes, J. F., and Tegtmeyer, P. (1993). p53 domains: identification and characterization of two autonomous DNA-binding regions. Genes & Dev. **7**, 2575-2586.

Wang, P., Reed, M., Wang, Y., Mayr, G., Stenger, J. E., Anderson, M. E., Schwedes, J. F., and Tegtemeyer, P. (1994a). p53 domains: structure, oligomerization, and transformation. Mol. Cell. Biol. **14**, 5182-5191.

Wang, X. W., Forrester, K., Yeh, H., Feitelson, M. A., Gu, J. R., and Harris, C. C. (1994b). Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding,

transcriptional activity, and association with transcription factor ERCC3. Proc. Natl. Acad. Sci. USA, **91**, 2230-2234.

Wang, X. W., Vermeulen, W., Coursen, J. D., Gibson, M., Lupold, S. E., Forrester, K., Xu, G., Elmore, L., Yeh, H., Hoeijmakers, J. H.J., and Harris, C. C. (1996b). The XPB and XPD helicases are components of the p53-mediated apoptosis pathway. Genes & Dev. **10**, 1219-1232.

Wang, X., Zhan, Q., Coursen, J. D., Khan, M. A., Kontny, H. U., Yu, L., Hollander, M. C., O'Connor, P. M., Fornace Jr., A. J., and Harris, C. C. (1999). GADD45 induction of G2/M cell cycle checkpoint. Proc. Natl. Acad. Sci. USA **96**, 3706-3711.

Wang, Y., Schwedes, J. F., Parks, D., Mann, K., and Tegtemeyer, P. (1995). Interaction of p53 with its consensus DNA-binding site. Mol. Cell. Biol. **15**, 2157-2165.

Waterman, M. J., Stavridi, E. S., Waterman, J., and Halazonetis, T. D. (1998). ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. Nat. Genet. **19**, 175-178.

Wilson, C. J., Chao, D. M., Imbalzano, A. N., Schnitzler, G. R., Kingston, R. E., and Young, R. A. (1996). RNA polymerase II holyenzyme contains SWI/SNF regulators involved in chromatin remodeling. Cell **84**, 235-244.

Wolffe, A. P., and Pruss, D. (1996). Targeting chromatin disruption: transcription regulators that acetylates histones. Cell **86**, 817-819.

Woodcock, C. L., and Horowiz, R. A. (1995). Chromatin organization re-viewed. TICB **5**, 272-277.

Wu, C. (1997). Chromatin remodeling and the control of gene expression. J. Biol. Chem. **272**, 28171-28174.

Wu, X., Bayle, J. H., Olson, D., and Levine, A. J. (1993). The p53-mdm-2 autoregulatory feedback loop. Genes & Dev. **7**,1126-1132.

Wu, X., and Levine, A. J. (1994). p53 and E2F-1 cooperate to mediate apoptosis. Proc. Natl. Acad. Sci. USA **91**, 3602-3606.

Wu, L., and Levine, A. J. (1997). Differential regulation of the p21/waf1 and mdm2 genes after high-dose UV irradiation: p53-dependent and p53-independent regulation of the mdm2 gene. Molec. Med. **3**, 441-451.

Xiao, Z-X., Chen, J., Levine, A. J., Modjtahedl, N., Xing, J., Sellers, W. R., and Livingston, D. M. (1995). Interaction between the retinoblastoma protein and the oncoprotein MDM2. Nature **375**, 694-698.

Xiao, G., White, D., and Bargonetti, J. (1998). p53 binds to a constitutively nucleosome free region of the mdm2 gene. Oncogene **16**, 1171-1181.

Yano, H., (1993). Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. J. Biol. Chem. **268**, 25846-25856.

Zauberman, A., Flusberg, D., Haupt, Y., Barak, Y., and Oren, M. (1995). A functional p53-responsive intronic promoter is contained within the human mdm2 gene. Nucleic Acids Res. **23**, 2584-2592.

Zhan, Q., Carrier, F., and Fornace, Jr, A. J. (1993). Induction of cellular p53 activity by DNA-damaging agents and growth arrest. Mol. Cell. Biol. **13**, 4242-4250.

Zhan, Q., Lord., K. A., Alamo, I. Jr., Hollander, M. C., Carrier, F., Ron, D., Kohn, K. W., Hoffman, B., Liebermann, D., and Fornace Jr., A J. (1994). The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. Mol. Cell. Biol. **14**, 2361-2371.

Zhan, Q., Fan, S., Smith, M. L., Bae, I., Yu., K., Alamo Jr. I., O'Connor, P. M., and Fornace, Jr, A. J. (1996). Abrogation of p53 function affects the response of gadd genes to DNA base damaging agents and medium starvation. DNA Cell Biol. **15**, 805-815.

Zhan, Q., Chen, I., Antinore, M. J., and Fornace Jr, A. J. (1998). Tumor suppressor p53 can participate in transcriptional induction of the gadd45 promoter in the absence of direct DNA binding. Mol. Cell. Biol. **18**, 2768-2778.

Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell **92**, 725-734.